Forschungszentrum Karlsruhe
in der Helmholtz-Gemeinschaft
Wissenschaftliche Berichte
FZKA 6857

Attenuation of IgE receptor signalling in mast cells as molecular basis for the anti-allergic action of glucocorticoids*

Alessandra Sancono

Institut für Toxikologie und Genetik

*Von der Fakultät für Chemie und Biowissenschaften
der Universität Karlsruhe (TH)
genehmigte Dissertation

Forschungszentrum Karlsruhe GmbH, Karlsruhe
2003

Impressum der Print-Ausgabe:

Als Manuskript gedruckt

Forschungszentrum Karlsruhe GmbH
Postfach 3640, 76021 Karlsruhe

Mitglied der Hermann von Helmholtz-Gemeinschaft
Deutscher Forschungszentren (HGF)

ISSN 0947-8620

Abstract

Glucocorticoids are well known for their potent anti-inflammatory, immune suppressive and anti-allergic activities. In mast cells, they inhibit the exocytotic release of granules containing pre-formed allergic mediators, the activation of arachidonic acid metabolism and the induction of expression of pro-inflammatory cytokines. These processes are brought about by activation of the receptor with high affinity for IgE (FcεRI), following IgE/antigen cross-linking. In the work presented here, the mode(s) of action of glucocorticoids in inhibiting FcεRI signalling have been analysed.

Glucocorticoids suppress the expression of FcεRI alpha chain gene by inhibiting its promoter activity. This effect requires *de novo* protein synthesis and regulatory elements at the FcεRI α-chain promoter that bind the transcription factors GATA-1, Elf-1, PU.1 and YY1. The downregulation of the FcεRI α-chain gene expression correlates with a reduced surface expression of the FcεRI. Furthermore, glucocorticoid treatment promotes inactivation of the lck/yes-related novel (Lyn) Src-like tyrosine kinase, responsible of phosphorylation and activation of the FcεRI, *via* enhanced phosphorylation of the regulatory tyrosine residue of Lyn. The reduction of surface expressed IgE receptor and the inactivation of Lyn would suppress the signal transduction events originating from the FcεRI and ending up with the activation of the downstream targets ERK1/2.

In addition, it has been shown here that glucocorticoids inhibit IgE receptor signalling by enhancing the expression of the MAPK phosphatase MKP-1, which in turn inhibits the activation of ERK1/2. The glucocorticoid-mediated enhancement in MKP-1 expression occurs at the MKP-1 promoter level. This regulation requires the glucocorticoid receptor (GR) dimerisation function and the presence of discrete elements on the promoter proximal sequence, suggesting that it is a direct action of the GR. A crucial role of MKP-1 in glucocorticoid-mediated repression of ERK1/2 phosphorylation was demonstrated in bone marrow derived mast cells from MKP-1-deficient mice. ERK1/2 in these cells are activated by IgE receptor cross-linking or by Stem Cell Factor through the c-kit receptor, and they were no longer inhibited by glucocorticoids, while this was the case in cells from wild-type mice. However, ERK1/2 activity in other cell types such as thymocytes and splenocytes of MKP-1 deficient mice, could still be inhibited by glucocorticoids. These results demonstrate that repression of ERK1/2 through MKP-1 is a specific process, that occurs in mast cells but not in other cell populations.

Taken together, the results presented here show that the glucocorticoid-mediated inhibition of expression of FcεRIα gene and increased expression of MKP-1 may possibly function together in the attenuation of IgE receptor signalling by glucocorticoids.

Verzögerung der Signaltransduktionskette des IgE-Rezeptors in Mastzellen als molekulare Basis für die antiallergische Wirkung von Glukokortikoiden

Zusammenfassung

Glukokortikoide sind für ihre starken entzündungshemmenden, immunsuppressiven und anti-allergischen Wirkungen bekannt. Sie verhindern sowohl das exozytotische Ausschütten der Granulae, welche bereits vorsynthetisierte allergische Mediatoren enthalten, als auch die Aktivierung des Arachidonsäurestoffwechsels und die Induktion der Expression von proinflammatorischen Zytokinen. Alle diese Prozesse kommen durch die Aktivierung von FcεRI, eines Rezeptors mit hoher Affinität zu IgE, zustande, gefolgt von Antigen-IgE-Antikörper-Kreuzreaktionen in Mastzellen.

In der hier vorgestellen Arbeit werden die Wirkung(en) der Glukokortikoide bei der Hemmung des FcεRI-Signaltransduktionsweges untersucht. Glukokortikoide unterdrücken die Expression des Genes, welches für die alpha-Kette des FcεRI kodiert, im Bereich seines Promoters. Dieser Effekt benötigt neue Proteinsynthese und regulatorische Elemente im Promoter des Genes für die FcεRI-alpha-Kette, an welche die Transkriptionsfaktoren GATA-1, Elf-1, PU.1 und YY1 binden können. Die Verringerung der Expression des Genes, welches für die FcεRI-alpha-Kette kodiert, korreliert mit einer verringerten Oberflächenexpression des gesamten FcεRI-Komplexes. Darüber hinaus fördert die Behandlung mit Glukokortikoiden die Inaktivierung der mit lck/yes-verwandten neuen Src-ähnlichen Tyrosinkinase Lyn, welche für die Phosphorylierung und Aktvierung von FcεRI verantwortlich ist, mittels einer verstärkten Phosphorylierung der regulatorischen Tyrosinreste von Lyn. Diese Reduktion der Oberflächenexpression des IgE-Rezeptors (FcεRI) und Inaktivierung von Lyn können die Signaltransduktionsereignisse unterdrücken, die vom FcεRI kommen und mit der Aktivierung des weiter unten in der Signaltransduktionskaskade befindlichen Zielmoleküls ERK1/2 enden.

Zusätzlich konnte in dieser Arbeit gezeigt werden, daß Glukokortikoide den durch den IgE-Rezeptor aktivierten Signaltransduktionsweg über eine Verstärkung der Expression der MAPK-Phosphatase MKP-1, welche ihrerseits die Aktivierung von ERK1/2 hemmt, vermindern können. Die über Glukokortikoide vermittelte Verstärkung der Aktivität von MKP-1 findet am Promoter von MKP-1 statt. Für diese Regulation ist sowohl eine Dimerisation des Glukokortikoidrezeptors (GR) notwendig, als auch das Vorhandensein bestimmter Elemente an der Promotorsequenz. Dies weist darauf hin, daß es sich um eine direkte Aktion des GR handelt. Gezeigt werden konnte eine entscheidende Rolle von MKP-1 bei der Glukokortikoid-vermittelten Unterdrückung der ERK1/2-Phosphorylierung in Mastzellen, die aus primären Knochenmarkszellen von MKP-1-defizienten Mäusen stammen. ERK1/2 werden in diesen Zellen durch die Kreuzreaktion des IgE-Rezeptors oder durch den Stammzellfaktor, welcher über den c-Kit-Rezetpor wirkt, aktiviert und nicht länger durch Glukokortikoide gehemmt, wie dies in Zellen von Wildtyp-Mäusen der Fall ist. Allerdings konnte die Aktivität von ERK1/2 in anderen Zelltypen wie Thymozyten und Splenozyten von MKP-1-defizienten Mäusen immer noch durch Glukokortikoide gehemmt werden. Diese Ergebnisse zeigen, daß die Repression von ERK1/2 durch MKP-1 ein spezifischer Prozeß ist, welcher in Mastzellen, aber nicht in anderen Zellpopulationen stattfindet.

Zusammenfassend zeigen die hier vorgestellten Ergebnisse, daß die über Glukokortikoide vermittelte Hemmung der Expression des FcεRIalpha-Genes und die verstärkte Expression von MKP-1 möglicherweise gemeinsam bei der Abschwächung des IgE-Rezeptor-Signaltransduktionsweges durch Glukokortikoide wirken könnten.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1 Glucocorticoids

Glucocorticoids are steroid hormones secreted by the adrenal cortex. They were identified in the 1930s (Reichstein and Shoppee, 1943) and described to be involved in the regulation of carbohydrate and protein metabolism and of stress response (Munck et al., 1984; Clark et al., 1992). Biosynthesis and release of glucocorticoids from the adrenal cortex is controlled by the adrenocorticotrophic hormone (ACTH), which is produced in the anterior pituitary gland. Release of ACTH depends, in turn, on the action of corticotropin releasing factor (CRF), and is regulated following a circadian rhythm and in response to stress stimuli.

Glucocorticoids, used at pharmacological doses, proved to be effective as therapeutic agents in the treatment of rheumatoid arthritis (Hench et al., 1949). The possibility to synthesise synthetic analogues of glucocorticoids rendered it possible to successfully use these compounds in the treatment of a wide range of allergic and inflammatory disorders such as asthma, allergic rhinitis, dermatitis and psoriasis. However, prolonged use of glucocorticoids is associated with a wide range of unwanted side-effects, including bone loss, dermal thinning, diabetes, psychotic disturbances and Cushing´s syndrome (Capewell et al., 1990; Abma et al., 2002; Dendukuri et al., 2002; Ismail et al., 2002; McLaughlin et al., 2002). A reduction of the adverse effects would allow a more effective use of these antiinflammatory compounds. To achieve this, a thorough understanding of their molecular mode of action is necessary.

1.2 Mode of action of glucocorticoids: the glucocorticoid receptor

Glucocorticoids reach their target cells via the blood. Due to their lipophilic character, they can freely diffuse through cellular membranes. Within the cell,

they bind to a cytoplasmic specific target, the glucocorticoid receptor (GR). The GR belongs to a large family of ligand-binding transcription factors and is structurally composed of three major domains: a DNA-binding domain (DBD) linked by a hinge region to a C-terminal hormone-binding domain (HBD) and a modulatory N-terminal domain (Wrange and Gustafsson, 1978; Carlstedt-Duke et al., 1987). In the cytoplasm, the GR is kept in an inactive state through interaction with a complex of chaperones and co-chaperones (reviewed in Pratt, 1993). Upon hormone binding, which occurs through interaction of the hormone with the HBD, the receptor is conformationally altered and the GR-chaperones complex is disrupted, allowing the receptor to translocate into the nucleus, where it can act as a modulator of gene expression (for a review on gene regulation by steroid hormones and on the steroid receptors, see Beato, 1989; Cato et al., 1992; Beato et al., 1995).

1.2.1 Positive regulation of gene expression by glucocorticoids

The hormone-liganded GR can homodimerise, and in this form recognise and bind with high affinity to specific sequence motifs (glucocorticoid response elements, GREs) in the promoter region of target genes. GREs consist of a palindrome (inverted repeat) of a pair of six base pairs spaced by a three base pairs spacer, corresponding to the structure 5´NAGAACANNNTGTTCTN 3´, where N is any nucleotide (Scheidereit et al., 1983). These recognition sites mediate hormone-dependent increase in the transcription rate of target genes. The process of transactivation by glucocorticoids was demonstrated using a model involving the hormone-dependent induction of expression of the mouse mammary tumour virus (MMTV). In a cultured cell line derived from mouse mammary tumour tissue, treatment with the synthetic glucocorticoid Dexamethasone stimulated the expression of integrated proviral copies of the MMTV, as early as 15 min after addition of hormone (Parks et al., 1974; Ringold et al., 1975). It could be shown that the MMTV promoter region contains a distinct GRE. GREs have since been found in other genes, including human metallothionein IIA gene, rat tyrosine aminotransferase gene, rat tryptophan oxygenase gene and rat acidic glycoprotein

gene (for a review, see Beato et al., 1989), indicating a common mechanism of positive regulation of gene expression by glucocorticoids.

1.2.2 Negative regulation of gene expression by glucocorticoids

In addition to positive regulation, the GR is also able to bring about negative regulation of gene expression. “Cross-talk” of the activated GR with positively acting transcription factors has been demonstrated to cause repression of transcriptional activity of target genes. This occurs through different mechanisms.

In some cases, negative modulation of gene expression by glucocorticoids is achieved when the GREs overlap binding sites of other trans-activating elements, so that the GR represses the action of other transcription activators by competing for DNA binding to their cognate binding sites. The transactivation function of the GR in these cases is likely to be less than that of the transcription factors binding to the overlapping site. An example of this mode of action is the glucocorticoid-mediated negative regulation of the expression of the human osteocalcin gene, where the GRE overlaps a TATA binding site, needed for the general activation of transcription (Stromstedt et al., 1991). Another model for repression of gene expression by glucocorticoid hormones involves sequence motifs called negative glucocorticoid response elements (nGREs). nGREs mediate enhanced promoter activity in the absence of glucocorticoids or GR, possibly through a factor that binds in the same region to promote transcription. The hormone-GR complex seems to abrogate this regulation by competing or by inactivating this factor. For instance, a region upstream of the bovine prolactin gene that confers repression by glucocorticoids through this mechanism has been characterised (Sakai et al., 1988). The binding of two factors, the transcription factor Pit-1/GHF-1 and a second protein, termed XTF, in this region was shown to be needed for increased basal expression of the prolactin gene but also for the GR to mediate transrepression (Subramaniam et al, 1997). However, the mechanism underlying this downregulation is yet to be unravelled.

The GR has also been shown to negatively regulates gene expression through protein-protein interaction, whereby the GR binds to trancription factors already

bound to DNA to inhibit their action. This negative regulation has been referred to as "tethering" and does not require binding of the GR itself to DNA or GR dimerisation. Examples of this type of negative regulation are the repression of function of nuclear factor-kappaB (NF-κB) (Scheinman et al., 1995; McKay and Cidlowski, 1998), and the repression of activity of activator protein-1 (AP-1) (Jonat et al., 1990; Yang-Yen et al., 1990; König et al., 1992; Heck et al., 1994). The mechanism of the interaction is still under investigation; involvement of intermediary proteins might be needed to mediate interaction between the GR and other transcription factors (Herrlich, 2001; Herrlich and Göttlicher, 2002). However, direct interaction does not seem to be the only mechanism through which glucocorticoids can repress these transcription factors' function. In the case of the GR-mediated inhibition of NF-κB, the GR is also thought to act in an indirect way, by increasing the synthesis of the NF-κB inhibitor (IκB) which traps activated NF-κB in inactive cytoplasmic complexes (Auphan et al., 1995). The need for increased IκB levels to repress NF-κB-regulated genes expression, however, has not been confirmed by other investigators (Heck et al, 1997; Stein and Yang, 1995). In the case of AP-1, competition between the GR and AP-1 for limiting amounts of the CREB-binding protein (CBP) and its homologue P300, cofactors mediating nuclear receptor-activated gene transcription, has been proposed to contribute to the glucocorticoid-mediated inhibition of AP-1 activity (Kamei et al., 1996). However, this model would not satisfactorily fit to several observations on GR/AP-1 cross-talk (Herrlich, 2001). It is very likely that different mechanisms co-exist.

Glucocorticoid-mediated negative regulation of gene expression is thought to be the molecular basis for the antiinflammatory properties of these hormones. This claim is based on the fact that the expression of several immunomodulatory genes, such as those encoding tumour necrosis factor (TNF)-α (Swantek et al., 1997), interleukin (IL)-1β (Lee et al., 1988), IL-2, IL-4 and interferon γ (Moynihan et al., 1998), IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) (Tobler et al., 1992), β interferon (Gessani et al., 1988), is negatively regulated by glucocorticoids. This repression is thought to occur through different mechanisms. In some of these cases (IL-1β, IL-8, IL-6, GM-CSF, β interferon; refer to above

mentioned literature) glucocorticoids have been shown to negatively regulate gene expression by decreasing the stability of the respective mRNAs. In addition, several pro-inflammatory cytokines and chemokines are functionally regulated by AP-1 and/or NF-κB (reviewed in Barnes and Adcock, 1998; Adcock et al., 2001) such as GM-CSF (Schreck and Baeuerle, 1990), TNF-α (Goldfeld et al., 1990), IL-6 (Libermann and Baltimore, 1990) and IL-2 (Kang et al., 1992), so that inhibition of AP-1 and NF-κB action by the GR has become a major mechanism for the anti-inflammatory and immunosuppressive action of glucocorticoids. Finally, besides the antagonism of the action of transcription factors the GR with transcription, cross-talk of the GR with signalling pathways has been proposed as yet another mechanism used by glucocorticoids to repress inflammatory processes.

1.2.3 Glucocorticoid-mediated interference with MAPK signalling pathways

Signalling pathways are important components in the intracellular regulatory network because they allow the cell to transduce extracellular cues to intracellular responses. An external signalling molecule (be it a hormone, neurotransmitter, cytokine, drug, etc.), often with the mediation of a specific receptor, can start a signal transduction cascade within the cell which ultimately elicits specific cellular responses. Typical cascades involve a hierarchical succession of phosphorylations and dephosphorylations of enzymes, as in the mitogen-activated protein kinase (MAPK) pathway. MAPK are a family of serine/threonine protein kinases and are involved in many cellular programs. Three major MAPKs cascades have been identified: the extracellular signal-regulated protein kinase (ERK1/2) pathway, the c-Jun N-terminal protein kinase (JNK) pathway and the p38 kinase pathway (reviewed in Robinson and Cobb, 1997). MAPKs are phosphorylated and activated by MAPK-kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs) (fig.1.2). The MAPKKK is in turn activated by interaction with small guanosine triphosphatases (GTPases) and/or other protein kinases connecting the MAPK cascade to a cell surface receptor or external stimulus.

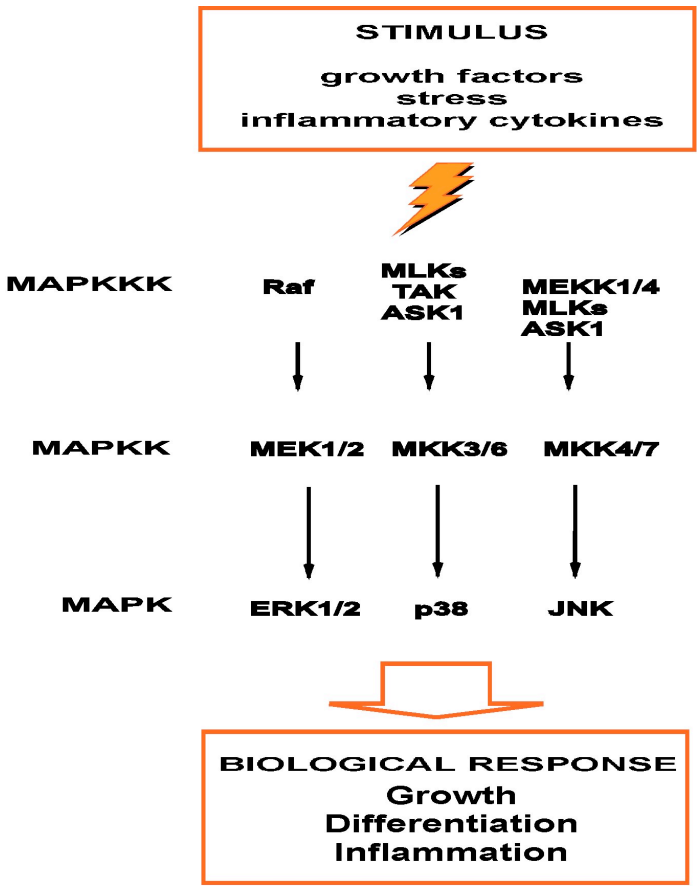

Figure 1.1 MAPK signalling cascades. Mitogen-activated protein kinases (MAPK) signalling cascades, which include the extracellular signal-regulated protein kinase (ERK1/2) pathway, the p38 kinase pathway and the c-Jun N-terminal protein kinase (JNK) pathway, transduce extracellular cues to intracellular responses. MAPKs are phosphorylated and activated by specific MAPK-kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs), in a hierarchical organisation.

MAPK pathways are involved in a wide variety of cellular functions, including the generation of proinflammatory responses. Secretion of inflammatory mediators (histamine, leukotriene C4, interleukin-4, and interleukin-13) by human basophils, for instance, is controlled by the p38 MAPK (Gibbs, 2002). The regulation of transcription of TNF-□ gene, a potent proinflammatory cytokine which plays a crucial role in early events of inflammation, requires the contribution of all three MAPK in T lymphocytes (Hoffmeyer et al., 1999) and in mast cells (Okabe et al., 2000; Azzolina et al., 2002). Release of IL-8 from cells

of the monocytic lineage was shown to require signalling along MAPK cascades (Bhattacharyya et al., 2002). The expression of the pro-inflammatory GM-CSF requires all three MAPK in bronchial epithelial cells (Cui et al., 2002). It is therefore to be expected that interfering with these signalling pathways would affect the inflammatory response of the cell.

Glucocorticoids were shown to inhibit the activity of all three MAP kinases. Inhibition of JNK activity by glucocorticoids has been shown and proposed as a mechanism for glucocorticoid-induced suppression of TNF-α production (Swantek et al., 1997). Glucocorticoid-mediated inhibition of activity of AP-1 may not only involve interaction of the GR with this transcription factor but in addition it would require inhibition of the JNK pathway. This would result in the suppression of phosphorylation of c-Jun, the major component of the transcription factor AP-1 (Caelles et al., 1997). Repression of the MAPK p38 pathway by glucocorticoids was as well observed and since this pathway controls the mRNA stability of cyclooxygenase-2 gene, it was suggested as a mean of negative regulation of gene expression by glucocorticoids (Lasa et al., 2001). In recent reports, repression of p38 MAP kinase activity by glucocorticoids was shown to occur through up-regulation of the activity of the MAP kinase phosphatase-1 (MKP-1) (Imasato et al., 2002; Lasa et al., 2002). Decreased activity of ERK1/2 upon glucocorticoid treatment was observed in a macrophage-like cell line where it was proposed as a mechanism to inhibit histamine production (Hirasawa et al., 2001). These observations, taken together, indicate that blockade of MAPK signalling pathways might play an important role in mediating the immunosuppressive and antiinflammatory action of glucocorticoids.

1.3 Mast cells as a model for studying glucocorticoid-mediated repression of signalling pathways

Mast cells constitute a suitable cellular model to study interference of glucocorticoids with signalling pathways in the context of glucocorticoid-mediated inhibition of allergic and inflammatory processes. Mast cells play a central role in eliciting allergic response and are also involved in host defence (reviewed in Nolte, 1996 and Broide, 2001). Their presence in most of vascularised peripheral tissues, especially at the interface with the external environment such as skin, digestive tract and respiratory system, enables them to readily respond to noxious stimuli. When mast cells are activated upon contact with allergen (antigen), distinct cellular responses are elicited. First, granule-stored mediators such as histamine, serotonin and neutral proteases, are released by exocytosis in the local extracellular environment (Schneider et al., 1992; Ishizaka et al, 1984; reviewed in Stevens and Austen, 1989). Second, the metabolism of arachidonic acid is activated, so that arachidonic acid itself and its derived products are released (Ishizaka et al, 1984; reviewed in Ishizaka and Ishizaka, 1984). Finally, synthesis of pro-inflammatory cytokines and chemokines is induced, such as IL-4, IL-13, IL-5, GM-CSF and TNF-α (Plaut et al., 1989; Wodnar-Filipowicz et el., 1989; Gordon and Galli, 1991; reviewed in Galli et el., 1991 and in Kobayashi et al., 2001).

The activation of mast cells is mediated through the receptor with high affinity for IgE (FcεRI), expressed on their surface. The FcεRI confers to mast cells the ability to bind antibodies. Whenever an antigen capable of cross-linking a specific antibody is present in the organism, it causes the aggregation of the receptor, resulting in expression of inflammatory functions (reviewed in Metzger, 1992). The FcεRI possesses a heterotetrameric structure composed of one IgE binding alpha (α) chain, one beta (β) chain and two gamma (γ) chains (Ra et al., 1989; reviewed in Ravetch and Kinet, 1991). Although the FcεRI lacks intrinsic tyrosine phosphorylation activity, the β and γ subunits possess definite cytoplasmatic immunoreceptor tyrosine based activation motifs (ITAMs; Cambier, 1995a; Cambier, 1995b), which are rapidly phosphorylated in response to aggregation

(Paolini et al., 1991) by the lck/yes-related novel (Lyn) Src-like tyrosine kinase (Quarto and Metzger, 1986; Eiseman and Bolen, 1992). Phosphorylation of the receptor ITAMs constitutes the starting point for the initiation of several signal transduction pathways (for a review see Turner and Kinet, 1999), among which the MAPK pathways (Ishizuka et al., 1996; Ishizuka et al., 1997) which ultimately contribute to eliciting mast cells biological responses (Hirasawa et al., 1995; Zhang et al.,1997; Song et al., 1999; Hirasawa et al., 2000) (Fig.1.2).

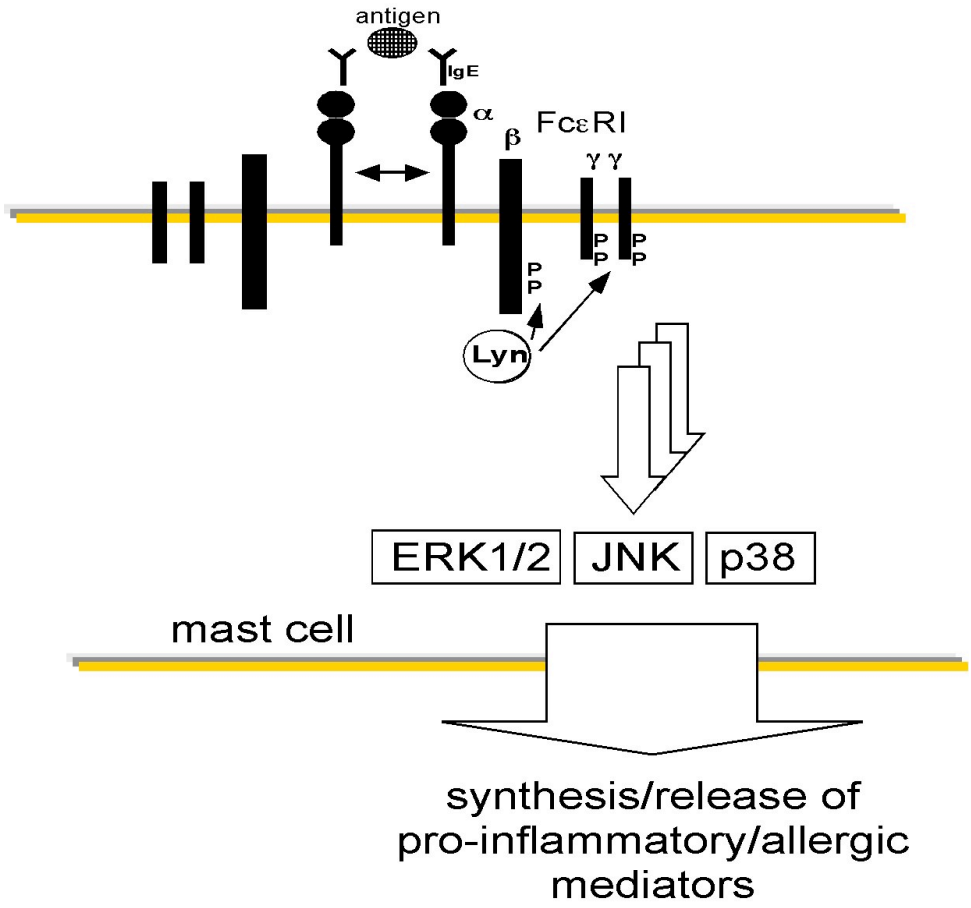

Figure 1.2 Aggregation of the FcεRI triggers a cascade of intracellular events leading to release and synthesis of pro-inflammatory mediators. Expression of the FcεRI on mast cells enables them to bind antibodies. Cross-linking of specific antibodies with antigen causes the aggregation of the receptor and phosphorylation of the β and γ subunits by the protein tyrosine kinase Lyn. Phosphorylation of the receptor constitutes the starting point for the initiation of several signal transduction pathways, among which are the JNK, ERK1/2, p38 MAPK pathways. These pathways in turn mediate synthesis and/or release of pro-inflammatory and allergic mediators.

Release of inflammatory mediators and synthesis of cytokines by mast cells are inhibited by glucocorticoids (Daeron et al., 1982; Rider et al., 1996; Eklund et al.,

1997; Sewell et al., 1998). This could occur through different mechanisms. As AP-1 and NF-κB control the activity of several proinflammatory cytokine genes, protein-protein interaction between GR and these specific transcription factors may play a role in the glucocorticoid-mediated repression of cytokine synthesis in mast cells. However, also interference of glucocorticoids with MAPK signalling pathways in mast cells might lead to inhibition of synthesis/release of pro-inflammatory/allergic mediators. Glucocorticoids action on MAPK pathways in mast cells was examined by several investigators. Glucocorticoids were shown to completely inhibit antigen-induced increase in JNK activity (Hirasawa et al., 1998), to suppress antigen-induced phosphorylation of the p38 MAP kinase (Hundley et al., 2001) and to inhibit the ERK1/2 pathway (Rider et al., 1996) in mast cells, but the mechanism(s) underlying these effects are still under investigation. With respect to the glucocorticoid-mediated inhibition of the ERK1/2 pathway, Cissel and Beaven (1999) have shown that it occurs at a step immediately before activation of the MAPKKK Raf-1 by disruption of the Raf-1/Heat shock protein (Hsp)90 complex, required for the activation of Raf-1 by the small GTPase Ras. A novel dual action of glucocorticoids has also been proposed for the inhibition of activity of ERK1/2 in mast cells. Glucocorticoids increase the expression of the MKP-1 gene at the promoter level. Furthermore, glucocorticoids are able to attenuate the proteasomal degradation of MKP-1 which takes place upon cell activation. Both induction of MKP-1 expression and inhibition of its degradation were shown to be necessary for glucocorticoid-mediated inhibition of ERK1/2 activation (Kassel et al., 2001). Because of the importance of the ERK1/2 pathway in mediating mast cells effector functions, it was suggested that blockade of this pathway by glucocorticoids might be potentially important for regulating mast cell activity in allergic reactions. It is therefore particularly interesting to try and analyse at which level(s) glucocorticoids are able to affect the ERK1/2 pathway.

AIM

The aim of this work is to characterise the different levels at which glucocorticoids inhibit IgE receptor signalling in mast cells. In particular, the effect of glucocorticoids at the level of the receptor with high affinity for IgE (FcεRI) is to be analysed. The FcεRI is essential in coupling contact with antigen to intracellular signal transduction events, such as activation of the ERK1/2 pathway. Furthermore, the effect of glucocorticoids at the level of the mitogen-activated protein kinase phosphatase (MKP)-1 is to be analysed, since this enzyme has been shown to function at the last step in one of the receptor-initiated signalling cascades, by dephosphorylating ERK1/2. Using mast cells as a model to investigate interference of glucocorticoids in signalling pathways, insights on the mechanisms by which glucocorticoids might exert their anti-inflammatory and anti-allergic action can be gained.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

All chemicals were of the highest quality, and were purchased from:

Agarose	Peqlab, Erlangen
Ammonium persulfate	Sigma, Deisenhofen
Aprotinin	Sigma, Deisenhofen
2-β Mercaptoethanol	Roth, Karlsruhe
Bromophenol blue	Roth, Karlsruhe
Bovine Serum Albumin	Sigma, Taufkirchen
Chloroform	Merck, Darmstadt
Dexamethasone	Sigma, Taufkirchen
Dimethyl sulfoxide	Fluka, Neu-Ulm
Dithiothreitol	Sigma, Taufkirchen
DMSO (Dimethyl sulfoxide)	Fluka, Neu-Ulm
EDTA	Merck, Darmstadt
Ethanol	Roth, Karlsruhe
Ethidium bromide	Sigma, Taufkirchen
Fish sperm-DNA	Sigma, Taufkirchen
Formamide	Merck, Darmstadt
G418 (Geneticin-sulfate)	GIBCO, Eggenstein
Glycerol	Merck, Darmstadt
HEPES	Roth, Karlsruhe
Isopropanol	Merck, Darmstadt
Methanol	Roth, Karlsruhe
Nonidet P-40	Boehringer, Mannheim
PMSF	Sigma, Taufkirchen
Proteinase K	Roche, Mannheim
Rotiphorese® Gel30: Acrylamide/bis-acrylamide (30% / 0.8%)	Roth, Karlsruhe
Sodium lauryl sulfate	Roth, Karlsruhe
Skimmed milk powder	Saliter, Obergünzburg
TEMED	Sigma, Taufkirchen
TPA	Sigma, Taufkirchen
Tris	Roth, Karlsruhe
Tris Hydrochloride	Roth, Karlsruhe

Triton-X100	BioRad, Müchen
Tween-20	Roth, Karlsruhe

All other chemicals were, unless otherwise stated, purchased from *Carl Roth GmbH+Co (Karlsruhe, Germany), Merck AG (Darmstadt, Germany), Sigma Aldrich (Taufkirchen, Germany), Roche Diagnostics (Munich, Germany).* All radiochemicals were supplied by *Amersham Pharmacia Biotech (Freiburg, Germany).* All antibiotics were obtained from *Roche Diagnostics (Munich, Germany).*

2.1.2 Oligonucleotides

All the oligonucleotides were made by *MWG Biotech GmbH (Germany)* and were all of an high-pressure liquid chromatography (HPLC)-purified grade.

Oligonucleotides for cloning into pCR 2.1 vector

Specific forward (for) and reverse (rev) oligonucleotides were used to amplify portions of genes of interest by PCR, using RBL-2H3 rat mast cells cDNA as a template, in order to clone the PCR products in a pCR 2.1 vector (TOPO cloning kit, *Invitrogen, The Nederlands)* as described in paragraph 2.1.13. In brackets, the names of the genes and the length of the PCR products are indicated.

ERK 3 (extracellular signal-regulated kinase-3, 623 bp)
for 5´CTGCTGTAGACAATGACTGTGAC3´
rev 5´CAGCTGCATCTGTTCAAGTTCATG3´;

Fc RI (receptor with high affinity for IgE, alpha chain, 687 bp)
for 5´CGTGATTAGATACTTACAGGAG3´
rev 5´CTCTTAAGCGATGACATTAGTTC3´;

Fc RI (receptor with high affinity for IgE, beta chain, 478 bp)
for 5´CTGACATTGAACTCTTGGAAGCG3´
rev 5´CCATAGTTAACTGTGGTGAGTG3´;

Fc RI (receptor with high affinity for IgE, gamma chain, 600 bp)
for 5´GACCCACAATCCTGGAAACTCTTCTACCCAGTG3´
rev 5´GGAGCCTTGTACTTTCCGACTGACAGGGATTTC3´;

FDPS (farnesyl diphosphate synthase, 659 bp)
for 5´CTGGAGTACAACACTGTAGGAGGCAAG3´
rev 5´CTGCATTTGTTGTCCTGGATGTCAGGC3´;

HePTP (haematopoietic protein tyrosine phosphatase, 707 bp)
for 5´GCTCCAGCCAAGAAGCATGTG3´
rev 5´CCGGCTGATTCTGGCGTCTG3´;

HOP (Hsp70/Hsp90 organiser protein, 340 bp)
for 5´CTATGCCAAGAAAGGAGACTACCAGAAG3´
rev 5´CTGTAGTTGCTCTATGAGTTCCCTGTAG3´;

Hsp70 (heat shock protein 70, 387 bp)
for 5´CTGTGGAACAATGCTATACCCTCTAC3´
rev 5´GATTTGCTGCTCTTGGATGTCACTC3´;

Hsp 90 (heat shock protein 90, 436 bp)
for 5´CTCATCATCACTGACTTCCTTGTCACGTTC3´
rev 5´TCACTATTGTGGATACTGGCATGCACATTAATC3´;

IL-18 (interleukin-18, 248 bp)
for 5´CTGTGTTCGAGGACATGCCTGATATC3´
rev 5´GTTGTGTCCTGGCACACGTTTCTG3´;

MCP-3 (mast cell protease-3, 741 bp)
for 5´CATGGCCCTTCTGAAGATCGTC3´
rev 5´GACAGGGTGAGTCAGGCTTTG3´;

p59 (immunophilin p59, 287 bp)
for 5´CAGCCACTTCTGTCTTGGCCTTATGTTC3´
rev 5´CTCAGCTGCCATCGAAAGCTGTAACCAAG3´;

ppRLX (preprorelaxin, 710 bp)
for 5´CGGAATTCTGAACCGCCCAGGAGCACCGCCCA3´
rev 5´GCTCTAGATTATTAACAGAACTACAACAATGCA3´;

RCX (rat proteasome subunit X, 319 bp)
for 5´CATTGCTTCCCAGACAGTGAAGAAAGTG3´
rev 5´CTCCTCCACTTGTAGGTCATAGGAGTAG3´;

R-delta (proteasome delta chain, 455 bp)
for 5´GTGGTTCTAGGAGCAGACTCCAGAACAAC3´
rev 5´GCCAAAGCAAGAGCATTGGCAGTGAATTG3´;

RN3 (rat proteasome beta-type subunit N3, 352 bp)
for 5´GTTTCCGCATTATCTCTCGTATTATGCGAGTC3´
rev 5´CAAGTATGCACCATAACCAGTGGCTAGTGAAG3´;

rVH6 (*alias* MKP-3/Pyst1) (dual specificity protein phosphatase-3, 749 bp)
for 5´GATAGATACGCTAGACCCGTG3´
rev 5´GCATTCTCAAACAGATTGGGCAG3´;

R-zeta (proteasome zeta chain, 483 bp)
for 5´CATAAGCTTGGTTCTACGGCCATTGGCACT3´
rev 5´CAGAGTCGTAGACTTGTGGTAAACTTCCTGC3´;

SHP-1 (Src homology 2-containing protein tyrosine phosphatase-1, 637 bp)
for 5´CTCAGCCAACCTGGTGATTTTGTG3´
rev 5´GTA GTCATGACGATGACACGAGTG3´.

Oligonucleotides for genotyping

Oligonucleotides used for genotyping to discriminate between wt and MKP-1(-/-) mice (the method is described in paragraph 2.2.1.18):

Erp7IIfw 5´CAGGTACTGTGTGTCGGTGGTGCTAATG 3´;

Erp7IIrev 5´CTATATCCTCCTGGCACAATCCTCCTAG 3´;

HHneo 5´ AAATGTGTCAGTTTCATAGCCTGAAGAACG 3´.

2.1.3 Plasmids and constructs

-1716MKP-1 luc was generated as follows. The plasmid pUC18erp7 (a gift from Dr. R. Bravo, containing >7 kb of MKP-1 promoter sequence) was linearised by *BsiEI* restriction digest. The *BsiEI* site was blunted by T4 DNA polymerase reaction. Digestion with *BamHI* followed, to generate a 2.7 kb fragment. This fragment was then inserted into the *BamHI-EcoRI* site of the pCR2.1 TOPO TA cloning vector *(Invitrogen, Groningen, The Nederlands)*. A fragment including the nt –1716/+88 region (where 0 is the transcription initiation site) of MKP-1 gene was recovered by digestion with *Asp718-XhoI* and cloned into the *Asp718-XhoI* site of pGl3 basic vector *(Promega, Mannheim, Germany)* such that the MKP-1 promoter controls the expression of the luciferase gene;

-116MKP-1luc was derived from the –1716MKP-1luc. By restriction digest with *Pml I*, a fragment of about 1600 bp in length was excised; the

remaining linearised vector was rendered blunt end by T4 DNA polymerase reaction and subsequetly re-circularised by T4 ligase reaction, so that it contains only –116/+88 nt of the MKP-1 promoter in front of the luciferase gene;

A458T (glucocorticoid receptor dimerisation defective mutant) has been described previously (Heck et al., 1994);

AI096961 contains a 1657 bp fragment of the human MKP-1 cDNA (GenBank Acc. AI096961; Clone Id. IMAGE:1702739 (3')) cloned into the *Not I* and *Eco RI* sites of a pT7T3D-Pac vector with a modified polylinker *(Pharmacia).* Kindly provided by Schering AG, Berlin. The MKP-1 fragment was used as a probe in Northern Blot hybridisation;

actinluc belonged to the plasmid collection of lab.114, ITG;

FcRIluc and M1-9 expression vectors are a kind gift of Dr. Chiharu Nishiyama and are described in Nishiyama et al., 1999 and Nishiyama et al., 2002. Briefly, pGV-B- HN1.3/KN2.4 were constructed by cloning the nt –1256/+29 and nt –2437/+29 regions of the FcRI -chain promoter into the pGV-B2 vector *(Promega, Madison, WI).* From these constructs, other plasmids containing a variety of 5´ truncations of the FcRI -chain promoter region connected upstream of the luciferase structural gene were constructed using several restriction endonucleases. The pGV-B2- NNO.6 (M1-9), are mutants derived from the -605FcRIluc costruct, in which different replacements of 3-6 nucleotides in the –605/-88 region were obtained by site-directed mutagenesis;

GR wt (glucocorticoid receptor wild type expression vector) has been described previously (Heck et al., 1994);

hGR(D4X) (glucocorticoid receptor dimerisation defective mutant) has been described previously (Heck et al., 1994);

pCR 2.1 TA vector (TOPO cloning kit) was purchased from *Invitrogen, (Munich, Germany);*

pGAPDH belonged to plasmid collection of lab.114, ITG. A fragment of 900 bp was excised by *PstI* restriction digestion to be used a probe for Northern Blot hybridisation;

pGl3 basic vector was obtained from *Promega (Mannheim, Germany);*

pGl3MMTV encodes the firefly luciferase gene under the control of the mouse mammary tumour virus (MMTV) long terminal repeat cloned as a *BamHI/BglII* fragment from the plasmid pHCwtCAT (Kaspar et al., 1993);

pGl3 tkluc contains the –109/+52 region of the thymidine-kinase promoter in the pGl3 basic vector *(Promega, Mannheim, Germany);*

pTOPO TA plasmids were generated in the pCR2.1 vector from the TOPO TA cloning kit *(Invitrogen, Munich, Germany).* A PCR product comprehending part of the coding region of the gene of interest was generated using the appropriate forward and reverse oligonucleotides and cloned in the pCR2.1 TA vector following the manufacturer's instructions. The identity of the inserts was ascertained by sequencing. When necessary, the inserts were excised by *EcoRI* restriction digestion and used as probes for Northern Blot analysis;

Renillatkluc was obtained from *Promega (Mannheim, Germany).*

2.1.4 Antibodies

Rabbit polyclonal **anti-Lyn** 56kDa; rabbit polyclonal **anti-phosphoLyn** (Tyr 507) 53/56 kDa isoforms; rabbit polyclonal **anti-phospho p44/42 MAP kinase** (Thr 202/Tyr 204) were purchased from *Cell Signalling Technology (Frankfurt am Main, Germany);*

R-Phycoerythrin R-(PE) conjugated rat **anti-mouse CD117 (c-kit)**; Fluorescein

isothiocyanate (FITC)-conjugated rat **anti-mouse IgE** monoclonal antibody: purified mouse **IgG_{2b} monoclonal immunoglubulin isotype** standard were purchased from *Pharmingen (BD) (Heidelberg, Germany);*

Rabbit polyclonal **anti-MKP-1** (M-18) and mouse monoclonal **anti-ERK 2** (CD-2) were purchased from *Santa Cruz Biotechnology Inc. (California);*

Mouse monoclonal IgG_{2b} **anti-phosphotyrosine** clone 4G10 and **anti-Ras** (clone RAS10, part of the Ras activation kit) were purchased from *Upstate Biotecnology (Biomol), (Hamburg, Germany);*

The rabbit polyclonal **anti-Hsp90** antibody was a gift by Dr. Etienne Baulieu;

Monoclonal **anti-dinitrophenol (DNP) IgE** clone SPE-7, for sensitisation of mast cells, were purchased from *Sigma Aldrich Chemie, (Taufkirchen, Germany).*

All secondary antibodies HRP conjugated were purchased from *DAKO Diagnostika GmbH, (Hamburg, Germany).*

2.1.5 Enzymes

Unless otherwise indicated, all restriction endonucleases and other enzymes were purchased from *New England Biolabs (Frankfurt am Main, Germany); Gibco (Karlsruhe, Germany); Takara (Taufkirchen, Germany); Roche (Mannheim, Germany).*

2.1.6 Cytokines

Interleukin-3 (IL-3) and Stem Cell Factor (SCF) were purchased by *Peprotech, London, UK.*

2.1.7 Bacteria

E.coli strain DH5: supE44lacU169(80lacZM15)hsdR17recA1 endA1 gyrA96 thi-1 relA1, belonged to the cell bank of ITG.

TOP10F´:F$^-$mcrA Δ(mrr-hsdRMS-mcrBC) ΔlacX74(Δ80lacZΔM15) recA1 endA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (Str^R) nupG, were part of the TOPO TA cloning kit from *Invitrogen (Groningen, The Nederlands)*.

2.1.8 Cell lines and media

Tissue culture media for mammalian cells were, unless otherwise stated, purchased from *Life Technologies GmbH (Karlsruhe, Germany)*. All media were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin *(Life Technologies, Karlsruhe, Germany)*. FCS of South American origin was purchased by *BioWhittaker (Taufkirchen, Germany)*. Trypsin was purchased from *Difco Laboratories (Detroit, USA)* and was diluted to 0.25% in 15 mM sodium citrate, 134 mM potassium chloride prior to use.

The 2H3 subline of rat basophilic leukaemia cells (RBL-2H3) was cultured in DMEM 15% FCS and belonged to the cell bank of ITG.

COS-7 monkey kidney cell line were cultured in DMEM, 10% FCS and belonged to the cell bank of ITG;

BMMCs were cultured in IMDM or RPMI 20% FCS; 1 mM Sodium Pyruvate; 2mM Glutamine supplemented with 20U/ml IL-3.

Splenocytes and Thymocytes were cultured in RPMI 10% FCS.

2.1.9 Research animals

All animals were bred in the ITG animal facility under full specific pathogen free (SPF) conditions and used for experiments when they had reached approximately 5 weeks of age.

C57Bl/6 mice belonged to the ITG animal facility.

Erp7/(MKP-1)(-/-) mice embryos were provided by *Jackson Laboratories (USA)*. Mice were generated by embryo transfer in the ITG animal facility.

2.1.10 Other materials

Protein A Agarose (packed beads); Ras activation assay kit were purchased from *Upstate Biotecnology (Biomol), (Hamburg, Germany).*

2.2 METHODS

The majority of protocols and recipes for commonly used buffers and routine procedures used in this work were taken from Molecular Cloning (Maniatis et al., 1989) and Current Protocols in Molecular Biology (Ausubel et al., 1989) unless otherwise stated.

2.2.1 NUCLEIC ACIDS METHODS

2.2.1.1 Phenol/Chloroform extraction of nucleic acids

To remove unwanted protein contaminants from nucleic acids solutions an equal volume of Tris-buffered phenol and chloroform at a ratio of 1:1 *(v/v)* was added and the mixture was vortexed. The two phases were separated by centrifugation at 12000 rpm for 10 min. The upper aqueous nucleic acid-containing phase was transferred to a fresh reaction tube and subjected to a further round of extraction with phenol/chloroform *(*1:1, *v/v).*

2.2.1.2 Ethanol precipitation of nucleic acids

In order to recover nucleic acids from solution, the salt concentration was brought to 200 mM with 3 M Na-acetate (pH 4.8-5.0) and 2.5 volumes of cold ethanol was added. After 30 min to overnight incubation at -20°C or 30 min at -80°C, the precipitate was centrifuged at 12000 rpm for 15 min. The pellet was washed with 70% ethanol, centrifuged for another 3 min to remove the salt and was then dried.

DNA was re-suspended in either H_2O or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.2.1.3 Determination of nucleic acid concentration

The concentration of nucleic acids was determined by measuring their optical density (OD) at 260 and 280 nm. An OD_{260} = 1 is equivalent to 50 μg/ml double stranded DNA or 40 μg/ml RNA or 20 μg/ml single-stranded oligonucleotide. A ratio of OD_{260}/OD_{280} ranging from 1.8 to 2.0 indicates an acceptable purity of the nucleic acid.

2.2.1.4 Large scale plasmid preparation

Plasmid DNA was prepared on a large scale using the Qiagen Plasmid Maxi Kit *(Qiagen)* following the manufacturer's instructions. Briefly, a volume of 200-250 ml of LB medium (10 g Tryptone; 5 g Yeast extract and 10 g NaCl for 1 l, autoclaved before use) supplemented with the appropriate antibiotic was inoculated with a single bacterial colony and incubated with shaking (220 rpm) at 37°C overnight until the bacteria had reached a stationary growth phase. The bacteria were pelleted by centrifugation in a fixed angle rotor at 6000 x g for 10 min and the pellet resuspended in 10 ml solution P1 (10 mM EDTA, 50 mM Tris-HCl pH 8.0 and 400 □g/ml RNAse A). Following 5-10 min incubation at RT, the cells were lysed by addition of 10 ml solution P2 (200 mM NaOH and 1% SDS). Once the solution had taken an opaque appearance, the mixture was neutralised with 10 ml of solution P3 (3 M Na acetate pH 4.8), the entire content inverted gently to aid mixing of the solutions. After additional 10-20 min on ice, the cell wall fragments and the bacterial chromosomal DNA were sedimented by centrifugation at 9000 x g for 20 min at 4°C in a fixed angle rotor. The retained supernatant was then added directly to a pre-equilibrated Qiagen-tip 500 column and the plasmid DNA was recovered in the provided elution buffer according to the manufacturer's instructions. The purified DNA was then precipitated using 0.8-1 volumes of isopropanol, washed twice in 70% ethanol before being re-

suspended to a final concentration of 1-3 mg/ml in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C.

2.2.1.5 Small scale plasmid preparation

Single bacterial colonies were picked, inoculated in 5 ml of LB medium (10 g Tryptone; 5 g Yeast extract and 10 g NaCl for 1 l, autoclaved before use) containing the appropriate antibiotic and grown overnight under constant shaking at 37°C. 1.5 ml of these cultures were transferred to a fresh vial and the bacteria were collected as a pellet by centrifugation at 6000 rpm for 10 min at RT. For isolation of plasmid DNA, a modified "large scale plasmid preparation" protocol was used. Briefly, the bacterial pellet was resuspended in 150 μl of solution P1 containing RNase A, from the MaxiPrep Kit *(Qiagen)* and let 5 min at RT. Then 300 μl of solution P2 were added and the mixture was left 5 min on ice. After addition of 225 μl of solution P3 followed by vortexing, the lysate was again left for 5 min on ice. After centrifugation at 12000 rpm for 10 min at 4°C, 400 μl of the supernatant were collected and transferred to a fresh tube containing 1 ml of ice-cold ethanol. Incubation for 30 min at -80°C would allow the nucleic acid to precipitate. The plasmid DNA pellet was collect by centrifugation at 12000 rpm for 10 min at 4°C and was washed once with 70% ethanol. The resulting pellet was air-dried and resuspended in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.2.1.6 Isolation/Purification of genomic DNA from eukaryotic cells or tissues

In order to isolate DNA, fresh tissue (approximately 1 g) was lysed by overnight incubation in 0.6 ml of lysis buffer (10 mM Tris pH 8.0, 10 mM EDTA, 150 mM NaCl, 0.2% SDS, 500 □g/mL Proteinase K) at 55°C with shaking. Tissue culture cells instead were grown in a 6-well plate until sub-confluency, washed once in PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and lysed by incubation with 0.9 ml of lysis buffer at 37°C overnight. Lysates were then transferred into a fresh vial and after addition of 1/3 total volume of saturated

NaCl (approximately 6-7 M NaCl) the tubes were shaken for another 15 min. The cell debris were removed by mild centrifugation at 5000 rpm for 10 min. The DNA-containing supernatant was transferred into a fresh vial and DNA was precipitated by addition of 2 volumes of ethanol and centrifugation at 14000 rpm for 10 min at room temperature. The DNA pellet was washed with 70% ethanol and re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C until used.

2.2.1.7 Restriction endonuclease digestion of DNA

Typically 2-3 units of a restriction enzyme were used per μg DNA. DNA was digested at a concentration of 1 μg/μl in the buffer recommended by the supplier. The reaction was carried out for 1 hour to overnight at 37°C (unless otherwise recommended by the supplier). The quality of the digest was controlled by gel electrophoresis.

2.2.1.8 Size separation of nucleic acid by agarose gel electrophoresis

Given amounts of agarose *(Peqlab, Erlangen, Germany)* final concentration between 0.7 and 2%) were dissolved in TAE buffer (0.04 M Tris pH 7.2, 0.02 M Sodium acetate, 1 mM EDTA). Ethidium bromide was added at a concentration of 0.3 μg/ml. The gel was set in horizontal electrophoresis chambers and was run at 35-45 mA (50-100 V) at RT for the required time. Samples were loaded onto the gel in loading buffer (0.5 M EDTA, 50% glycerol, 0.01 g bromophenol blue). DNA was visualised by transillumination with 320 nm UV light and photographs were taken by using an Eagle Eye *(Stratagene)* photocamera system.

2.2.1.9 Isolation/purification of DNA from agarose gels

To isolate an appropriate DNA fragment from an agarose gel, Easy Pure DNA Purification Kit *(Biozym Diagnostik GmbH, Oldendorf, Germany)* was used as recommended by the manufacturer. Briefly, the DNA band of choice was cut out from the gel under long wave UV light with the aid of a scalpel. The gel piece

containing DNA was melted at 55°C in the salt buffer (provided) and the DNA-binding resin (provided) was added. After two subsequent washing steps the resin with bound DNA was air-dried and the DNA eluted by addition of bi-distilled H_2O. The presence of the DNA fragment in solution was confirmed by agarose gel electrophoresis.

2.2.1.10 DNA ligation

All ligation reactions, except for the TOPO cloning (see 2.2.1.13) were performed using T4 ligase and appropriate buffer supplied by the manufacturer *(Life Technologies, Karlsruhe, Germany)* and incubated either overnight at 16°C or 2-3 h at RT. When a fragment was intended to be inserted in a vector, usually the reaction mixture would contain a 10:1 ratio insert:vector. The reaction was stopped by inactivation of the enzyme for 10 min at 70°C.

2.2.1.11 Sub-cloning

Typically, the cloned fragment of DNA was released from the vector using appropriate restriction endonucleases, resolved on an agarose gel by electrophoresis, isolated from the gel and subsequently cloned into the new vector using compatible sites or through blunt end ligation.

2.2.1.12 Polymerase Chain Reaction (PCR)

All PCR reactions were carried out in a Thermal Cycler machine *(Perkin Elmer 9600/2400)*. Usually the PCR reaction in a total volume 20 µl contained 10 ng of plasmid or 100 ng of genomic template DNA, 200 µM dNTP, 10 pmol of primers, 0.25 U to 1 U of Taq Polymerase and 1× supplier's buffer. Addition of 5-10% Glycerol was sometimes necessary to optimise the reaction. Depending on the application specific cycling parameters were used for each individual PCR reaction. Usually 1 cycle (94°C, 1 min) followed by 30 cycles (94°C, 1 min; 45°C, 1 min; 72°C, 1 min) were carried out. The annealing temperature was

changed according to the different primers' T_m. In case of TOPO TA cloning in pCR2.1 vector, an additional cycle of 72°C for 10 minutes was performed to ensure the generation of A-overhangs by Taq Polymerase in DNA templates for cloning.

2.2.1.13 Cloning into pCR2.1-TOPO TA vector

The TOPO (TA) cloning Kit *(Invitrogen, Groningen, The Nederlands)* was used according to the manufacturer's instructions. The plasmid vector (pCR2.1) is supplied linearised with single 3´-thymidine(T) overhangs for TA cloning and with a covalently bound topoisomerase I. Because *Taq* polymerase has a non-template dependent terminal transferase activity, it adds single deoxyadenosine (A) to the 3´end of PCR products. This allows PCR inserts to efficiently ligate with the vector´s overhanging 3´-deoxythymidine residues. The Topoisomerase I from *Vaccinia* virus functions like both a restriction enzyme and a ligase. It binds to duplex DNA at specific sites and forms a covalent bond with the phosphate group of the 3´thymidine. It cleaves one DNA strand, enabling the DNA to unwind. The enzyme then religates the ends of the cleaved strand and releases itself from the DNA. The reaction mixture consists of 0.5 to 4 µl of fresh PCR product and 1 µl of pCR2.1 TOPO TA vector; sterile water is added to a total volume of 6 µl. The reaction mixture is mixed gently and incubated for 5 min at RT. The ligation is thus complete and 2 µl are used for transformation into chemically competent *E.Coli* TOP10F´.

2.2.1.14 Transformation of chemically competent bacteria

This type of transformation was used for propagation of different plasmids. The DNA sample, usually 0.01-1 µg, dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or water, was added to 100 µl of the ice-thawed chemically competent cells. After brief vortexing and incubation on ice for 5-30 min the cells were heat-shocked for 30-60 s at 42°C and immediately transferred on ice. The transformed cells were finally mixed with 250 µl room temperature SOC medium

(2% tryptone; 0.5% yeast extract; 10mM NaCl; 2.5mM KCl; 10mM $MgCl_2$; 10mM $MgSO_4$; 20mM glucose), incubated at 37°C with shaking (200 rpm) for 1 hour, spread onto pre-warmed selective agar plates (typically, supplemented with ampicillin (100 μg/ml) and grown overnight at 37°C.

2.2.1.15 Colony PCR

For rapid screening of plasmids carried in bacteria, single transformed bacterial colonies were grown overnight and lysed in an equal volume (typically 25 μl) of distilled water. The lysed bacteria were then boiled for 5 min and 5 µl of the lysate was used as a template for PCR with appropriate primer pairs and cycling conditions.

2.2.1.16 Automated (fluorescence) DNA sequencing method

The VISTRA Thermo Sequenase Pre-mixed Cycle Sequencing *Kit (Amersham Pharmacia Biotech, Braunschweig, Germany)* was used to routinely sequence cloned DNA on both strands. Briefly, 1 μg DNA was mixed with 4 pmol of an appropriate Texas-red-labelled primer (usually T7 Forward universal primer). From this mixture 6 μl was aliquoted into each termination vial ('G', 'A', 'T', 'C') containing 2 μl of the corresponding ddNTP mix (i.e. ddGTP, ddATP, ddTTP and ddCTP) containing all the necessary reaction components such as polymerase and dNTPs plus the actual ddNTPs. The reaction was cycled in a PCR cycler using the following parameters: 94°C, 1 min, 1 cycle; 94°C, 30 s, 50°C, 30 s and 72°C, 30 s for a total of 25 cycles. Upon completion, 3 μl loading buffer was added to each reaction mix and the volume of the reaction was reduced to 3 μl by drying in a vacuum centrifuge. The samples were loaded on a RapidGel-XL-6% gel *(Amersham Pharmacia Biotech, Braunschweig, Germany)* and run in TBE (90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA, pH 8.3) buffer in a DNA Sequencer 725 *(Molecular Dynamics & Amersham)* for 12 hours. The sequencing data were analysed using Molecular Dynamics software.

2.2.1.17 Manual (radioactive) DNA sequencing method

The Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit *(Amersham Pharmacia Biotech, Braunschweig, Germany)* was used for the sequencing of DNA templates. The label is incorporated into the DNA reaction products by the use of four (^{33}P)-ddNTP terminators. By use of an engineered Thermo Sequenase DNA polymerase, efficient incorporation in cycling sequencing protocols is allowed. Sequencing of desired templates was carried out according to the manufacturer's guidelines. Approximately 1 μg of plasmid DNA was taken together with 3 pmol of an appropriate sequencing primer with 8 U of the Thermo Sequenase polymerase in a total volume of 20 μl. From this mixture 4.5 μl was aliquoted into each 'termination' PCR vial ('G', 'A', 'T', 'C') containing a mixture of 2 μl dGTP and 0.5 μl (^{33}P)-ddNTP and cycled in a PCR machine using the following parameters: 95°C, 30 s, 55°C, 30 s and 72°C, 1 min for a total of 40 cycles. The reactions were stopped by the addition of stop solution (provided). After a denaturation step (*i.e.* incubation at 70°C for 5 min), 3 μl samples were loaded in each lane and resolved over a 6% polyacrylamide 6 M urea/TBE (90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA, pH 8.3) gel. Once the run had reached the desired length, the gel was removed, dried on Whatmann 3MM paper at 80°C for 2 hours on a vacuum gel dryer before autoradiography. Films were developed after 18-36 hours exposure.

2.2.1.18 Genotyping of MKP-1 (-/-) vs wt mice

Genomic DNA was obtained from tail biopsies and used as a template in PCR reactions. Specific oligonucleotides were designed in order to amplify by PCR distinct DNA fragments that would allow to distinguish between different genotypes. The oligonucleotides erp7IIfw (forward) and erp7IIrev (reverse) were chosen so that they anneal within the genomic sequence of the MKP-1/*erp7* gene, outside the left and right border, respectively, of the *neo cassette* that was originally inserted in the second exon of the MKP-1 gene to disrupt it (Dorfman et al., 1996); the primer HHneo, instead, anneals within the *neo cassette* sequence (see Fig.2.1). Two separate PCR reactions were set for each template DNA. In the

first PCR reaction, erp7IIfw and erp7IIrev were used as primers; in this case, a PCR product of 288 bp would be obtained only if the template DNA contained a wt allele. In the second PCR reaction, erp7IIrev and HHneo were used as primers; in this case, a PCR product of about 230 bp would be obtained only if the template contained the mutated allele.

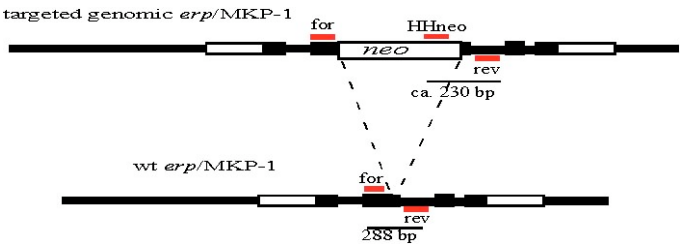

Figure 2.1 Schematic representation of the oligonucleotides designed for genotyping. The oligonucleotides erp7IIforward (fw) and erp7IIreverse (rev) lie outside of the insertion site for the *neo cassette*, on the left and right border, respectively, while the oligo HHneo lies within the *neo cassette*, originally inserted in the II exon of the MKP-1 gene to impede gene expression in the MKP-1(-/-) mice. Using in a PCR amplification reaction the couple of primers erp7IIforward (fw) and erp7IIreverse (rev), a 288 bp PCR product will be obtained if the genomic DNA template contains a wt allele. Conversely, using in a PCR amplification reaction the couple of primers erp7IIreverse (rev) and HHneo, a 230 bp (ca.) PCR product will be obtained if the genomic DNA template contains a mutated allele.

2.2.1.19 Isolation of total RNA from tissue or cultured cells

Cultured cells were grown to sub-confluency. After removing the medium, cells were lysed by addition of 1 ml (per 3,5 cm dish) of the TRIFAST Reagent *(Peqlab, Erlangen, Germany)*. The cell lysate was homogenised by passing it several times through the pipette tip. Upon completion of homogenisation the lysates were incubated for 5-10 min at room temperature in order to allow dissociation of nucleoproteins. After addition of chloroform (0.2 ml per 1 ml of TRIFAST reagent used) and vigorous shaking by hand the samples were allowed to stand at room temperature for another 5-10 min and centrifuged at 12000 rpm for 15 min at RT. Centrifugation resulted in phase separation. The top aqueous RNA-containing layer was carefully decanted and transferred to a fresh tube. In order to precipitate the RNA, an equal volume of isopropanol was added,

followed by centrifugation for 15 min at 12000 rpm at 4°C. The RNA pellet was washed twice in 70% ethanol before being air-dried. Finally, RNA was re-suspended in bi-distilled water at a final concentration of 1 mg/ml and stored at -80°C.

2.2.1.20 Separation of RNA by agarose gel electrophoresis

To prepare the gel, 1 g of Pure Agarose *(Peqlab, Erlangen, Germany)* was re-suspended in 100 ml of MOPS buffer (0.02 M 4-morpholinepropanesulfonic acid, 5 mM Na-acetate, 1 mM Na_2EDTA, pH 7.0) and autoclaved. Just before pouring the gel the agarose/MOPS mixture was melted in a microwave. After brief cooling, 3 ml of 37% formaldehyde were added. Gels were electrophoresed in a RNAse-free horizontal chamber at 90 V for 2.5 h in 1× MOPS buffer. Prior to loading RNA samples were denatured for 15 minutes at 55°C in formamide loading buffer (1× MOPS, 22.6% formaldehyde, 64.5% formamide, 3 µg/ml Ethidium bromide, 0.01 g bromophenol-blue) and loaded onto the gel. The integrity of the mRNA was visualised by illumination with UV light and photographs were taken by using an Eagle Eye *(Stratagene)* photocamera system.

2.2.1.21 Northern Blot

The RNA was blotted from the gel by overnight capillary transfer to a Hybond-N+ nylon membrane *(Amersham, Braunschweig, Germany)* using 10X SSC buffer (derived from a stock solution of 20X SSC, NaCl 3 M, Na_3-citrate 0.3 M). The dehydrated gel was then discarded and the RNA was covalently cross-linked to the membrane using a UV Stratalinker, type 2400 (*Stratagene, La Jolla, USA*). The filters were stored in sealed bags at RT.

2.2.1.22 Radioactive labelling of DNA/cDNA probes

For the radioactive labelling of nucleic acids, the Rediprime Kit (*Amersham Pharmacia Biotech, Braunschweig, Germany)* random primer labelling system

was used. This system makes use of random sequence hexanucleotides to prime DNA synthesis at numerous sites along its length. The primer-template complex is a substrate for the Klenow fragment of DNA Polymerase I. By replacing a non-radioactive nucleotide with the radio-labelled equivalent in the reaction mixture, newly synthesised DNA is made radioactive. The Rediprime Kit was used according to the manufacturer's instructions. Briefly, 60-100 ng of the gel-purified plasmid DNA fragment in a volume of 45 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were heat-denatured at 95°C for 5 min. After rapid chilling on ice the solution was added to a Redi Prime reaction tube and 5 µl [α-^{32}P]-dCTP *(Amersham Buchler GmbH, Braunschweig, Germany* 370 MBq/mL, 10 mCi/ml) were added. After incubation for 10 minutes at 37°C, unincorporated nucleotides were removed from the labelled DNA by use of Nick columns (*Amersham Pharmacia Biotech, Braunschweig, Germany)* according to the manufacturer's guidelines. Finally, the labelled DNA was eluted in a volume of 400 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and used for hybridisation.

2.2.1.23 Northern Blot hybridisation

An amount of labelled probe corresponding to $12 \cdot 10^6$ total counts/10 ml solution, as determined by use of a Scintillation counter, was heat-denatured together with 100 µl of 10 mg/ml sonicated fish sperm DNA at 95°C for 5 minutes immediately before use. Hybridisation was performed using Quick Hyb hybridisation solution *(Stratagene, Heidelberg, Germany)* according to the supplier's instructions. Briefly, the blotted membrane was pre-hybridised in a glass tube with 10-20 ml of Quick Hyb solution so that the membrane was constantly covered by a thin layer of solution for 20 min a 68°C in an hybridisation oven under constant rotation. The heat-denatured probe was then added into the hybridisation solution and incubated at 68°C for 1 h under rotation. The membrane was then washed twice in 100 ml 2X SSC (derived from a stock solution of 20X SSC, NaCl 3 M, Na_3-citrate 0.3 M) 0.1% SDS buffer for 15 min at RT and once in 100 ml 0.5X SSC 0.1% SDS buffer at 60°C always under constant shaking.

2.2.1.24 Stripping the Northern Blot membranes

To remove the hybridised probe from a Northern Blot filter, the filter was washed twice at 95°C for 15 min under constant shaking in 0.1X SSC (derived from a stock solution of 20X SSC, NaCl 3 M, Na_3-citrate 0.3 M) 0.1% SDS buffer.

2.2.1.25 Detection of radioactive signal

The hybridised filter was sealed in a plastic bag and exposed to Amersham hyperfilm or exposed to a Phosphorimager screen *(Fujifilm, Japan)* and subsequently analysed with the AIDA 200.1 software *(Raytest GmbH, Straubenhardt, Germany).*

2.2.1.26 Reverse transcription polymerase chain reaction (RT-PCR)

To create the first strand of cDNA, total RNA (1-5 μg) was mixed with 200 pmol random hexamer primers to give a 12 μl reaction volume. After incubation of the mixture at 70°C for 10 min and quick chilling on ice, 4 μl 5x First Strand buffer, 2 μl 0.1 M DTT and 1 μl 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP) were added to the reaction. The reaction tube was then incubated for 2 min at 42°C, followed by the addition of 1 μl (200 U) SuperScript II *(Life Technologies, Karlsruhe, Germany).* Finally, the reaction was incubated at 42°C for 50 min. To inactivate the reaction, the tube was heated at 70°C for 15 min. In order to amplify a specific cDNA fragment the appropriate PCR was performed using 1-3 μl of the first strand reaction and a specific set of amplification primers.

2.2.2 PROTEIN METHODS

2.2.2.1 Determination of protein concentration

Protein concentration was determined using the Lowry method. 100 volumes of Lowry I reagent (2% Na_2CO_3 in 0.1 N NaOH) were added to 1 volume of Lowry II reagent (2% Na-K-tartrate) and to 1 volume of Lowry III reagent (1% Cu sulfate) immediately before use and strictly in the mentioned order. To 495 µl of this mixture, called Lowry IV reagent, 5 µl of the protein solution to be measured were added, vortexed and let 10 min at RT in the dark. 0.1 ml of Lowry V (50% Folin solution) reagent were then added. The mixture was immediately vortexed and let at RT for 30 min in the dark. Finally, it was vortexed and 300 µl were taken and transferred to a 96 wells plate. Absorbance at 600 nm was measured. To establish the exact amount of protein present in the probe a standard curve was obtained by measuring the absorbance of known amounts of BSA (typically from 0 to 50 µg).

2.2.2.2 Protein lysates preparation

Cells at 80-90% confluency, typically grown in a 6 wells plate, were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4), then collected in 300-400 µl of appropriate lysis buffer.

According to experimental need, different lysis buffers were used:

2X Laemmli buffer (160 mM Tris HCl pH 6.8, 4% SDS, 16% glycerol and 0.005% bromophenol blue with addition of 10% β-mercaptoethanol);

Radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl pH 7.4, 1% Nonidet P-40 substitute, 0.25% Na-deoxycholate, 150 mM NaCl, 1mM EGTA);

PY buffer, optimised to allow precipitation of tyrosine phosphorylated proteins (50mM Tris-HCl (pH 7.4), 1% Nonidet P-40 substitute, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA);

When desired, 1 mM PMSF, 1 µg/ml each aprotinin, leupeptin and pepstatin, 1 mM Na_3VO_4 and 1 mM NaF where added prior to use.

Cells were removed from the dish by scraping with a rubber policeman or by pipetting and transferred to a fresh vial. Samples were sonified for 5 to 10 s (Branson cell disruptor B15, output 6) to reduce viscosity by disrupting chromosomal DNA. Prior to loading onto a gel, samples were heat-denatured at 95°C for 4 min and Laemmli buffer to a final concentration of 1 X was added when necessary.

2.2.2.3 Separation of proteins by SDS polyacrylamide gel electrophoresis

Usually 10% SDS-Polyacrilamide gels were used: 9.9 ml acrylamide/*bis*-acrylamide 30:0.8, *(Carl Roth GmbH & Co, Karlsruhe, Germany)*; 11.7 ml 1 M Tris-HCl pH 8.8; 8.4 ml bi-distilled H_2O; 150 µl 20% *(w/v)* SDS 20%; 300 µl APS; 15 µl TEMED. The Penguin Doppelgelsystem P9DS apparatus *(Peqlab, Erlangen, Germany)* was used to cast the gel. Twenty ml of resolving gel were poured and overlaid with Rotisol. After polymerisation Rotisol was washed off with deionised water. Ten ml of stacking gel were then added (7.3 ml bi-distilled H_2O; 1.68 ml acrylamide/bis-acrylamide; 0.69 ml 1 M Tris-HCl, pH 6.8; 56 µl 20% SDS; 45 µl 10% APS and 15 µl TEMED). After polymerisation, the gel was fixed within the gel tank. 1X Laemmli buffer was used as running buffer (25 mM Tris, 200 mM glycine, 0.1% (w/v) SDS). Typically 40-60 µl of cell lysate were loaded per lane onto a SDS-PAGE. The run was performed at 80 V until the probes had reached the resolving portion of the gel and then at 150 V.

2.2.2.4 Staining the SDS-PAGE gels *(Coomassie staining)*

Gels were incubated with Coomassie stain solution (0.5 g/l Coomassie brilliant blue, 45% methanol and 10% acetic acid) for 1 hour. To de-stain, the gels were incubated in 10% acetic acid, 10% methanol for 24 hours or longer with several changes of the de-stain solution until the background appeared clear.

2.2.2.5 Western blotting

After proteins were separated by SDS-PAGE, they were electrically transferred onto Immobilon membrane (*Millipore*, type PVDF, pre-soaked in methanol) at 250-300 mA for at least 6 hours in transfer buffer (25 mM Tris, 190 mM glycine, 20% (*v*/*v*) methanol and 0.05% *(w/v)* SDS). Immunoblotting was performed according to individual instructions for each antibody. Typically, in order to reduce unspecific binding of the antibodies to the membrane, the blot was incubated in blocking solution: TBST (10 mM Tris-HCl, pH 8.1, 100 mM NaCl, 0.05% Tween 20) or PBST (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.05% Tween 20) supplemented with either 3-5% *(w/v)* non-fat dried milk or 5% BSA, according to the different antibodies that were going to be used, at RT for 1 h with shaking. For detection of proteins of interest the membrane was incubated in blocking solution containing the appropriate primary antibody (optimal working dilution was determined empirically) at 4°C overnight. After 3 washes of 10 min each in the appropriate buffer (TBST or PBST), the membrane was incubated 1 hour in blocking solution containing a 1:2000 dilution of HRP-conjugated secondary antibody. Once the membrane had been washed 3 times 10 min each, detection of specific proteins was achieved by enhanced chemiluminescence using ECL Western blotting detection reagents as recommended by the manufacturer *(Amersham, Braunschweig, Germany)* and exposure to ECL Hyperfilm *(Amersham, Braunschweig, Germany)* .

2.2.2.6 Stripping of the Western blot membranes

To allow more than a single use of Western blot membranes, the membranes were incubated with a stripping solution (62.5 mM Tris, pH 6.8, 2% SDS, 0.75% 2-mercaptoethanol) at 50°C for 30 min with shaking. The membranes were then washed three times in the 1X PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) for 15 min each time.

2.2.3 OTHER EXPERIMENTAL PROCEDURES

2.2.3.1 Immunoprecipitation

Cells were washed in 1X PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and then lysed with the desired buffer to an approximate protein concentration of 1mg/ml. The soluble fraction of the lysate was incubated with varying amounts of antibody (typically 10 times the amount recommended for immunoblotting) with rotation overnight at 4 °C. In case of precipitation of tyrosine phosphorylated proteins, the lysate was incubated with 0.1 *v/v* 50% Protein A agarose beads suspension for at least 2 hours at at 4 °C with rotation, to capture the antibody complex. The beads were then collected by centrifugation pulse (5-10 s, 12000 rpm), washed twice with lysis buffer containing proteases inhibitors and finally resuspended in an equal volume of 2X Laemmli sample buffer, heated at 95°C for 5 min and loaded onto a protein gel.

2.2.3.2 Ras activation assay

This assay was purchased from *Upstate Biotechnology, NY* and was performed as suggested by the manufacturer. Cells were grown to 75-80% confluency, treated as desired and deprived of serum overnight. Cells were lysed in 1X MLB buffer (a 5 X stock solution is supplied with the kit and contains: 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 50 mM EDTA and 10% glycerol). The lysates (about 1 mg protein in 1 ml of solution) were pre-cleared with glutathione agarose prior to the addition of 10 µg of Raf-1 RBD agarose (provided). The reaction mixture was rocked at 4 °C for at least 1 h to specifically allow capture of activated Ras by the beads. The beads were collected by pulsing (5 s in the microcentrifuge at 12000 x g) and the supernatant was drained off. Three washing in 1 X MLB steps followed, before resuspending the beads in 20 µl 2X Laemmli sample buffer. The beads were collected by a microcentrifuge pulse and SDS-PAGE was performed on the supernatants. After transfer of the

proteins to a nitrocellulose membrane, the activated Ras was visualised by immunoblotting with a anti-Ras, clone RAS10, antibody (provided).

2.2.3.3 Flow cytometry

Adherent cells were detached from the Petri dishes by scraping, collected by centrifugation at 1000 rpm for 3 min. $2 \cdot 10^5$ cells/point were resuspended in 200 μl FACS buffer (1X PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4); 2% FCS) containing a 1:50 dilution of FITC- or PE-conjugated antibody, transferred into one well of a 96 wells plate and incubated for 20 min at 4°C in the dark. After three washes with FACS buffer, the cells were finally re-suspended in 200 μl FACS buffer and analysed with the aid of a FACStar flow cytometer *(Becton Dickinson).* When desired, cells were fixed by incubation in 100 μl of 10% formalin for 10 min at room temperature prior to FACS (Fluorescence Activated Cell Sorter) analysis. For detection of FcεRI, cells were incubated with IgE (0.5 μg/ml) for 1 hour at 4°C, then the medium containing IgE was removed and the cells washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4), prior to being collected and incubated with an anti-IgE FITC conjugated antibody as described.

2.2.3.4 FcεRI-dependent activation of mast cells

Sub-confluent RBL-2H3 cells were sensitised for 1 h with IgE anti-DNP (0.5 μg/ml) at 37 °C, washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4), and incubated in fresh growth medium warmed up at 37°C containing DNP-BSA *(Sigma, Taufkirchen, Germany),* final concentration 0.3-0.5 μg/ml, for the desired time. BMMCs were cultured at a density of $5 \cdot 10^5$ cells/ml; before activation, they were deprived of IL-3 overnight, then were sensitised with IgE anti-DNP 0.5 μg/ml at 37 °C for at least 4 h, washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and incubated in fresh growth medium warmed up at 37°C containing DNP-BSA (final concentration 0.3-0.5 μg/ml) for the desired time.

2.2.3.5 Transfection of cells by FuGene6™ reagent

FuGene6™ transfection reagent was used according to the manufacturer's instructions. Briefly, proliferating cells were sub-cultured the day before transfection so that they could reach 50-80% confluency on the day of the experiment. For a 35 mm culture dish, 3-6 µl of FuGene6™ reagent were diluted in serum-free medium to a total volume of 100 µl and incubated at RT for 5 min. The diluted FuGene6™ solution was then added dropwise to a second sterile tube containing 1-2 µg of DNA and incubated at RT for 15 min. The mixture was finally transferred to the cells dropwise and by gentle swirling to ensure an even distribution. In case of transient transfection, typically 1.2 µg of desired reporter plasmid, 0.2 µg of GR expression vector (when necessary) and 0.3 µg Renilla Luciferase (as an internal standard standard to normalise firefly luciferase values) were transfected per dish.

2.2.3.6 Transfection of cells by electroporation

Electroporation was used exclusively to transfect RBL-2H3 cells. Cells were grown to confluency and split 1:3 the day before transfection. The day of transfection $5 \cdot 10^6$ cells pro cuvette were collected by scraping with a rubber policeman and centrifugation at 1000 rpm, 3 min, and resuspended in 200 µl of medium plus the plasmid DNA to be transfected. Cells were then electroporated at 500 µF/300 V and re-suspended in 10 ml of growth medium by pipetting to separate cell clumps in a 10 cm Petri dish. For transient transfection, typically 15 µg of specific plasmid and 5 µg of Renilla Luciferase (as an internal standard to normalise firefly luciferase activity values) were transfected per cuvette.

2.2.3.7 Measurement of firefly luciferase activity

After transfection, cells were let grow for at least 24-48 h to allow expression of the transfected reporter gene. Typically they were kept in 6-wells plates. Cells were then washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4,

1.5 mM KH_2PO_4), strictly without Ca^{2+} and Mg^{2+}. PBS was then removed and the cells harvested in 300 µl 1X lysis buffer (Passive Lysis Buffer, *Promega, Mannheim, Germany)*. The cells were kept on ice for ca. 10 min and occasionally rocked to distribute the buffer evenly and favour the detachment of the cells from the plates. The cell lysates were pipetted up and down several times, collected with a rubber policeman and finally transferred to pre-cooled vials. 100 µl of cell lysate were transferred to a reading tube with autoinjection of 350 µl of assay buffer (1mM DTT, 1 mM ATP in glycylglycine buffer (25 mM glycylglycine, 15 mM $MgSO_4$ and 4 mM EGTA) and 100 µl of luciferin assay solution (1mM luciferin; stock solution: 0.28 mg/ml) were then added. Luciferase activity was measured by use of a luminometer *(Berthold, Wildbad, Germany)*.

2.2.3.8 Measurement of Renilla luciferase activity

100 µl of cell lysates obtained as described in the precedent paragraph were mixed with 500 µl of coelenterasin buffer (0.1 M KPi-buffer (0.2 M KH_2PO_4 and 0.2 M K_2HPO_4, pH 7,6), 0.5 NaCl, 1 mM EDTA, pH 7.6) together with substrate solution (25 nM end concentration coelenterazine, *Byosinth AG, Gstaad, Switzerland)*. Luminescence was measured by use of a luminometer *(Berthold, Wildbad, Germany)*.

2.2.4 CELL CULTURE

All mammalian cells were maintained at 37°C in an incubator (*Forma Scientific, Labortect GmbH, Göttingen, Germany)* in 5% CO_2 and 95% air humidity. All cells were grown in Petri dishes or flasks *(Greiner, Frickenhausen, Germany)* of varying sizes depending on the application. The cells were allowed to grow until a confluence of 80-90% had been reached, whereupon the cells were subsequently split by trypsinisation and re-seeded at a lower density. Trypsin treatment of cells was performed by removal of the culture medium from the cells, followed by one wash with 1X PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM

KH_2PO_4). After removal of PBS, 0.25% trypsin was applied to the cells and the cells incubated at 37°C until they became detached. Fresh medium was then directly applied and the cells re-plated at the desired density in new Petri dishes.

2.2.4.1 Long term storage of cells (freezing and thawing of cells)

To prepare cells for long term storage, logarithmically growing cells were trypsinised and collected by centrifugation at 800-1500 rpm. The medium was removed and the cells re-suspended in culture medium with 50-90% FCS and 10% DMSO (*Fluka Chemie AG. Switzerland)* and placed in 1 ml aliquots in cryovials. After incubation on ice for 1 hour, the cells were placed at -80°C for 16-24 h before finally being stored in liquid nitrogen. To re-propagate cells, the vials were removed from the liquid nitrogen and placed at 37°C until the cells had thawed. The cells were then removed and mixed with 10 ml fresh medium; cells were then collected by centrifugation and seeded on Petri dishes in fresh medium.

2.2.4.2 Isolation and culturing of primary mouse cells

Mice of about 5 weeks of age were sacrificed by CO_2 inhalation. Tissues were removed on the bench with ethanol-rinsed dissecting instruments and transferred into sterile PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) or serum-free medium. Single cells suspensions were prepared under sterile conditions in a hood. For cell culture, all instruments were autoclaved before use.

2.2.4.2.1 Bone Marrow Derived Mast Cells (BMMC)

The skeletal muscle was removed from the bones of the posterior limbs; femurs and tibias were collected. The extremities of the bones were cut under sterile conditions so that the bone marrow could be flushed with 2-3 ml of serum-free medium using a syringe and needles of appropriate size. Single cell suspension was prepared by repeated pipetting and transferred to a 50 ml conical tube. The cells were collected by centrifugation at 1300 rpm for 10 min at 4°C. Cells were

resuspended in 5 ml of ACK lysis buffer (0.15 M NH_4Cl, 1mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4, sterile filtered) and incubated for no more than 5 min at RT to allow disruption of red blood cells. The reaction was stopped by addition of 10 ml of cold growth medium. After collecting the cells by centrifugation, the medium was discarded and 10 ml of fresh medium were added. The cells were re-suspended by pipetting, re-collected by centrifugation and finally seeded at a density of $5 \cdot 10^5$ cells/ml in growth medium. These cells were then cultured for 4 weeks in presence of IL-3 in order to allow specific differentiation of mast cells. Every 3-4 days half volume of old medium was removed and replaced with fresh one; when necessary cells were transferred to a new plate in order to enrich the population in mast cells (which grow in suspension) and eliminate adhering cell populations. The cells were examined for expression of typical mast cells markers (Fc RI and c-kit receptor), by flow cytometry.

2.2.4.2.2 Splenocytes and Thymocytes

Organs (spleens or thymi) were removed and pushed through a nylon mesh (70 m pores, *Falcon 2350)* into a 50 ml conical tube. The sieve was rinsed with 20 ml of serum-free medium to collect most of the cells. Single cell suspension was prepared by repeated pipetting. The cells were collected by centrifugation at 1500 rpm for 10 min at 4°C. Cells were re-suspended in 5 ml of ACK lysis buffer (0.15 M NH_4Cl, 1mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4, sterile filtered) and incubated for no more than 5 min at RT to allow disruption of red blood cells. The reaction was stopped by addition of 10 ml of cold serum-free medium. After collecting the cells by centrifugation, the medium was discarded and 10 ml of fresh medium were added. The cells were re-suspended by pipetting, re-collected by centrifugation and finally seeded at a density of $1 \cdot 10^6$ cells/ml in growth medium.

3. RESULTS

3.1 Glucocorticoids repress ERK1/2 phosphorylation in mast cells

ERK1/2 control several functions indispensable for immediate allergic response in mast cells (Hirasawa et al., 1995; Zhang et al., 1997; Kimata et al., 2000). ERK1/2 phosphorylation was shown to be inhibited by a prolonged treatment with glucocorticoids (Rider et al., 1996), and this inhibition had been proposed as a mechanism through which glucocorticoids exert an anti-allergic action on mast cells. However, the exact mechanism by which glucocorticoids repress ERK1/2 activity is not fully understood.

The ERK1/2 pathway in mast cells is activated by calcium mobilising agents as well as by IgE/antigen trigger and by phorbol esters. The capability of the synthetic glucocorticoid Dexamethasone to repress ERK1/2 phosphorylation following stimulation with different agents was examined. RBL-2H3 mast cells were treated with Dexamethasone or with solvent alone (ethanol) for 48 h then activated using different stimulants. Activation by IgE/antigen-dependent cross-linking of the receptor with high affinity for IgE (FcεRI) was achieved by sensitisation of mast cells with antigen-specific immunoglobulins E (IgE anti-dinitrophenyl), which bind the FcεRI, followed by activation with specific antigen (dinitrophenyl-bovine serum albumin, DNP-BSA). In addition, RBL-2H3 mast cells were activated also *via* phorbol ester (TPA) treatment that activates ERK1/2 through the protein kinase C (PKC) pathway or *via* incubation with Thapsigargin which activates ERK1/2 through mobilisation of intracellular calcium. ERK1/2 activation is achieved by dual phosphorylation on tyrosine and threonine residues (Cano and Mahadevan, 1995). Phosphorylated ERK1/2 were detected by immunoblotting using a specific antibody for dual phosphorylated ERK1/2. Activation of ERK1/2 is repressed in Dexamethasone-treated cells whether they are stimulated by either of these agents (Fig. 3.1/pERK1/2, compare lane 3-4, 5-6,

7-8). A phosphorylation state-independent anti-ERK2 antibody was used as a control to ensure that any effect observed on ERK1/2 phosphorylation was not due to differences in the level of these proteins (Fig. 3.1/ERK2). Hsp90 protein levels are also shown in Fig. 3.1 as control for equal protein loading.

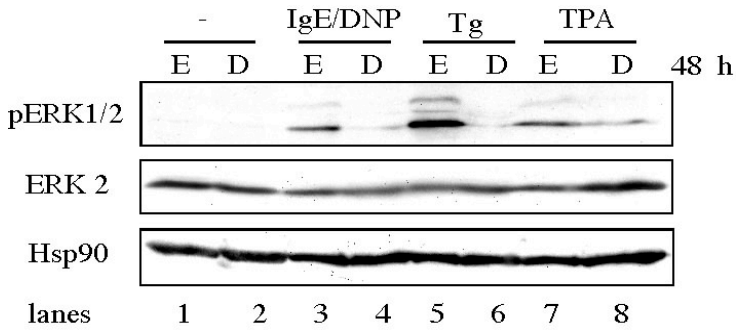

Figure 3.1 Glucocorticoids inhibit ERK1/2 phosphorylation in RBL-2H3 mast cells. Sub-confluent RBL-2H3 mast cells were incubated with Dexamethasone (D, 10^{-7} M) or with solvent alone (ethanol, E) for 48 h, then sensitised with IgE anti-DNP (0.5 □g/ml) for 1 h and activated with DNP-BSA(0.3 □g/ml) for 10 min or activated with Thapsigargin (Tg) 150 mM for 10 min or activated with TPA 80 ng/ml for 10 min. Cells were harvested, subjected to SDS-PAGE and Western blotting with an antibody against phosphorylated ERK1/2. The membrane was stripped and re-probed with an antibody that recognises ERK 2 independently of its phosphorylation state. The membrane was also immunoblotted for Hsp90 to show equal protein loading.

A prolonged treatment with Dexamethasone is therefore able to repress ERK1/2 phosphorylation. As a first step towards understanding the mechanism by which glucocorticoids inhibit ERK1/2 activity in mast cells, the kinetics of ERK1/2 inhibition upon glucocoticoid treatment were examined. RBL-2H3 mast cells were treated with Dexamethasone for different times (1-24 h) and then activated by IgE/antigen triggering. This type of activation was chosen because it is the most similar to the physiological activation of mast cells. Treatment with solvent alone (ethanol) or Dexamethasone for 1 to 24 h had no effect on the basal levels of phosphorylated ERK1/2 (Fig. 3.2, compare lane 1 corresponding to untreated cells, with lanes 3, 4, 7, 8, 11, 12, 15 and 16). Cells activated with IgE/DNP-BSA showed a robust phosphorylation of ERK1/2 (Fig. 3.2, lanes 5, 9, 13 and 17). The slight phosphorylation of ERK1/2 following IgE sensitisation (Fig. 3.2, lane 2), has been observed by other investigators (Brondello et al., 1999). Dexamethasone treatment for 1 or 5 h prior to activation of the cells with IgE/DNP-BSA did not

have any effect on ERK1/2 phosphorylation (Fig. 3.2, compare lanes 5-6 and 9-10). On the contrary, repression of ERK1/2 phosphorylation was clearly detectable if cells were treated with hormone for 16-24 h (Fig. 3.2, compare lanes 13-14 and 17-18). As a control for protein loading of the gel, Western immunoblotting was carried out with an anti-Hsp90 antibody (Fig. 3.2/ Hsp90).

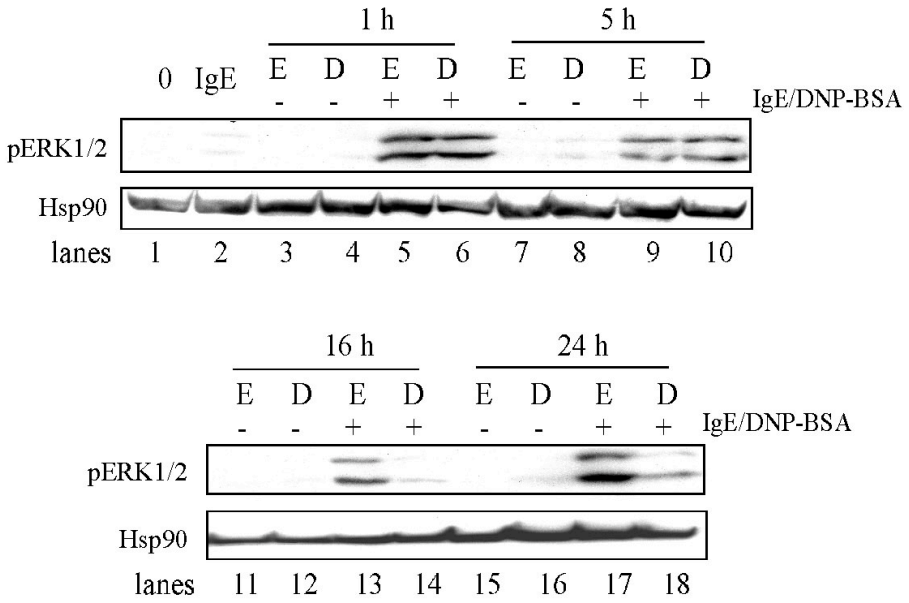

Figure 3.2 ERK1/2 phosphorylation in RBL-2H3 mast cells is repressed only after a prolonged treatment with glucocorticoids. Sub-confluent RBL-2H3 mast cells were incubated with Dexamethasone (D, 10^{-7} M) or with an equal volume of solvent alone (ethanol, E) for the indicated times, sensitised for 1 h with IgE anti-DNP (0.5 µg/ml) and finally activated by DNP-BSA (0.3 µg/ml) for 10 min (indicated with a +) or not (indicated with a -). Cell lysates were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Phosphorylated ERK1/2 were detected in immunoblotting using an anti-phosphoERK1/2 specific antibody. An antibody against Hsp90 was used to demonstrate equal protein loading. Lane 1 (0) corresponds to untreated cells. Lane 2 (IgE) corresponds to cells only sensitised 1 h with IgE anti-DNP (0.5 µg/ml).

Since direct action of glucocorticoids on gene expression is a rather rapid process, which occurs within 0.5-2 h (König et al., 1992; Nissen and Yamamoto 2000), the kinetics of Dexamethasone-mediated inhibition of ERK1/2 phosphorylation suggested that ERK1/2 inhibition might not, or not exclusively, depend on direct gene regulation. Previous studies had shown that glucocorticoid-mediated repression of ERK1/2 activity requires *de novo* protein synthesis (Kassel et al.,

2001), thus suggesting that inhibition of ERK1/2 phosphorylation by Dexamethasone might require the mediation of secondary factor(s).

3.2 Candidate mediators of negative regulation of ERK1/2 activity by glucocorticoids

Identification of possible factors responsible for the action of glucocorticoids on ERK1/2 phosphorylation was achieved by analysis of changes in gene expression following glucocorticoid treatment, using the Affymetrix rat genome U34A array (the screening was kindly performed at Schering AG, Berlin, by Dr. Jörn Krätzschmar). Briefly, total RNA derived from RBL-2H3 mast cells treated with or without Dexamethasone for 16 h or 24 h was reverse-transcribed into complementary DNA (cDNA) and used for hybridisation onto a gene array chip. Two independent experiments were performed. One hundred seventy-five genes/EST showed altered expression in response to 16 h of Dexamethasone treatment; of these, 89 were upregulated and 86 downregulated upon hormone administration; upon 24 h of Dexamethasone treatment, 120 genes/EST showed altered expression; of these, 58 were upregulated and 62 were downregulated upon hormone administration (partial results of this analysis are reported in Appendix).

Several genes likely to be involved in the regulation of inflammatory processes in mast cells, were selected for further analysis. The genes chosen were: the immunoglobulins-binding chain (chain) of the high affinity IgE receptor (Fc RI), indispensable for the activation of mast cells upon IgE/antigen trigger (Dombrowicz et al., 1993); signalling molecules like the extracellular signal-regulated kinase (ERK)-3 (Boulton et al., 1991) and two dual specificity phosphatases, MKP-1 and MKP-3, which had been shown to be able to dephosphorylate activated MAP kinase (Alessi et al., 1993; Sun et al., 1993; Groom et al., 1996); a series of chaperones that could possibly be involved in the regulation of the activity of the glucocorticoid receptor; several proteasomal subunits which could be important in regulation of degradation of proteins involved in proinflammatory processes; the enzyme Farnesyl diphosphate

synthase (FPPS), required for protein prenylation and shown to be implicated in post-translational modification of proteins involved in growth control signalling pathways (reviewed in Goldstein and Brown, 1990); mast cells specific mediators like the granule-associated serine protease MCP-3, possibly involved in tissue remodelling during inflammatory processes (Lazaar et al., 2002), a peptide hormone, Relaxin, that had been shown to inhibit histamine release in mast cells (Masini et al., 1995) and IL-18, which had been shown to induce histamine release by mast cells (Konishi et al., 2002).

The regulation of the expression of the above mentioned genes upon Dexamethasone treatment was reassessed by Northern Blot analysis. The gene expression array analysis results could be confirmed only for 4 genes out of 17. The results of these analyses are summarised in Table 3.1.

gene	Affymetrix gene profiling analysis 16 h /24 h	Northern Blot analysis 16 h /24 h
receptor		
Fc RI	**(-2.4; -14) / (-2.3; -3.3)**	/
signalling molecules		
ERK3	**(-2.6; -6.4) / (-2.5; -1.7)**	/
phosphatases		
MKP-1	**(6.1;undefined)/(>13; >4.6)**	/
MKP-3	**(3.9; 1.8) / (3.3; 2.4)**	/
chaperones		
Hsp90	**(no change) / (-1.9 ; <-2.3)**	**no change**
Hsp70	**(-1.3 ; >-23) / (no change)**	**no change**
p59	**(-2; -3.5) / (no change)**	**no change**
Hop	**(-1.5; -5.6) / (no change)**	**no change**
proteasome subunits		
R-zeta	**(no change) / (-1.5; -2.8)**	**no change**
R-delta	**(no change) / (-1.7; -5.1)**	**no change**
RCX	**(-1.4; -7.6) / (no change)**	**no change**
RN3	**(-2; -16) / (-1.7; -3.5)**	**no change**
MSS1	**(-2.7; -2.1) / (no change)**	**no change**
other enzymes		
FPPS	**(-1.7; -19) / (no change)**	**no change**
mediators		
IL-18	**(-2.2; -4.6) / (no change)**	**no change**
ppRLX	**(19; 5.1) / (19; 30)**	/
MCP-3	**(2.5; 3.7) / (no change)**	**no change**

Table 3.1 Regulation of expression of 17 selected genes upon glucocorticoid treatment in RBL-2H3 mast cells as assessed by gene expression array analysis and Northern Blot analysis. Gene expression array analysis: the Affymetrix rat genome U34A array was used (the screening was kindly performed at Schering AG, Berlin, by Dr. Jörn Krätzschmar). Briefly, total RNA was derived from RBL-2H3 mast cells treated with or without Dexamethasone for 16 h or 24 h. The RNA was reverse-transcribed into complementary DNA (cDNA) and used for hybridisation onto a gene array chip. For each time point, two independent experiments were performed. In brackets, the fold induction after 16 h/24 h of hormone treatment in two independent experiments is indicated. Northern Blot: total RNA was derived from RBL-2H3 mast cells treated with Dexamethasone (10^{-7} M) or with solvent alone for 16 or 24 h , resolved on MOPS/formaldehyde 1% agarose gel (15 g/lane) and subjected to Northern Blot hybridisation. The probes for Northern Blot hybridisation were generated by PCR amplification of specific segments of RBL-2H3 cells cDNA and cloning into pCR 2.1 (TOPO TA cloning kit) vector. The inserted fragments were excised from the vector and labelled with [^{32}P]dCTPs by random hexamer primer labelling before being used in Northern Blot hybrisation. An ascending arrow indicates upregulation of the expression of the corresponding gene upon Dexamethasone treatment; a descending arrow indicates downregulation. In some cases, no significant change in the basal level of expression was observed, as indicated. The membranes were stripped and re-hybridised with a GAPDH probe to ascertain equal loading. Abbreviations used are: Fc RI (high-affinity IgE receptor, alpha chain); ERK3 (extracellular signal-regulated kinase 3); MKP-1 (mitogen-activated protein kinase (MAPK) phosphatase-1); MKP-3 (mitogen-activated protein kinase (MAPK) phosphatase-3); Hsp90 (90-kDa heat shock protein); Hsp70 (heat shock protein 70); p59 (immunophilin p59); Hop (Hsp70-Hsp90 organizing protein); R-zeta (proteasome zeta chain); R-delta (proteasome delta chain); RCX (rat proteasome subunit X); RN3 (rat proteasome beta-type subunit, N3); MSS1 (mammalian suppressor of sgv1); FPPS (farnesyl-diphosphate synthase); IL-18 (interleukin-18); ppRLX (preprorelaxin, precursor mRNA for Relaxin); MCP-3 (mast cell protease-3).

3.3 Specific regulation of phosphatases gene expression in mast cells upon glucocorticoid treatment

The occurrence of MAP kinase phosphatase (MKP)-1 and MKP-3 among the genes transcriptionally up-regulated upon glucocorticoid treatment in mast cells was particularly interesting. MKP-1 and MKP-3 belong to a family of dual specificity phosphatases which consists of more than nine members in mammalian cells. These phosphatases catalyse the removal of the phosphoryl group from the phosphorylated tyrosine (Y) as well as from the phosphorylated threonine (T) in the activation loop motif TXY of MAP kinases (Alessi et al., 1993; Sun et al., 1993; Groom et al., 1996; Camps et al., 2000; Keyse, 2000). MKP-1 and MKP-3 were therefore considered as potential candidates for the glucocorticoid-mediated dephosphorylation of ERK1/2. The regulation of their expression upon glucocorticoid treatment was therefore further investigated. Northern B1ot analysis showed that the mRNA levels of both genes were upregulated as early as 30 min (MKP-1) and 60 min (MKP-3) after addition of hormone (Fig. 3.3A, compare lanes 4-5 and 6-7). Glucocorticoid-mediated mRNA level enhancement persisted up to 24 h, and was more sustained in the case of MKP-3 (Fig. 3.3A, compare lanes 14-15).

As controls, the mRNA levels of two other phosphatases, the haematopoietic protein-tyrosine phosphatase (HePTP) and the Src homology 2-containing protein tyrosine phosphatase (SHP)-1, which have also been shown to interfere with ERK1/2 signalling (Plas et al., 1996; Saxena et al., 1999), were examined and found not to be significantly altered by glucocorticoid treatment (Fig. 3.3B, lanes 1-13). Note that lanes 12(B) and 13(B) contain more RNA. These data, taken together, indicate that enhanced expression of the genes encoding MKP-1 and MKP-3 by glucocorticoids in RBL-2H3 mast cells is specific.

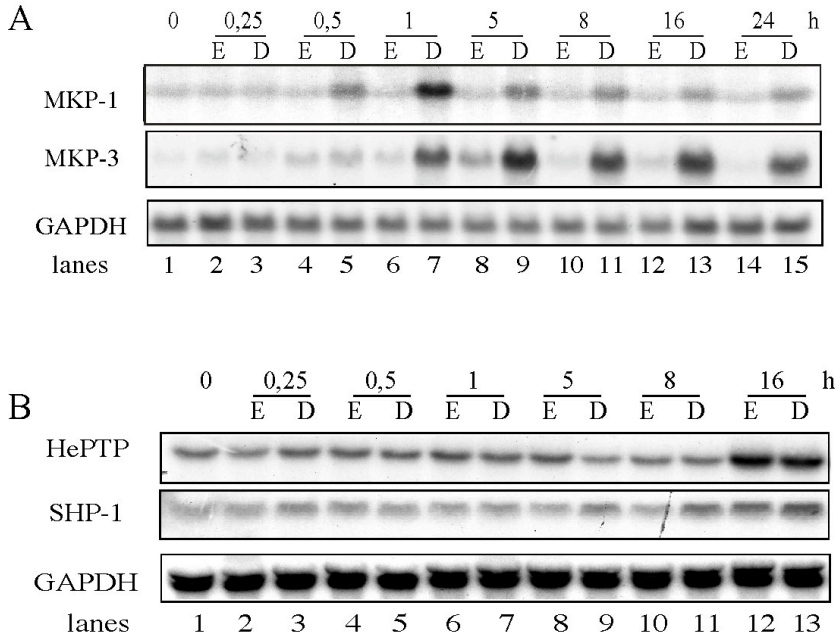

Figure 3.3(A,B) Glucocorticoids specifically upregulate the mRNA level of the phosphatases MKP-1 and MKP-3 in RBL-2H3 mast cells. Sub-confluent RBL-2H3 mast cells were treated with Dexamethasone (D, 10^{-7} M) or solvent (ethanol, E) for the indicated times. Lane 1 (0) indicates untreated cells. Total RNA was extracted, resolved on MOPS/formaldehyde 1% agarose gel (20 □g/lane), subjected to Northern Blot hybridisation using specific [□^{32}P]-dCTP labelled cDNA probes for either MKP-1 or MKP-3 or HePTP or SHP-1. The membranes were stripped and re-hybridised with a probe for GAPDH as a RNA loading control.

3.3.1 Cell type-specific regulation of MKP-1 and MKP-3 expression by glucocorticoids

Previous data from our group had shown that glucocorticoid-mediated repression of ERK1/2 phosphorylation is a cell type specific phenomenon (Kassel and Cato, 2002). It was shown to occur in the monocytic leukaemia cell line U937 and T lymphoma cells S49.1, as well as in RBL-2H3 mast cells, but not in NIH3T3 fibroblasts, primary lung fibroblasts (6PHII) and bronchial epithelial carcinoma A549 cells. In all the cells in which glucocorticoids downregulated ERK1/2 activity, this effect correlated with a glucocorticoid-mediated upregulation of MKP-1 gene expression (Kassel and Cato, 2002). It was therefore important to determine whether the down-regulation of activity of ERK1/2 by glucocoticoids also correlated with an increase in expression of MKP-3. In RBL-2H3 mast cells, increase in the levels of MKP-3 mRNA upon glucocorticoid treatment were detectable after 1 h of hormone treatment and still elevated after 8 h. Therefore,

these time points were chosen to treat U937, S49.1, A549, NIH3T3 and 6PHII cells, together with RBL-2H3 as a control, with Dexamethasone or solvent. Levels of specific transcripts were analysed *via* Northern Blot. Enhancement of levels of MKP-3 mRNA was induced upon Dexamethasone treatment in RBL-2H3 mast cells, as expected (Fig. 3.4, compare lanes 1-2 and 3-4) but not in U937 and NIH3T3 cells (Fig. 3.4, lanes 9-12; and 17-20). No basal expression or regulation by glucocorticoids was detected in S49.1, A549 and 6PHII cells (Fig. 3.4, lanes 5-8, 13-16 and 21-24). This demonstrated a cell specific expression and regulation of expression of MKP-3 gene by glucocorticoids. Only in RBL-2H3 mast cells, were glucocorticoids able to increase the levels of MKP-3 transcript.

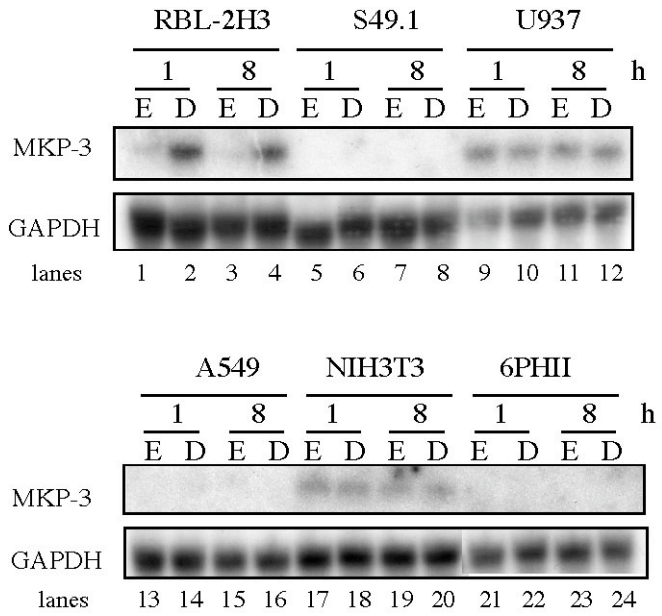

Figure 3.4 Glucocorticoids specifically up-regulate MKP-3 MRNA levels in RBL-2H3 mast cells. RBL-2H3, S49.1, U937, A549, NIH-3T3 and 6PHII cells were treated with Dexamethasone (D, 10^{-7} M) or solvent (ethanol, E) for the indicated times. Total RNA was estracted, resolved on MOPS/formaldehyde 1% agarose gel (10 □g/lane), subjected to Northern Blot hybridisation using a specific [□^{32}P]-labelled cDNA probe for MKP-3. The membrane was stripped and re-hybridised with a probe for GAPDH as a RNA loading control.

To extend the analysis of expression of MKP-3 in mast cells other than the rat cell line RBL-2H3, the effect of Dexamethasone on mRNA levels of MKP-3 in mouse bone marrow-derived mast cells (BMMCs) was studied *via* Northern Blot

analysis. The expression of MKP-3 was not upregulated upon Dexamethasone treatment for 1 or 8 h (Fig. 3.5/MKP-3, compare lanes 1-2 and 3-4), whereas the MKP-1 gene expression was upregulated in these cells (Fig. 3.5/MKP-1, compare lanes 1-2 and 3-4). Further work on MKP-3 was therefore suspended since the results regarding regulation of MKP-3 expression upon glucocorticoid treatment in RBL-2H3 mast cells could not be extended to other mast cells populations. However, further studies were undertaken to possibly establish a role for MKP-1 in glucocorticoid-mediated ERK1/2 dephosphorylation.

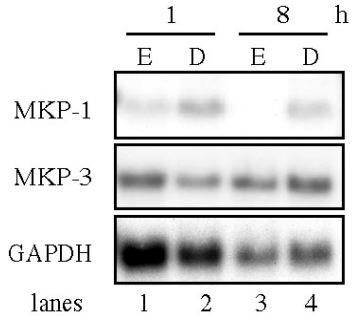

Figure 3.5 Glucocorticoids upregulate mRNA levels of MKP-1 but not of MKP-3 in BMMCs. BMMCs were treated with Dexamethsone (D, 10^{-7} M) or solvent (ethanol, E) for 1 and 8 h. Total RNA (kindly supplied by Dr. Michael Stassen) was resolved on MOPS/formaldehyde 1% agarose gel (20 □g/lane), subjected to Northern Blot hybridisation using specific [□^{32}P]-labelled cDNA probes for MKP-1 or MKP-3. The membrane was stripped and re-hybridised with a probe for GAPDH as a RNA loading control.

3.3.2 Glucocorticoids regulate the expression of MKP-1 gene at the promoter level

The rapid increase in MKP-1 mRNA level upon glucocorticoid treatment was compatible with the hypothesis of a direct regulation of MKP-1 gene transcription by the glucocorticoid receptor (GR). To investigate this claim, the effect of Dexamethasone on MKP-1 promoter activity was examined, making use of transient transfection assays. A reporter construct (-1716MKP-1luc) consisting of the sequence -1716 to +88 (where 0 is the transcription initiation site) of the MKP-1 promoter cloned in front of firefly luciferase gene was transfected in GR-negative simian kidney COS-7 cells together with a wild type (wt) GR expression

vector. A Renilla luciferase construct was also co-transfected in all the samples as an internal control for the normalisation of transfection efficiency. Treatment with Dexamethasone or solvent alone was performed 4 h after transfection and the cells harvested 48 h later for luciferase activity measurement. The results of these experiments showed that incubation with Dexamethasone enhanced the expression of the -1716 MKP-1luc reporter gene when co-transfected with the wt GR about 4 fold (Fig. 3.6 compare lanes 5-6). The expression of an indicator gene containing a Dexamethasone insensitive promoter (the simian virus thymidine kinase promoter) cloned in front of the firefly luciferase gene, transfected in the same cells together with the GR expression vector, was hardly affected by glucocorticoid treatment, as expected (Fig. 3.6, lanes 1-2). Interestingly, co-transfection of the -1716MKP-1luc construct together with GR mutants lacking dimerisation capability, A458T or hGR(D4X) (both described in Heck et al., 1994), abrogated Dexamethasone-mediated increase in promoter activity (in Fig. 3.6 compare lanes 7-8 and 9-10). Since dimerisation is required by the GR to bind DNA, this result suggests that DNA binding by the GR is necessary for the enhanced activation of expression at the MKP-1 promoter.

The sequence -1716 to +88 of the MKP-1 promoter contains three putative glucocorticoid response elements (GREs). Dexamethasone-mediated enhancement of activity of a MKP-1 promoter fragment (-116MKP-1luc) which lacks these three GREs, was significantly lower as compared to the -1716MKP-1luc (Fig. 3.6 compare lanes 3-4 and 5-6). It can be noted that the basal level of expression of the -116MKP-1luc reporter gene is lower as respect to the -1716MKP-1luc (Fig. 3.6 compare lane 3 with 5). Considering that the former construct is shorter than the latter, it is likely that this depends on the absence of regulatory elements necessary for promoter activity. These data show that glucocorticoids can specifically enhance the activity of the promoter of MKP-1 gene and that dimerisation of the GR is required to mediate transcriptional activation, as well as the presence of discrete nucleotide sequences on the MKP-1 promoter, presumably GREs.

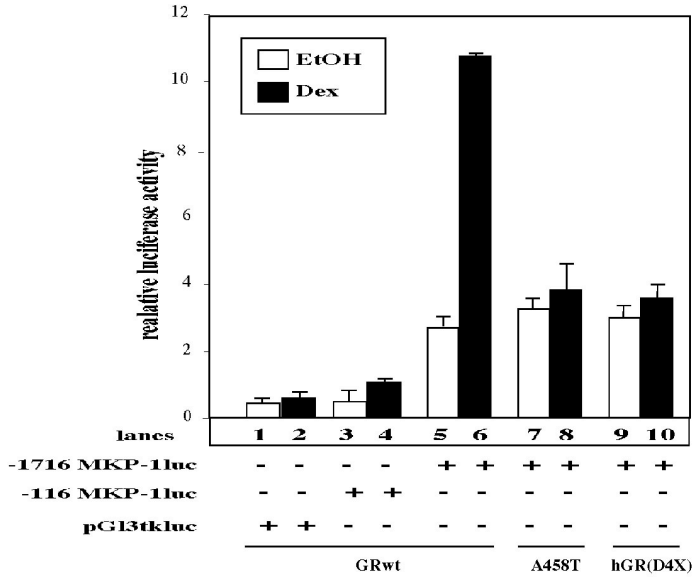

Figure 3.6 Glucocorticoids induce MKP-1 gene expression at the promoter level. Sub-confluent COS-7 cells were transiently transfected with FuGene6™ reagent either with the MKP-1 promoter-firefly luciferase construct -1716MKP-1luc or with the MKP-1 promoter-firefly luciferase construct -116MKP-1luc or with a thimidine kinase promoter-luciferase indicator plasmid (pGl3tkluc). In addition the cells were co-transfected with either wt GR or two different dimerisation/transactivation deficient GR mutants expression vectors, A458T or hGR(D4X), as indicated. A Renilla luciferase construct was also co-transfected in all samples as an internal control for transfection efficiency. Treatment with Dexamethasone (Dex, 10^{-7} M) or solvent alone (ethanol, EtOH) was performed 4 h after transfection and the cells harvested 48 h later for luciferase activity measurement. The results are expressed as levels of firefly luciferase expression after correcting for the transfection efficiency by Renilla luciferase measurement (relative luciferase activity) and are presented as the mean ± SD of three independent experiments.

3.3.3 Dual mechanism of regulation of MKP-1 expression by glucocorticoids

In the comparison between glucocorticoid-induced MKP-1 increased gene expression and glucocorticoid-mediated inhibition of ERK1/2 phosphorylation, it was noticed that MKP-1 mRNA levels were upregulated already at 30 min of hormone incubation (Fig. 3.3A, compare lanes 4-5), but ERK1/2 dephosphorylation occurred only after a treatment with hormone for 16 h (Fig. 3.2, compare lanes 13-14). To clarify this discrepancy, the effect of glucocorticoids on MKP-1 protein was examined. Basal level of MKP-1 protein was detected in unstimulated RBL-2H3 mast cells (Fig. 3.7, lane 1). Upon 1 to 24 h of Dexamethasone incubation, MKP-1 protein levels were enhanced in non-activated cells (Fig. 3.7, compare lanes 3-4, 7-8, 11-12 and 15-16), in agreement with the increased mRNA level of MKP-1 (refer to Fig. 3.3A). However, upon activation of ERK1/2 *via* Fc□RI triggering, MKP-1 protein was not anymore

detectable (Fig. 3.7, lanes 5, 9, 13 and 17). Even the slight activation caused by IgE sensitisation alone (refer to Fig. 3.2 lane 2) was sufficient to lead to the disappearance of MKP-1 protein (Fig. 3.7, lane 2). In activated mast cells MKP-1 protein levels were still not detectable after treatment with Dexamethasone for 1 or 5 h (Fig. 3.7, lanes 6 and 10). However, upon prolonged treatment (16-24 h), MKP-1 protein levels were again elevated (Fig. 3.7, lanes 14 and 18). This shows that glucocorticoids protect against the loss of MKP-1 protein following activation of mast cells. This result is consistent with findings previously published (Kassel et al., 2001) in which it is shown that upon cell activation, MKP-1 protein is rapidly degraded by the ubiquitin/proteasome pathway. A 16-24 h treatment with glucocorticoid attenuates the degradation process, so that MKP-1 protein is available to eventually repress ERK1/2.

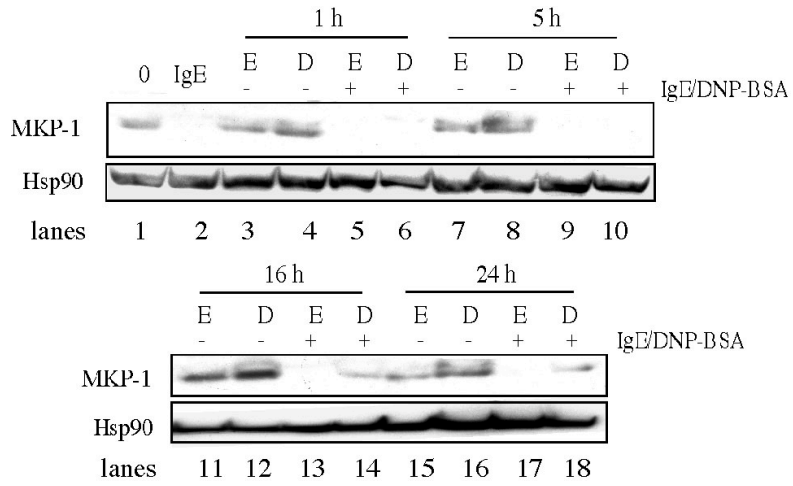

Figure 3.7 Glucocorticoids protect against degradation of MKP-1 protein in activated RBL-2H3 mast cells. Sub-confluent RBL-2H3 mast cells were incubated with Dexamethasone (D, 10^{-7} M) or with solvent alone (ethanol, E) for the indicated times, then sensitised by incubation for 1 h with IgE anti-DNP (0.5 μg/ml) and activated by DNP-BSA (0.3 μg/ml) for 10 min (+) or not (-). Cell lysates were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and MKP-1 protein was detected by immunoblotting with a specific antibody. An antibody against Hsp90 was used in immunoblotting to demonstrate equal protein loading. Lane 1 (0) corresponds to untreated cells. Lane 2 (IgE) corresponds to cells only sensitised 1 h with IgE anti-DNP (0.5 μg/ml).

3.4 Glucocorticoids do not alter the mRNA levels of R-zeta, R-delta, MSS1, RCX and RN3 proteasomal subunits

Glucocorticoid attenuation of the proteasomal degradation of MKP-1 could occur at different levels. Dexamethasone could prevent the initial steps of the proteasomal degradation pathway (i.e. ubiquitination or proteasomal enzymes activity) or it could also affect the expression of the proteasomal subunits, thus interfering with the function of the proteasomal machinery. In the gene expression array analysis, the expression of the zeta chain (R-zeta), delta chain (R-delta), X subunit (RCX), mammalian suppressor of sgv1 (MSS1) and beta-type N3 (RN3) proteasomal subunits were altered upon Dexamethasone treatment (refer to Table 3.1). To confirm the effect of Dexamethasone on the expression of these genes, RBL-2H3 mast cells were treated with Dexamethasone or solvent alone for 15 min to 24 h and RN3, R-delta, R-zeta, RCX and MSS1 transcripts levels were detected in Northern Blot analysis. In all cases, glucocorticoids failed to cause any significant alteration of mRNA levels at any of the time points checked (Fig. 3.8). These analyses therefore could not confirm the Affymetrix gene expression array analysis results. From these data, it appears unlikely that attenuation of degradation of MKP-1 protein by glucocorticoids occurs *via* alteration of expression of the proteasomal subunits analysed. Other mechanisms may instead account for it (for instance, interference with the initial steps of the proteasomal degradation pathway) and these remain to be identified.

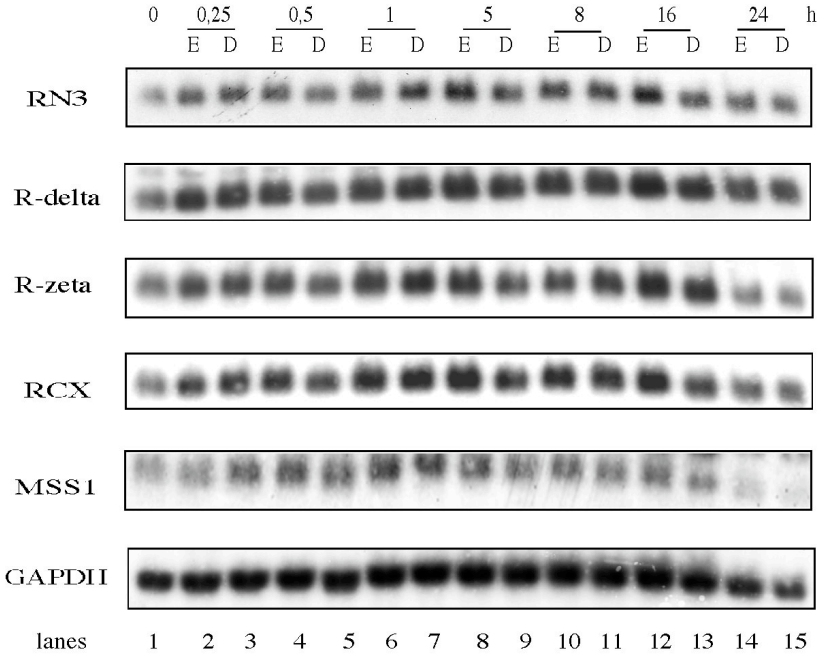

Figure 3.8 Glucocorticoid treatment does not alter the mRNA levels of RN3, R-delta, R-zeta, RCX and MSS1 proteasomal subunits in RBL-2H3 mast cells. RBL-2H3 mast cells were treated with Dexamethasone (D, 10^{-7} M) or solvent alone (ethanol, E) for the indicated times. Total RNA was extracted, resolved on MOPS/formaldehyde 1% agarose gel (15 □g/lane), subjected to Northern Blot hybridisation using specific [□^{32}P]-labelled cDNA probes for RN3, R-delta, R-zeta, RCX and MSS1. Lane 1 (0) refers to untreated cells. The membrane was stripped and re-hybridised with a probe for GAPDH as a RNA loading control.

3.5 Effect of glucocorticoids on ERK1/2 phosphorylation in cells derived from MKP-1 knock-out mice

A confirmation of the role of MKP-1 in the mediation of ERK1/2 inhibition by glucocorticoids in mast cells can be obtained from the analysis of the effect of glucocorticoids on ERK1/2 activity in mast cells derived from mice lacking MKP-1. Bone marrow-derived mast cells (BMMCs) were isolated from both wild type (C57Bl/6) mice and C57Bl/6 mice in which MKP-1 gene had been disrupted (Dorfman et al., 1996). BMMCs were pre-treated with Dexamethasone or with solvent alone for 16 h, followed by treatment with different stimuli leading to ERK1/2 activation such as Stem Cell Factor/c-kit ligand (SCF) or IgE/antigen

treatment. In Fig. 3.9(A,B) representative Western blots are shown, for cells activated with SCF or with IgE/DNP-BSA, respectively. Incubation of BMMCs with SCF caused phosphorylation of ERK1/2 in both wild type (wt) and MKP-1(-/-) cells (Fig. 3.9A, compare lane 1 with 3 for wt mast cells; lane 5 with 7 for MKP-1(-/-) mast cells). Activation of ERK1/2 is inhibited if wt cells are treated with Dexamethasone for 16 h prior to activation (Fig. 3.9A, compare lanes 3-4). This repression is instead totally absent in the MKP-1(-/-) BMMCs (Fig. 3.9A, compare lanes 7-8). The lack of Dexamethasone-mediated ERK1/2 repression in the MKP-1(-/-) BMMCs can be likewise observed in case IgE/DNP-BSA is used to activate ERK1/2 (Fig. 3.9B, compare lanes 3-4 for wt mast cells and 7-8 for MKP-1(-/-) mast cells). These studies confirm that MKP-1 plays an essential role in Dexamethasone-mediated repression of ERK1/2.

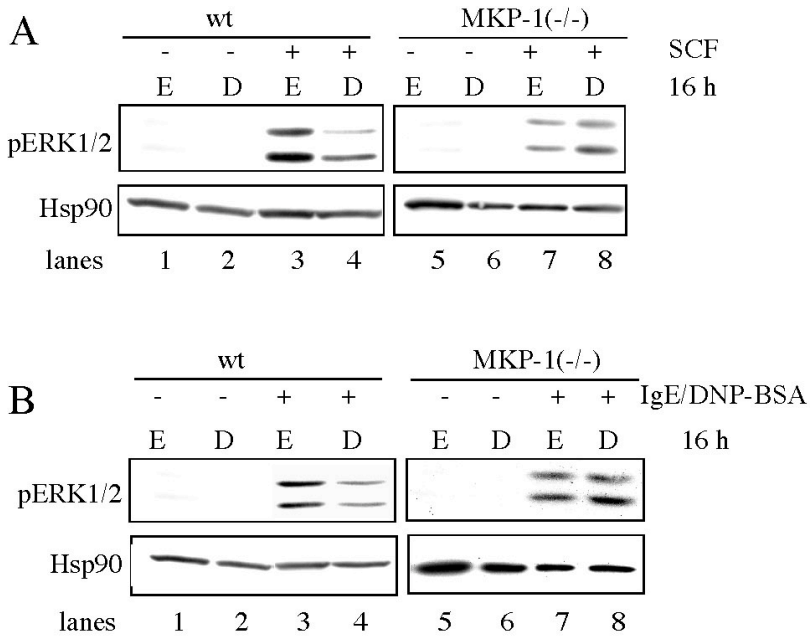

Figure 3.9(A, B) Glucocorticoids repress ERK1/2 activity in wt BMMCs but not in MKP-1(-/-) BMMCs. BMMCs were starved overnight by withdrawal of IL-3 from the culture medium and during this period treated with Dexamethasone (D, 10^{-7} M) or with solvent alone (ethanol, E) for the indicated time; the cells were then activated with SCF/c-kit ligand (100 ng/ml) for 10 min **(A)** or sensitised with IgE anti-DNP (0.5 µg/ml) for 4 h and activated with DNP-BSA (0.3 µg/ml) for 10 min **(B)**. Cells were subsequently harvested, lysed and the cell lysates were subjected to SDS-PAGE and immunoblottig with an antibody against dual phosphorylated ERK1/2. The membranes were incubated with an antibody anti-Hsp90 to demonstrate equal protein loading.

The effect of glucocorticoids on ERK1/2 phosphorylation in thymocytes and splenocytes derived from MKP-1(-/-) mice was also examined and found to be different as compared to mast cells. Thymocytes and splenocytes were pre-treated for 5-24 h with Dexamethasone or with solvent alone. Treatment with ethanol or with Dexamethasone of non activated cells had no effect on the basal level of phosphorylated ERK1/2 both in wt and in MKP-1(-/-) cells (Fig. 3.10(A,B), compare lanes: 1-2, 5-6, 9-10, 13-14). ERK1/2 activation was achieved by administration of phorbol ester TPA in splenocytes and thymocytes both wt and MKP-1(-/-) (Fig. 3.10(A,B), lanes 3, 7, 11 and 15). In activated cells from the wt mice, 5 h of treatment with Dexamethasone had no effect on ERK1/2 phosphorylation (Fig. 3.10(A,B), compare lanes 7-8). Instead, inhibition of phosphorylation of ERK1/2 was clearly detectable after 24 h of hormone treatment, and also after 16 h though to a lesser extent, in both thymocytes and splenocytes wt (Fig. 3.10(A,B), wt, compare lanes 11-12 and 15-16). In the activated MKP-1(-/-) thymocytes and splenocytes, a very slight repression, if any, of ERK1/2 phosphorylation upon 5 h of hormone treatment was observed (Fig. 3.10(A,B), compare lane 7-8). However, in contrast to what observed in BMMCs from the same animals, treatment with Dexamethasone for 16-24 h could still repress ERK1/2 phosphorylation both in MKP-1(-/-) thymocytes and MKP-1(-/-) splenocytes, though to a lesser extent in splenocytes (Fig. 3.10(A,B), compare lane 11-12 and 15-16). This finding shows that the MKP-1-mediated inhibition of ERK1/2 phosphorylation is cell-type specific and that in cell types other than mast cells, mechanisms different from MKP-1 up-regulation must account for the glucocorticoid-mediated repression of ERK1/2 activity.

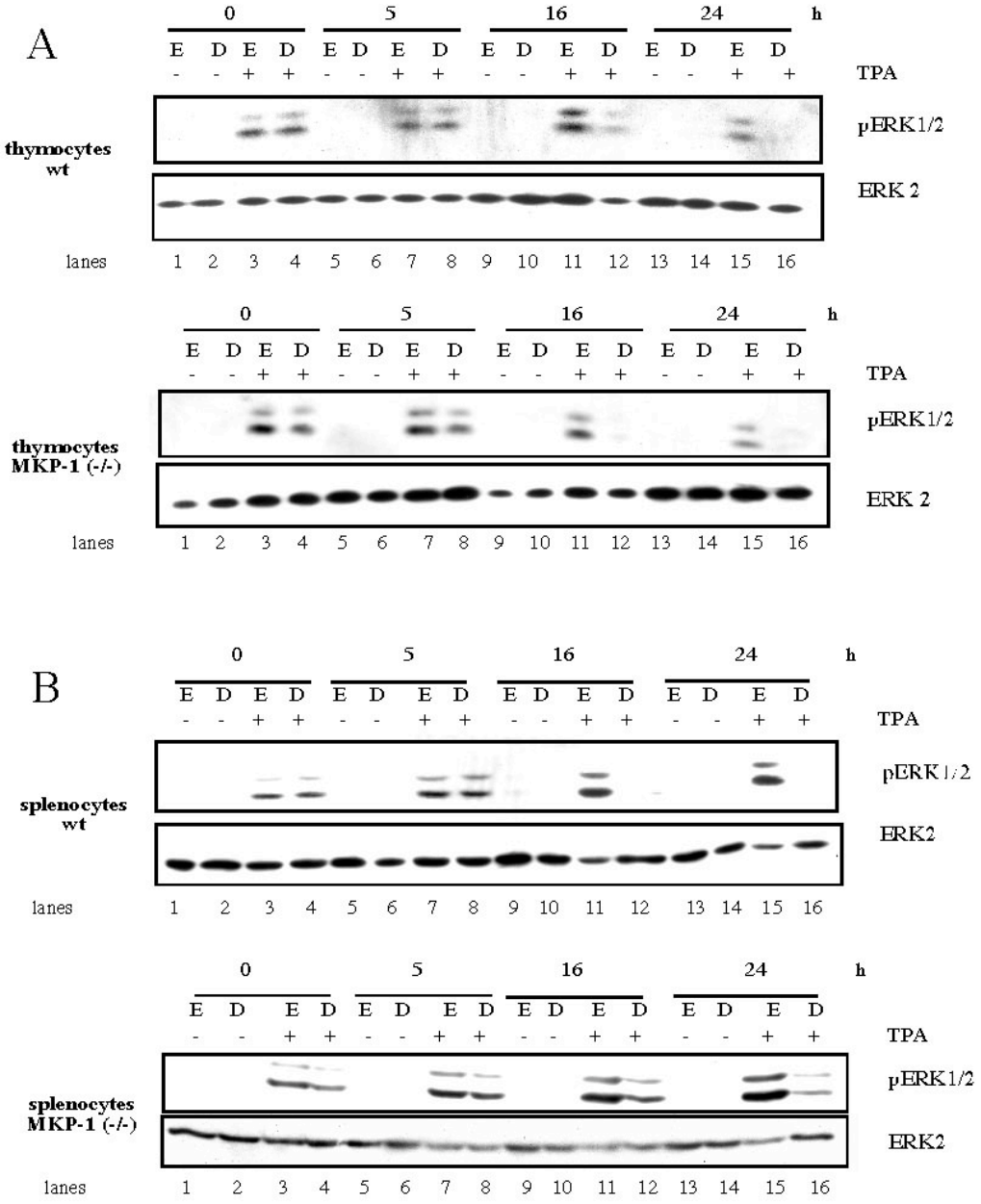

Figure 3.10(A, B) Glucocorticoids repress ERK1/2 activity in both wt and MKP-1(-/-) primary thymocytes and primary splenocytes. Thymi or spleens were removed from wt (C57Bl/6) and MKP-1(-/-) mice and cell suspensions were obtained by mechanical disruption of the organs. Erythrocytes were eliminated from the cell suspension using ACK lysis buffer. Cells were kept in culture for 24-48 h, pre-treated for the indicated times with Dexamethasone (D, 10^{-7} M) or with solvent alone (ethanol, E) and then activated with the phorbol ester TPA (80 ng/ml) for 10 min. Cells were then harvested, lysed and subjected to SDS-PAGE and immunoblotting with an anti-phosphoERK1/2 antibody. The membranes were stripped and incubated with an antibody that recognises ERK 2 independently from its phosphorylation state, to demonstrate equal protein loading.

3.6 Glucocorticoids down-regulate mRNA levels of the alpha chain of the high affinity receptor for IgE

In addition to MKP-1, the alpha chain of the receptor with high affinity for IgE (FcεRIα) was also identified as a target for regulation by glucocorticoids in the Affymetrix gene expression array analysis. This was particularly interesting, since FcεRI is the key molecule in the initiation of IgE-mediated allergic reactions and mast cells functional response to antigen can be influenced significantly by its level of expression (Yamaguchi et al., 1997; Yamaguchi et al., 1999). As a first step to analyse the action of glucocorticoids on FcεRIα, Northern Blot analyses were carried out to confirm the gene expression array analysis results. It could be confirmed that the mRNA levels of FcεRIα in RBL-2H3 mast cells decreased upon 16-24 h of Dexamethasone treatment (Fig. 3.11A, compare lanes 12-13, 14-15), whereas no alteration of mRNA levels was detectable after shorter times of hormone treatment (Fig. 3.11A, lanes 1-11). As a control, the mRNA levels of the beta chain (FcεRIβ) and of the gamma chain (FcεRIγ) were similarly examined, but they were not significantly affected by Dexamethasone treatment at any of the time points examined (Fig. 3.11B and Fig. 3.11C). Note that lanes 12-13 in Fig. 3.11(A,B) were loaded with more RNA. The apparent downregulation of the FcεRIγ in lane 5 Fig. 3.11B is an artifact due to the bad quality of the blotting/transfer process. These results indicate that glucocorticoid treatment selectively decreases the levels of FcεRIα mRNA in RBL-2H3 mast cells.

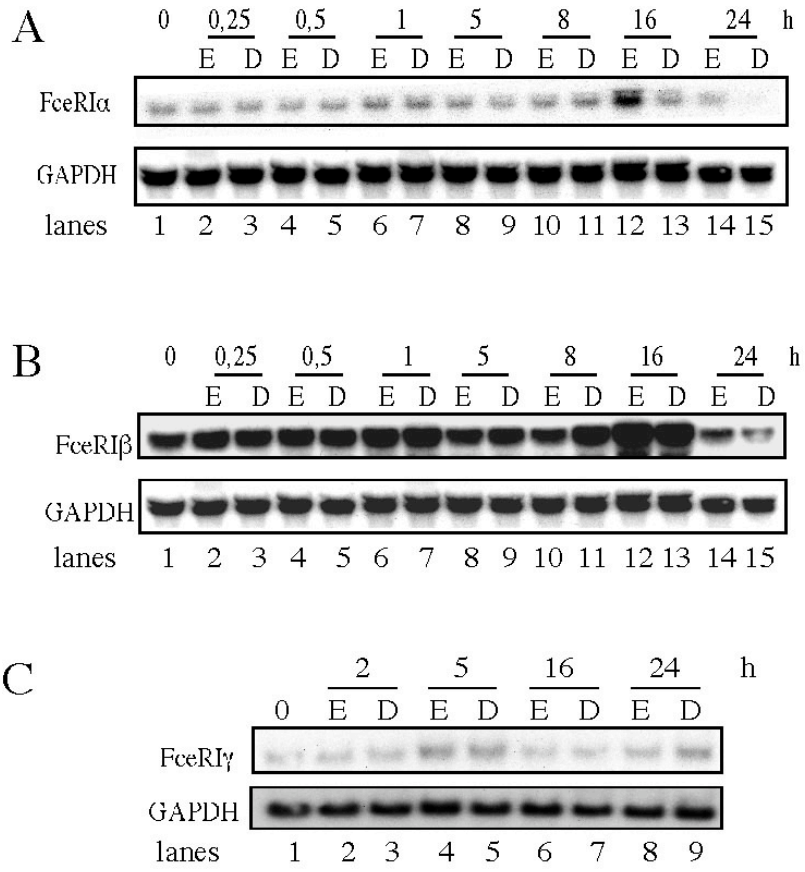

Figure 3.11 Glucocorticoids down-regulate mRNA levels of FcεRIα (A) but not of FcεRIβ (B) and FcεRIγ (C) in RBL-2H3 mast cells. Sub-confluent RBL-2H3 mast cells were treated with Dexamethasone (D, 10^{-7} M) or solvent alone (ethanol, E) for the indicated times. Lanes 1 (0) indicate untreated cells. Total RNA was extracted, resolved on a 1% agarose/MOPS gel (15 µg/lane) and subjected to Northern Blot analysis using specific [α-^{32}P]-labelled cDNA probes for either FcεRIα or FcεRIβ or FcεRIγ. The membranes were stripped and re-hybridised with a probe for GAPDH to show equal RNA loading.

3.6.1 Glucocorticoid-mediated down-regulation of mRNA levels of the FcεRI alpha chain gene requires *de novo* protein synthesis

The long time needed by glucocorticoids to bring about downregulation of FcεRIα mRNA levels suggested that it was not a primary response of the GR but could instead be mediated by an unknown factor. To ascertain whether ongoing protein synthesis was required for glucocorticoid-mediated decrease in FcεRIα mRNA, experiments were performed in which RBL-2H3 mast cells were treated simultaneously with Dexamethasone and the protein synthesis inhibitor cycloheximide (CHX) for 16 h. In the absence of cycloheximide, glucocorticoids

downregulated the level of FcεRIα specific trascript (Fig. 3.12, compare lanes 2-3). On the contrary, in presence of cycloheximide, Dexamethasone-mediated down-regulation of FcεRIα mRNA level was abrogated (Fig. 3.12, compare lanes 5-6), showing that new protein synthesis is required for the glucocorticoid-mediated negative regulation of FcεRIα gene expression.

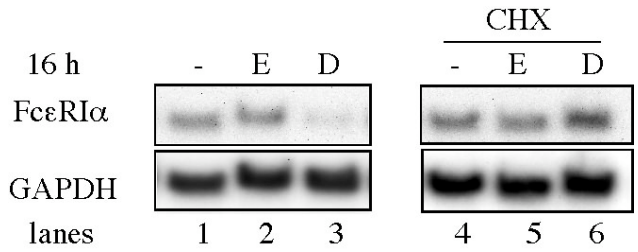

Figure 3.12 *De novo* protein synthesis is required for glucocorticoid-mediated down-regulation of FcεRIα mRNA levels in RBL-2H3 mast cells. Sub-confluent RBL-2H3 mast cells were treated with Dexamethasone (D, 10^{-7} M) or solvent alone (ethanol, E) for 16 h in presence or absence of cycloheximide (CHX) (5 μg/ml) as indicated. Total RNA was extracted, resolved on 1% agarose/MOPS gel (15 μg/lane) and subjected to Northern Blot analysis using a specific [α-^{32}P]-labelled cDNA probe for FcεRIα. The membranes were stripped and re-probed for GAPDH as a RNA loading control.

3.6.2 Glucocorticoids downregulate the expression of the FcεRIα gene at the promoter level

The requirement for *de novo* protein synthesis could indicate that glucocorticoids mediate the negative regulation of the FcεRIα gene expression by inducing the synthesis of a protein that could in turn either affect the stability of FcεRIα mRNA or the expression of the FcεRIα gene at the promoter level. To discriminate between these two possibilities, it was first examined whether the stability of the FcεRIα mRNA was decreased following glucocorticoid treatment. RBL-2H3 mast cells were treated with Dexamethasone for 12 h prior to block of transcription by administration of Actinomycin D (ActD). The amount of FcεRIα mRNA remaining at different time points after block of transcription was compared to that of a control group treated with solvent alone by Northern Blot analysis. Twelve hours of Dexamethasone incubation reduced the level of specific FcεRIα transcript (Fig. 3.13A, compare lane 1 with 8). However, the rate of decay

of the RNA following block of transcription did not significantly vary between the Dexamethasone-treated and -untreated samples (Fig.3.13A, compare lanes 1-7 with lanes 8-14). For clarification, the signals of the Northern Blot analysis were quantified by exposure of the filters to a Phosphorimager screen and analysis with the AIDA 200.1 software. These values were corrected relative to the intensity of the internal GAPDH control and reported on the graph in Fig. 3.13B. Best-fitting lines were obtained with the software Cricket Graph. It can be seen on the graphic (dotted lines) that the time needed for achieving a 50% reduction of the initial amount of mRNA, in the time window examined, did not significantly vary between the Dexamethasone-treated and -untreated samples. It appears therefore unlikely that glucocorticoids destabilise Fc RI mRNA.

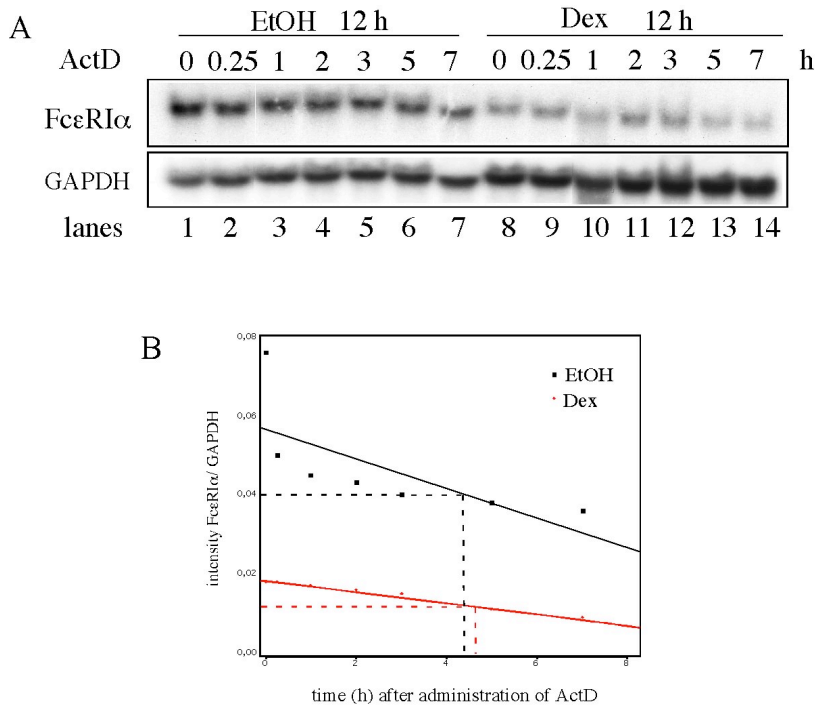

Figure 3.13(A,B) Glucocorticoids do not significantly destabilise Fc RI mRNA in RBL-2H3 mast cells. (A) RBL-2H3 mast cells were treated with Dexamethasone (D, 10^{-7} M) or solvent alone (ethanol, EtOH) for 12 h and subsequently with Actinomycin D (ActD, 10 g/ml) to block transcription. Total RNA was extracted at the times indicated, resolved on 1% agarose/MOPS gel (15 g/lane) and subjected to Northern Blot analysis using a specific [^{32}P]-labelled cDNA probe for Fc RI. The membrane was stripped and re-probed for GAPDH as a loading control. **(B)** The hybridised filter of fig.3.13A was exposed to a Phosphorimager screen and analysed with the AIDA 200.1 software, in order to quantitate the intensity of the [^{32}P] signal of specific mRNA, normalised for the internal GAPDH control. The obtained values were reported on the graphic and best fitting lines for Dexamethasone-treated and -untreated groups were obtained with the software Cricket Graph.

In addition to the analysis of FcεRIα mRNA stability, we investigated whether FcεRIα gene transcription was affected by glucocorticoid treatment. To do this, promoter activity of the FcεRIα gene upon Dexamethasone treatment was determined in transient transfection experiments. Studies with transgenic mice suggested that the sequences 1.3 kb upstream of the FcεRIα-coding region contained all the elements necessary for cell type-specific expression of the FcεRIα in mouse cells (Fung-Leung et al., 1996). RBL-2H3 mast cells were therefore transfected with a series of reporter constructs containing progressive 5´deletion mutants of the FcεRIα promoter, from -2192 to -13 nucleotides upstream of the transcriptional start site, driving the expression of the firefly luciferase gene (described in Nishiyama et al., 1999). An expression vector encoding Renilla luciferase was also co-transfected in all samples as an internal control to normalise the firefly luciferase expression values. In absence of hormone, different levels of activity were determined for the different promoter constructs (Fig.3.14B, lanes 7, 9, 11, 13, 15, 17 and 19). Upon Dexamethasone treatment, the expression of the reporter gene was repressed for all constructs up to the construct containing -88 nucleotides upstream of the promoter (Fig. 3.14B, lanes 9-20). The expression of the construct containing only the -13 bp portion of the promoter was comparable to that of the empty vector and unaffected by hormone treatment (Fig. 3.14B, compare lanes 7-8 with 1), as expected because this construct does not contain any transcription modulatory element. Dexamethasone had no effect on the expression of plasmid containing a glucocorticoid-insensitive promoter (such as the β-actin promoter) in front of the luciferase gene (Fig 3.14A, compare lanes 5-6), demonstrating the specificity of glucocorticoids regulation on the FcεRIα promoter. As a positive control for Dexamethasone efficacy, the expression of luciferase driven by the mouse mammary tumour virus (MMTV) promoter, whose activity is known to be enhanced upon glucocorticoid treatment, was as expected positively regulated by Dexamethasone (Fig. 3.14A, compare lanes 3-4). These results show that glucocorticoids specifically repress the activity of the FcεRIα promoter, and that

the promoter proximal sequences between -88 and -13 appear to be sufficient to mediate the negative regulation by glucocorticoids.

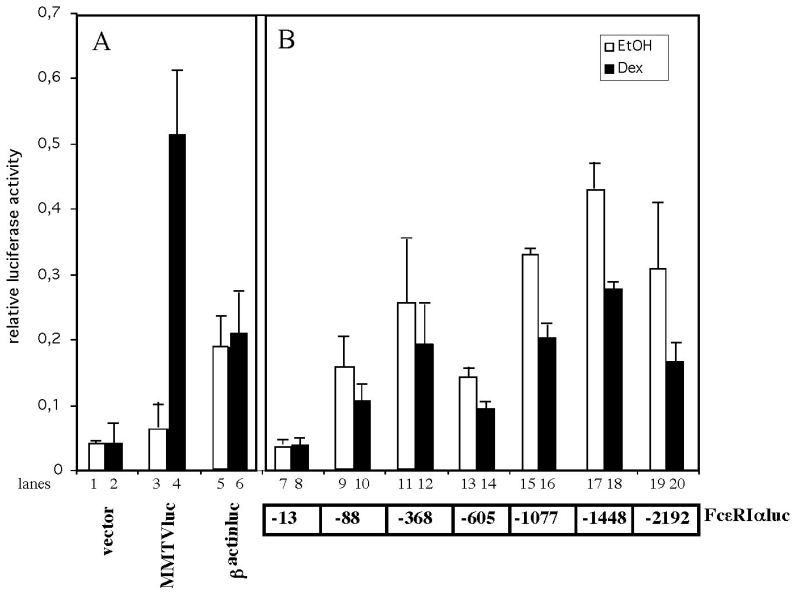

Figure 3.14(A,B) Glucocorticoids repress the activity of the FcεRIα promoter. RBL-2H3 cells ($5 \cdot 10^6$cells /point) were transiently transfected with 15 μg of either of the constructs containing a portion of the FcεRIα promoter of variable length (indicated at the bottom of the chart) controlling the expression of the firefly luciferase gene (FcεRIαluc) or with a promoterless luciferase expression vector (vector) or with an MMTV promoter-luciferase expression plasmid (MMTVluc) or with a βactin promoter-luciferase expression plasmid (βactinluc). Each sample was co-transfected with 5 μg of a Renilla luciferase expression vector whose expression level was used as an internal standard to normalise the values of firely luciferase expression. Transfection was performed by electroporation at 500 μF and 300 V in a total volume of 200 μl. After transfection the cells were resuspended in 8 ml fresh medium and divided into two groups. The cells were allowed to recover for 24 h and then treated with Dexamethasone (Dex) or with solvent alone (ethanol, EtOH). After additional 30 h, the cells were harvested and luciferase activity was measured. The results are expressed as levels of firefly luciferase expression after normalising for the Renilla luciferase expression (relative luciferase activity) and are presented as the mean ± SD of three independent experiments.

In order to determine whether specific sequences within the -88/-13 segment were required to mediate glucocorticoids action, further analysis where undertaken. The -88/-13 segment of the FcεRIα promoter contains binding sites for transcription factors such as GATA-1, Elf-1, PU.1 and YY1 which were shown to be involved in the regulation of expression of the FcεRIα gene (Nishiyama et al., 2002; Nishiyama et al., 1999). To determine whether these elements are necessary to mediate Dexamethasone-induced repression of promoter activity, reporter constructs were used, in which the binding sites of these transcription factors within a -605 FcεRIα promoter fragment cloned in front of the firefly luciferase

gene had been disrupted by site directed mutagenesis (Nishiyama et al., 2002). In Fig.3.15 the nucleotide sequence from -91 to -20 of the FcεRIβ promoter is shown. In the same picture, the mutated sequences for each of the expression vectors used, named M1-9, are also indicated.

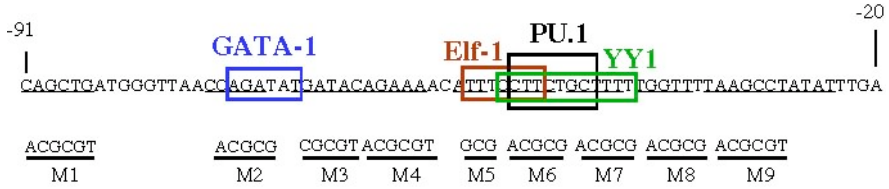

Figure 3.15 The M1-9 reporter constructs contain mutated portions of the FcεRIβ promoter. In the M1-9 constructs, the region of the FcεRIβ promoter comprising -605 bp from the transcriptional initiation site has been cloned in front of the firefly luciferase gene. This region has been diversely mutated by site directed mutagenesis (Nishiyama et al., 2002). Some of these mutations specifically disrupt the regulatory elements known to be necessary for the expression of the FcεRIβ gene, such as the binding sites for the transcription factors GATA-1, Elf-1, PU.1 and YY1.

Each of the M1-9 indicator plasmids (or the wt) was transfected into RBL-2H3 cells. In the absence of hormone, the promoter activity of the different constructs varied. While the expression of M1, M3, M4, M8 and M9 reporter genes was comparable to the wt (Fig. 3.16, compare lane 1 to lanes 3, 7, 9, 17, 19), the promoter activity of M2, M5, M6 and M7 (particularly the latter three) was reduced (Fig. 3.16, lanes 5, 11, 13 and 15). This was observed also by Nishiyama et al., (2002). It should be noted that the mutation in M2 disrupts the GATA-1 binding site, in M5 disrupts Elf-1 binding site, in M6 disrupts PU.1, Elf-1 and YY1 binding sites, in M7 disrupts YY1 and PU.1 binding site. These are transcription factors known to be important for the expression of the FcεRIβ gene (Nishiyama et al., 2002). In the presence of glucocorticoids, all the constructs, with the exception of M2, M6, M7 and possibly M5, showed dimished promoter activity (Fig. 3.16, compare lanes in pairs). It must be noted that the low expression level of M5, M6 and M7 makes the interpretation of glucocorticoids' effect less obvious. These studies indicate that the wt sequences which have been mutated in M2, M6 and M7 and possibly M5 are required by glucocorticoids to accomplish negative regulation of the FcεRIβ promoter activity. It appears that

disruption of each of the M2, M6 and M7 sequences alone is sufficient to abrogate Dexamethasone-mediated reduction of FcεRIα promoter activity. At this stage, these results suggest that glucocorticoids might regulate the activity of the FcεRIα promoter through regulation of the expression and/or activity of GATA-1 and/or PU.1 and/or YY1 and/or Elf-1. To establish the relative contribution of each of these factors to Dexamethasone-mediated repression of FcεRIα promoter activity, further studies are necessary.

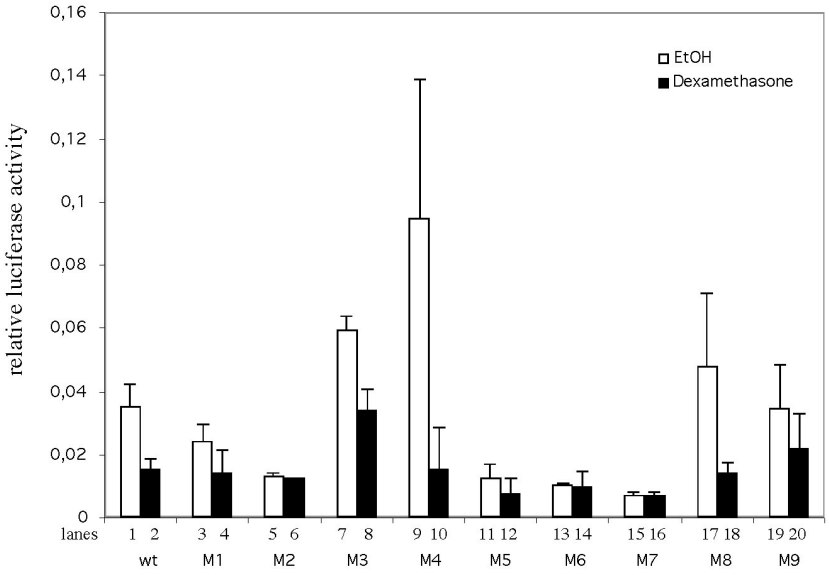

Figure 3.16 Disruption of regulatory elements in the -88/-13 region of the FcεRIα promoter abrogates glucocorticoid-mediated repression of promoter activity. RBL-2H3 mast cells ($5 \cdot 10^6$ cells/point) were transiently transfected with 15 μg of either of the M1-9 luciferase expression plasmids containing 605 bp upstream of the transcriptional initiation site of the FcεRIα promoter variously mutated in front of the firefly luciferase gene. A Renilla luciferase expression plasmid (5 μg) was also transfected in all samples and its expression was used as a measure of transfection efficiency. Transfection was performed by electroporation at 500 μF and 300 V in a total volume of 200 μl. After transfection the cells were resuspended in 8 ml fresh medium and divided into two groups. The cells were allowed to recover for 24 h and then treated with Dexamethasone or with solvent alone (ethanol, EtOH). After additional 30 h, the cells were harvested and luciferase activity was measured. The results are expressed as levels of firefly luciferase expression after normalising for the Renilla luciferase expression (relative luciferase activity) and are presented as the mean ± SD of three independent experiments.

3.7 Glucocorticoids down-regulate FcεRI surface expression

The expression of the FcεRI on cell surface is optimally fulfilled when all the subunits (α, β and γ) are expressed adequately (Blank U. et al., 1989; Ra C. et al., 1989). Therefore, a decreased expression of any of the subunits could in principle

affect the function of the whole receptor. It has been shown that homozygous mice with disrupted FcεRIα gene, do not express cell surface FcεRI (Dombrowicz et al., 1993). The glucocorticoid-mediated downregulation of expression of the FcεRI α gene in mast cells is therefore likely to have dramatic consequences on the surface expression of the entire receptor. This notion was investigated by the use of Fluorescence Activated Cell Sorter (FACS) analysis, to detect variations in surface expression of FcεRI upon glucocorticoid treatment.

RBL-2H3 mast cells were incubated with Dexamethasone for different periods of time, from 24 to 72 h. To detect surface expressed FcεRI, the cells were incubated with IgE and subsequently with a anti-IgE FITC-conjugated antibody and analysed by FACS. In Fig. 3.17A, representative FcεRI surface expression profiles after three different periods of incubation with Dexamethasone are shown. Upon 24, 48 and 72 h of Dexamethasone treatment, the fluorescence signal clearly decreases (shifts towards the left) indicating a decrease in FcεRI surface expression in a time dependent manner. In the bar chart of Fig 3.17B, the mean ± Standard Deviation of five independent FACS analyses is presented. In these experiments, along with 24, 48 and 72 h, a treatment of 1 h with Dexamethasone was included, which proved not to be sufficient to cause a decrease in surface expression of FcεRI. This correlates with the mRNA data regarding the alpha chain (it was previously presented that downregulation of FcεRI alpha chain expression in mast cells requires at least 12-16 h of glucocorticoid treatment). Dexamethasone treatment caused a reduction of FcεRI surface expression of about 10% after 24 h of hormone treatment, which progressed to 40% reduction after 48 h hormone treatment and to 60% by 72 h. To show that the glucocorticoid receptor (GR) is necessary for the Dexamethasone-mediated downregulation of surface expression of the FcεRI, in the same experiments RBL-2H3 cells were treated simultaneously with hormone and the glucocorticoid-receptor antagonist RU-486 in a ten-fold molar excess or with RU-486 alone as a control for specificity of the effect. Dexamethasone-induced repression of FcεRI surface expression was partially reversed by simultaneous treatment with RU-486, indicating the involvement of the GR in the inhibitory process. Taken together, these results show that the transcriptional

downregulation of the alpha chain of the FcεRI by glucocorticoids correlates with a reduction of functional FcεRI on the cell surface. Furthermore, the decrease in surface expression of the FcεRI is mediated by the GR.

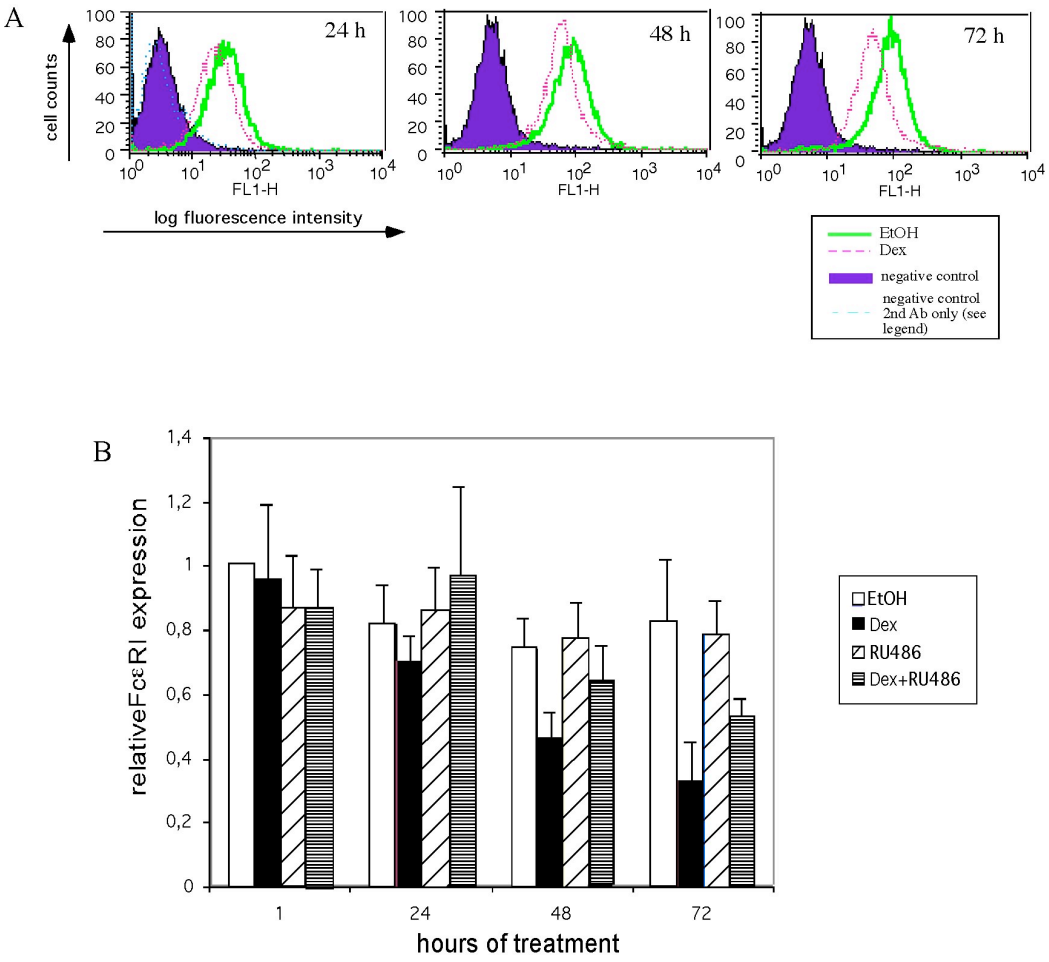

Figure 3.17(A,B) Glucocorticoids reduce the surface expression of FcεRI in RBL-2H3 mast cells. (A) Representative FcεRI surface expression profiles upon Dexamethasone or ethanol treatment for the indicated times. RBL-2H3 mast cells were treated with Dexamethasone (Dex, 10^{-7}M) or with solvent alone (ethanol, EtOH) for the indicated times, incubated with IgE (0.5 µg/µl) for 1 h at 4°C, washed, harvested, $5 \cdot 10^5$ cells/point were resuspended in 200 µl of FACS buffer (PBS, 2% FCS), incubated for 20 min at 4°C in the dark with anti-IgE FITC conjugated antibody (dilution 1:50) and analysed on a Fluorescence Activated Cell Sorter (FACS). Ten thousand cells were analysed per point. The solid violet curve represents the fluorescence detected on untreated and unstained cells and is considered as zero. The dotted blue line (shown only in the 24 h profile) represents the fluorescence detected on untreated cells stained only with anti-IgE FITC antibody and is a measure of unspecific staining. **(B)** RBL-2H3 mast cells were treated with Dexamethasone (Dex, 10^{-7}M) or with solvent alone (ethanol, EtOH) or with RU486 in a ten-fold molar excess relative to Dexamethasone or with RU486 and Dexamethasone for the indicated times. The cells were then incubated with IgE for 1 h at 4°C, washed, harvested, $5 \cdot 10^5$ cells/point were resuspended in 200 µl of FACS buffer (PBS, 2% FCS), incubated for 20 min at 4°C in the dark with anti-IgE FITC conjugated antibody and analysed on a Fluorescence Activated Cell Sorter. Number of cells gated positive at one hour of ethanol treatment was set to 1 and the other values were calculated as relative to this control value. The bar chart represents the mean ± SD of five independent experiments.

3.8 Early signalling events following FcεRI cross-linking

Downregulation of the surface expression of FcεRI would be expected to have a dramatic effect on subsequent downstream signalling events. Thus, signalling molecules situated downstream of the receptor were analysed, to ascertain whether glucocorticoid treatment would have an effect on the signal transduction events originating at the receptor level. Following IgE/antigen-mediated cross-linking of the FcεRI, the first molecular event which occurs is the activation of the lck/yes-related novel (Lyn) kinase, a member of the Src family of protein-tyrosine kinases (Yamashita et al., 1994; Eiseman and Bolen, 1992). Lyn, like other members of the Src family kinases, possesses a negative regulatory phosphotyrosine (Tyr 507) located near the C-terminus. This phosphotyrosine interacts with the SH2 domain at the N terminus of the same protein to prevent the catalytic site of the kinase from accessing the external kinase substrates. In non activated cells, Lyn is kept in an inactive state by phosphorylation of Tyr (507), at its C-terminal regulatory domain, by the c-src tyrosine kinase (Csk) (Ohtake et al., 2002). Upon receptor aggregation, Lyn is dephosphorylated on Tyr(507) by CD45 phosphatase and autophosphorylated on Tyr(396), thus becoming activated (Thomas et al., 1999). Lyn subsequently phosphorylates the β and γ chains of the receptor (Wofsy et al., 1999; Pribluda et al., 1994). These phosphorylation events allow further propagation of the signal to ERK1/2.

3.8.1 Glucocorticoids enhance phosphorylation of Lyn on the regulatory tyrosine (507)

In order to examine whether glucocorticoid-mediated reduction in surface expressed FcεRI correlated with reduced activity of the tyrosine kinase Lyn, experiments were performed in which the phosphorylation state of Lyn upon Dexamethasone treatment was examined. RBL-2H3 mast cells were incubated with Dexamethasone or solvent alone for 48 h. This time point was chosen

because it was shown to be sufficient to cause downregulation of the surface levels of the IgE receptor (Fig. 3.17). Since the events leading to activation of Lyn occur very rapidly after receptor aggregation (Eiseman and Bolen, 1992), the kinetics of Tyr(507)Lyn phosphorylation upon cell activation from 15 s to 10 min were examined. Tyrosine-phosphorylated proteins were precipitated from the lysates by incubation with a specific anti-phosphotyrosine antibody. Lyn is present in mast cells in two isoforms, of 53 and 56 kDa, which represent the products of two alternatively spliced *lyn* transcripts, previously described in murine myeloid cells (Yi et al., 1991). The cell lysates were examined in immunoblotting with an antibody that specifically recognises Tyr(507) phosphorylated 53/56 kDa Lyn isoforms. Basal Lyn phosphorylation was detectable and was not affected by IgE sensitisation (Fig.3.18, lanes 1 and 2). Upon cell activation in ethanol-treated samples, a transient enhancement of Lyn phosphorylation could be detected (Fig. 3.18, compare lane 1 with 3-8). This is in agreement with a report that showed that upon Fc RI aggregation, the Csk-binding protein (Cbp/PAG) relocates Csk, the tyrosine kinase responsible of Tyr(507) phosphorylation on Lyn, in the vicinity of aggregated Fc RI, thus repressing the protein tyrosine kinase activity of the receptor-associated Lyn and consequently mediating feedback inhibition of Fc RI signalling (Ohtake et al., 2002). Dexamethasone treatment, however, further enhanced Tyr(507) Lyn phosphorylation in activated cells very rapidly after cell activation (Fig. 3.18, compare lanes 3-8 with respective time points in lanes 9-14).

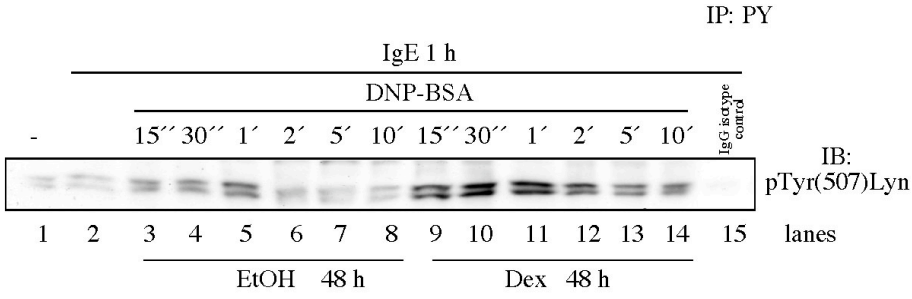

Figure 3.18 Glucocorticoids enhance phosphorylation of the regulatory tyrosine Tyr(507) of Lyn kinase in RBL-2H3 mast cells. Sub-confluent RBL-2H3 cells were incubated with Dexamethasone (Dex, 10^{-7} M) or with solvent alone (ethanol, EtOH) for 48 h, sensitised for 1 h with IgE anti-DNP (0.5 g/ml) and activated by DNP-BSA (0.3 g/ml) for the indicated times. Cells were harvested in PY lysis buffer and tyrosine (Y) phosphorylated proteins were precipitated from

the cell lysates by incubation with a specific mouse monoclonal anti-phosphotyrosine(PY) antibody followed by incubation with protein A agarose beads. The precipitated beads were resuspended in 2X Laemmli buffer, subjected to SDS-PAGE and immunoblotting with an antibody that recognises the Tyr(507) phosphorylated 53/56 kDa isoforms of Lyn. Lane 15 represents the negative control; an isotype-matched (IgG_{2b}) antibody was used in immunoprecipitation instead of the anti-PY to ascertain the specificity of the immunoprecipitation process.

When the levels of Lyn protein were examined in cell lysates from RBL-2H3 mast cells incubated with Dexamethasone or solvent alone for 48 h and activated with IgE/DNP-BSA for various times, no significant change upon Dexamethasone treatment was observed (Fig. 3.19, lanes 1-16). Taken together, these results indicate that glucocorticoid treatment leads to increased Tyr (507)phosphorylation of Lyn without affecting Lyn protein levels. This might in turn lead to a reduced activity of Lyn.

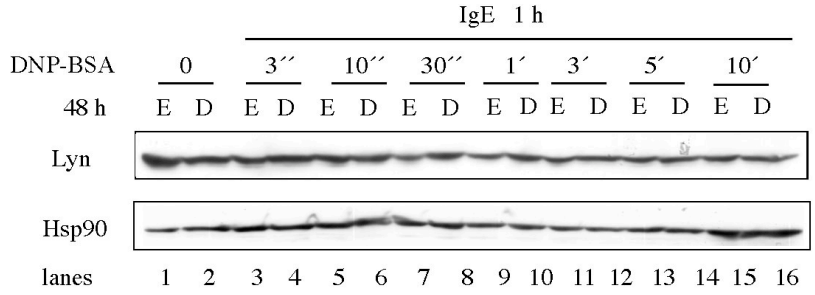

Figure 3.19 Glucocorticoid treatment does not significantly alter protein levels of Lyn kinase in RBL-2H3 mast cells. Sub-confluent RBL-2H3 cells were incubated with Dexamethasone (D, 10^{-7} M) or with solvent alone (ethanol, E) for 48 h, sensitised for 1 h with IgE anti-DNP (0.5 µg/ml) and activated by DNP-BSA (0.3 µg/ml) for the indicated times. Cells were harvested, subjected to SDS-PAGE and immunoblotting with an antibody that recognises the 56 kDa isoform of Lyn. The membranes were also incubated with an antibody anti-Hsp90 to demonstrate equal protein loading.

3.8.2 Glucocorticoids repress Ras activity

The results presented so far show that glucocorticoids downregulate the surface expression of the receptor for IgE on mast cells and promote the inactivation of the tyrosine kinase Lyn. As FcεRI phosphorylation by Lyn primes signal transduction, it is likely that also the activity of molecules situated downstream of the FcεRI would be affected by Dexamethasone treatment. One of these downstream molecules is Ras, which was shown to be activated following antigen

incubation in mast cells (Graham et al., 1998). The Ras protein belongs to a large super-family of proteins known as low-molecular weight guanosine tri-phosphatases. These proteins bind guanine nucleotides (GTP/GDP). Unstimulated Ras is found complexed with GDP, which is exchanged for a GTP upon stimulation *via* exchange factors (reviewed in Geyer and Wittinghofer, 1997). Active GTP-Ras is required to bring the serine/threonine kinase Raf to the membrane. Raf is then activated and phosphorylates MEK (Morrison and Cutler,1997), which is in turn responsible for further signal trasduction to ERK1/2 (reviewed in Williams and Roberts, 1994).

The effect of glucocorticoid treatment on the activity of Ras was therefore examined. RBL-2H3 cells were incubated with Dexamethasone or with solvent alone for 1 or 48 h, starved overnight to reduce basal level of activated Ras, sensitised for 1 h at 37°C with IgE anti-DNP and activated by DNP-BSA for 10 min or not. Because only activated Ras binds Raf-1, activated Ras was separated from inactive Ras by affinity precipitation from whole cell lysates with a Raf-1-GST (Ras-binding domain) peptide conjugated to agarose and thereafter detected by Western immunoblotting with a specific anti-Ras antibody. Upon cell activation, there was an increase in activated Ras of about two-fold (Fig. 3.20, compare lane 1 with 3). This is in agreement with observations made by other authors (Graham et al., 1998). Dexamethasone treatment did not change the basal level of activated Ras in non-activated cells after 1 h but could decrease it after 48 h of treatment (Fig. 3.20, compare lanes 1-2 and 5-6). Similarly, in activated cells Dexamethasone treatment at 1 h could not repress the level of activated Ras, but clearly repressed it upon 48 h incubation (Fig. 3.20, compare lanes 3-4 and 7-8). It appears, therefore, that a prolonged treatment with Dexamethasone is able to repress Ras activation. It is to be expected that this repression would impair further signal transduction towards ERK1/2, indicating yet another check-point where glucocorticoids inhibit signal transduction in mast cells.

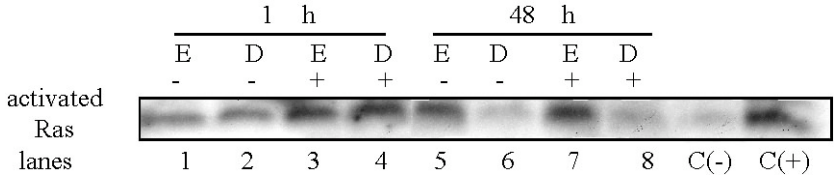

Figure 3.20 Glucocorticoids reduce the levels of activated Ras in RBL-2H3 cells. RBL-2H3 mast cells were incubated with Dexamethasone (D, 10^{-7} M) or with solvent alone (ethanol, E) for 1 or 48 h as indicated, starved overnight in DMEM without FCS, sensitised for 1 h at 37°C with IgE anti-DNP (0.5 µg/ml) and activated by DNP-BSA (0.3 µg/ml) for 10 min (indicated by a +) or not (indicated by a -). Cells were harvested in MLB buffer (*Upstate, Hamburg,* Ras activation Kit) and activated Ras was precipitated using a specific Raf-1 RBD agarose-bound (*Upstate, Hamburg,* Ras activation Kit) antibody. After incubation at 4°C for 1 h, the beads were collected and resuspended in Laemmli sample buffer. The supernatants were subjected to SDS-PAGE and immunoblotting with an anti-Ras antibody bound (*Upstate, Hamburg,* Ras activation Kit). Lane C(+) was obtained by in vitro GTPγS protein loading of Ras of cell lysates from untreated RBL-2H3 mast cells, which activates almost 100% of the Ras that can be activated in the cell lysate. Lane C(-) instead was obtained by in vitro GDP protein loading of the same lysates.

3.9 Correlation of decreased surface expressed FcεRI and diminished activated Ras levels/enhanced Lyn inactivation

Decreased surface expressed FcεRI seemed to correlate with decreased levels of activated Ras and with increased levels of inactivated Lyn upon 48 h of glucocorticoids treatment. To clarify whether there was indeed a correlation, levels of activated Ras and of Tyr(507)-phosphorylated Lyn were examined after 24 h of hormone treatment, a time point at which reduction of surface receptor is still minimal. RBL-2H3 cells were incubated with Dexamethasone or with solvent alone for 24 h, sensitised for 1 h at 37°C with IgE anti-DNP and activated by DNP-BSA for 10 min. Several observations can be made. First, ERK1/2 are activated upon IgE/antigen trigger (Fig. 3.21A/pERK1/2, compare lane 1 with 3) and ERK1/2 phosphorylation is repressed upon Dexamethasone treatment, as expected (Fig. 3.21A/pERK1/2, lanes 3-4). Second, Tyr(507) phosphorylation of Lyn is not affected by 24 h of Dexamethasone treatment (Fig. 3.21A/p(Tyr507)Lyn, lanes 3-4). Lyn protein levels are shown to exclude that any effect observed on phospho(Tyr507)Lyn is due to unequal loading (Fig. 3.21A/Lyn). Third, levels of activated Ras are upregulated upon cell activation as expected (Fig. 3.21B, compare lane 1 with 3) and downregulated upon 24 h of Dexamethasone treatment (Fig. 3.21B, lanes 3-4). It seems therefore that while inactivation of Lyn correlates with the kinetics of surface expressed FcεRI

downregulation by glucocorticoids, repression of Ras activity does not. Moreover, it appears that downregulation of surface expressed FcεRI and inactivation of Lyn are not indispensable for repression of ERK1/2 activity by glucocorticoids. This seems to point to the conclusion that glucocorticoids are able to act at multiple levels in mast cells in order to achieve an effective repression of ERK1/2 phosphorylation.

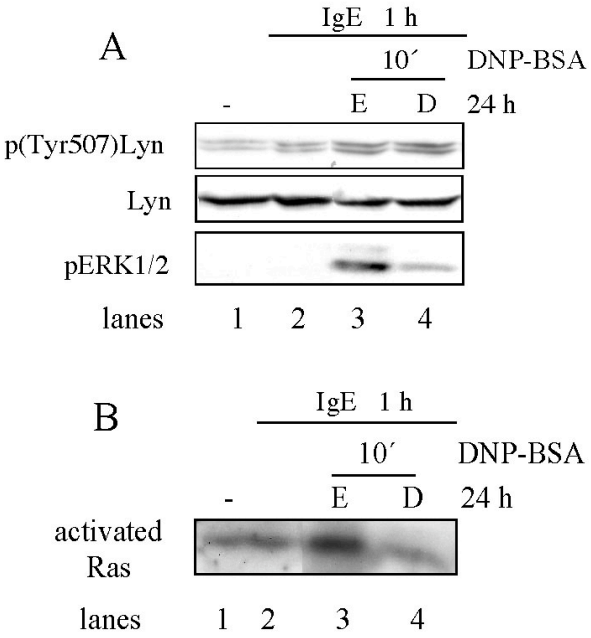

Figure 3.21 (A,B) Glucocorticoid treatment of 24 h reduces levels of activated Ras but is not sufficient to enhance Tyr(507) phosphorylation of Lyn in RBL-2H3 mast cells. RBL-2H3 mast cells were incubated with Dexamethasone (D, 10^{-7} M) or with solvent alone (ethanol, E) for 24 h, sensitised for 1 h at 37°C with IgE anti-DNP (0.5 μg/ml) and activated by DNP-BSA (0.3 μg/ml) for 10 min. For Ras activation assay, cells were deprived of serum overnight. In panel **A**, cells were harvested in lysis buffer, subjected to SDS-PAGE and immunoblotting with an antibody that recognises the Tyr(507) phosphorylated 53/56 kDa isoforms of Lyn, stripped and reprobed with an antibody that recognises the 56 kDa isoform of Lyn. The membrane were also incubated with an antibody anti-phosphoERK1/2. In panel **B**, were harvested in MLB buffer (*Upstate, Hamburg,* Ras activation Kit) and activated Ras was precipitated using a specific Raf-1 RBD agarose-bound (*Upstate, Hamburg,* Ras activation Kit) antibody. After incubation at 4°C for 1 h, the beads were collected and resuspended in Laemmli sample buffer. The supernatants were subjected to SDS-PAGE and immunoblotting with an anti-Ras (*Upstate, Hamburg,* Ras activation Kit) antibody.

4. DISCUSSION

In this work, mast cells were chosen as a system to elucidate the mechanisms of glucocorticoids interference with signalling pathways. It was presented here that a prolonged treatment of mast cells with the synthetic glucocorticoid Dexamethasone represses the phosphorylation of the ERK1/2 MAP kinases.

In mast cells, MAP kinases (MAPK) cascades are initiated upon aggregation of the receptor with high affinity for IgE (FcεRI) (Ishizuka et al., 1996; Ishizuka et al., 1997) and variously contribute to eliciting mast cells biological responses such as release and synthesis of pro-inflammatory mediators (Hirasawa et al., 1995; Zhang et al.,1997; Song et al., 1999; Hirasawa et al., 2000), all processes which have been shown to be repressed by glucocortiocoids (Daeron et al., 1982; Rider et al., 1996; Eklund et al., 1997; Sewell et al., 1998). Repression of MAPK pathways could be therefore reasonably proposed as one of the mechanisms glucocorticoids make use of to repress mast cells effector functions. It has already been shown, for instance, that expression of several proinflammatory cytokine genes in response to IgE is markedly reduced in embryonic stem cell-derived mast cells with targeted disruption of the MAPKK kinase that regulates the JNK and ERK5 pathways, MEKK-2 (Garrington et al., 2000). Inhibition of JNK activity by glucocorticoids has been shown and proposed as a mechanism for glucocorticoid-induced suppression of TNF-α production (Swantek et al., 1997).

ERK1/2 had also been identified as targets for negative regulation by glucocorticoids (Rider et al., 1996). However, how the inhibition was accomplished had not been clarified. Here, evidence is provided that glucocorticoids are able to repress ERK1/2 in mast cells following activation with the calcium mobilising agent Thapsigargin, with the phorbol ester TPA which activates ERK1/2 *via* protein kinase C (PKC) and by IgE/antigen-induced cross-linking of the FcεRI. The first point of convergence for all these pathways is the MAPKK kinase Raf, so that it can be argued that repression of ERK1/2 is brought

about by glucocorticoids at the level of Raf or further downstream. This claim was in agreement with the experimental observations by other authors (Rider et al., 1996; Cissel and Beaven, 1999).

Activation of ERK1/2 with IgE/antigen challenge was chosen for further studies, because it is the one that mimicks the physiological mode of activation of mast cells during allergic processes. Kinetic studies of ERK1/2 repression upon glucocorticoid treatment were performed, which revealed that 16-24 h of glucocorticoid treatment could repress ERK1/2 phosphorylation, whereas shorter incubations (1-5 h) could not. This finding, together with the reported need for *de novo* protein synthesis (Kassel at al., 2001), suggested that the mediation of other factor(s) was required for glucocorticoids to accomplish ERK1/2 repression.

4.1 Inhibition of ERK1/2 phosphorylation via the MAPK phosphatase (MKP)-1 in mast cells

Glucocorticoid treatment in RBL-2H3 mast cells induced the expression of the dual specificity MAP kinase phosphatase MKP-1. MKP-1 had previously been identified as the phosphatase responsible of dephosphorylation of ERK1/2 in mast cells (Kassel et al., 2001). In conditions of diminished levels of MKP-1, glucocorticoid-mediated ERK1/2 dephosphorylation was impaired (Kassel et al., 2001). A confirmation of the crucial role of MKP-1 in the dephosphorylation of ERK1/2 is presented in this study making use of MKP-1 knock-out mice. In these mice, targeted disruption of the MKP-1 gene had not revealed any apparent developmental defect (Dorfman et al., 1996). However, in primary mast cells from these animals, prolonged treatment with glucocorticoids failed to induce ERK1/2 dephosphorylation, whereas this was still the case in wild type mice. Treatment with glucocorticoids could repress ERK1/2 phosphorylation both if the cells were activated with IgE/antigen trigger and with stem cell factor (SCF), which acts through the c-kit receptor and can activate ERK1/2 in mast cells (Wershil et al., 1992). Again, these two pathways converge at the level of Raf (Ishizuka et al., 1999), indicating that MKP-1-mediated dephosphorylation of ERK1/2 might depend from events taking place at this level or downstream.

On the contrary, when cell populations derived from lymphoid tissue (spleen and thymus) of MKP-1(-/-) animals were analysed, glucocorticoid treatment with Dexamethasone was still able to cause dephosphorylation of ERK1/2, as effectively as in the wild type. This finding shows that the inhibition of ERK1/2 phosphorylation by glucocorticoids is cell-type specific and agrees with previous observations made with established cell lines (Kassel et al., 2001). To describe which molecules are instead responsible for glucocorticoid-mediated repression of ERK1/2 activity in thymocytes, a gene expression array analysis on thymocytes cDNA will be performed in which the differential regulation of genes upon administration of glucocorticoids will be compared in wild type versus MKP-1(-/-) cells.

Studies aimed at describing the mechanism through which glucocorticoids modulate MKP-1 expression showed that enhanced expression of MKP-1 gene by glucocorticoids is accomplished through enhancement of MKP-1 promoter activity. Careful analysis of the MKP-1 promoter sequence (Noguchi et al., 1993) revealed the presence of several putative glucocorticoid response elements (GREs). GREs are specific palindromic sequences, which were shown to mediate increased transcription of target genes upon glucocorticoid treatment (reviewed in Beato et al., 1989). Deletion of three of these sequences from the MKP-1 promoter abolished glucocorticoid-mediated increased promoter activity, as observed in transient transfection assays. It is known that the hormone-liganded GR binds to GREs as a homodimer. In transient transfection experiments it could be shown that dimerisation-defective GR mutants were no longer able to increase MKP-1 promoter activity. These results, together with the observation that treatment with the glucocorticoid antagonist RU486 abolished the Dexamethasone-mediated induction of MKP-1 expression (Kassel et al., 2001) presumably indicate that MKP-1 is a classical glucocorticoid-responsive gene.

How specific is the regulation of ERK1/2 phosphorylation by MKP-1? Also the mRNA levels of another phosphatase, MKP-3, belonging to the same family of dual specificity phosphatases as MKP-1 and shown to be able to inactivate ERK1/2 (Muda et al., 1996a and 1996b; Groom et al., 1996), were increased in

RBL-2H3 mast cells upon glucocorticoid-treatment. However, differently from MKP-1, regulation of MKP-3 gene expression by glucocorticoids was not observed in primary mast cells, thus could not be considered a general mechanism of regulation by glucocorticoids in mast cells. Furthermore, the kinetics of MKP-3 protein expression upon hormone treatment did not correlate with the kinetics of ERK1/2 dephosphorylation (data not shown), thus allowing to exclude the involvement of this phosphatase in glucocorticoid-mediated repression of ERK1/2 activity in mast cells.

The regulation of expression of other phosphatase genes with an established role in regulation of signalling pathways which involve ERK1/2 upon glucocorticoid administration was also examined. The haematopoietic protein-tyrosine phosphatase (HePTP) had been shown to act directly on ERK1/2 and to reduce the magnitude and duration of their catalytic activation in intact T cells (Saxena et al., 1999). The Src homology 2-containing protein tyrosine phosphatase-1 (SHP-1) had been shown to function as a negative regulator of the T cell antigen receptor and to dephosphorylate and inactivate tyrosine kinases such as ZAP-70 (Plas et al., 1996). The gene expression of both these phosphatases was not altered in response to glucocorticoids in RBL-2H3 mast cells, thus showing that the regulation of gene expression by glucocorticoids is limited only to selected tyrosine phosphatases.

4.2 Dual mechanism of glucocorticoid-mediated MKP-1 up-regulation

MKP-1 was shown to be the main effector of glucocorticoid-mediated ERK1/2 dephosphorylation. However, the up-regulation of MKP-1 mRNA levels is apparent as early as after 0.5 h from hormone administration, while ERK1/2 dephosphorylation requires a much longer glucocorticoid incubation. When the level of MKP-1 protein was examined, interestingly it was found out that upon cell activation, MKP-1 is rapidly degraded. A prolonged treatment with glucocorticoids, however, protects against the degradation and restores MKP-1 levels thus leading to repression of ERK1/2. This is in agreement with previous findings that showed that levels of MKP-1 protein are influenced by

glucocorticoids in RBL-2H3 mast cells through a dual mechanism which includes both transcriptional upregulation of MKP-1 gene expression and attenuation of degradation of MKP-1 protein through the ubiquitin/proteasome pathway (Kassel et al., 2001). Both mechanisms are essential for glucocorticoid-mediated inhibition of ERK1/2 activity.

It has been not ruled out yet how glucocorticoids attenuate the proteosomal degradation of MKP-1. One possible mode of action of glucocorticoids in the attenuation of MKP-1 degradation by the proteasome, might be the repression of expression of certain components of the proteasome machinery. The 26S proteasome is a large proteolytic complex consisting of at least 31 subunits which form a proteolytic core (20S proteasome) capped at one or both ends by a regulatory 19S complex. Precise functions for many of the individual subunits remain undefined (reviewed in Bochtler et al., 1999 and Voges et al., 1999). The expression of several proteasomal subunits genes upon glucocorticoid treatment was examined. Of these, MSS1 (mammalian suppressor of sgv1), is a putative ATPase of the 19S regulatory complex, which has been shown, in addition to proteolysis, to play a role in DNA metabolism including transcriptional regulation (Yanagi et al., 2000). R- zeta is an alpha-type subunit of the 20S proteasome and RN3 is a beta-type subunit of the 20S proteasome displaying hydrolase activity (Kopp et al., 1995). The R-delta and RCX subunits have been cloned but no functional data are available (Tamura et al., 1992: Tanaka, 1995). These subunits appeared to be downregulated upon glucocorticoid treatment according to the gene expression array analysis. However, reassessment of these results via Northern Blot analysis failed to show any alteration of gene expression of any of them. It is therefore unlikely that attenuation of degradation of MKP-1 by glucocorticoids occurs through alteration of expression of the proteasomal subunits analysed. It cannot be excluded, however, that glucocorticoids affect the expression of other subunits; alternatively, glucocorticoids might prevent the initial steps of the proteasomal degradation pathway (i.e. ubiquitination) or inhibit proteasomal enzymes' activity.

4.3 Regulation of gene expression of the receptor with high affinity for IgE (FcεRI) by glucocorticoids

From the data presented untill now, glucocorticoid-mediated dual regulation of MKP-1 expression appears sufficient to cause repression of ERK1/2 after 16 h of hormone treatment. However, upon longer treatment, additional effects of glucocorticoids on mast cells can be observed, which might also play a role in inhibitig mast cells' effector functions. Glucocorticoids downregulate the surface expression of the receptor with high affinity for IgE (FcεRI) on mast cells. Upon 24 h of glucocorticoid treatment, only a very slight reduction of FcεRI is to be seen, which progresses in a time-dependent manner to a 60% reduction upon 72 h hormone application. It has been shown that there is a correlation between the abundance of receptor for IgE on cell surface and the extent of response to antigen stimulation by mast cells (Yano et al., 1997; Yamaguchi et al., 1999). Local or systemic anaphylactic reactions are abolished in FcεRI-deficient mice, showing that *in vivo* no other mechanism can compensate for the absence of FcεRI to permit IgE-mediated allergic reaction (Dombrowicz et al., 1993). Hence, downregulation of the FcεRI might have dramatic consequences on mast cells' biological response to allergens. Inhibition of surface expression of the FcεRI occurs with the mediation of the GR, as confirmed by the fact that the use of a GR antagonist (RU486) partially abrogated Dexamethasone-mediated inhibition of FcεRI surface expression. This possibly indicates that the GR mediates down-regulation of the FcεRI by acting on a direct target at earlier time points.

Glucocorticoid were shown to selectively downregulate the expression of the alpha chain of the receptor with high affinity for IgE (FcεRIα), by repressing its promoter activity, whereas the mRNA levels of the beta and gamma chains were not altered by glucocorticoid treatment. Other investigators observed no difference in the levels of any of the FcεRI subunits mRNA levels upon glucocorticoid treatment and therefore suggested that a post-translational mechanism might be responsible for the reduction of surface expressed receptors (Yamaguchi et al., 2001). It must be noted that in the work of Yamaguchi et al., levels of FcεRIα mRNA were examined after 4-12 h of hormone treatment, a time

perhaps not sufficient for an appreciable decrease in FcεRIα transcript level to be detected. It cannot be excluded, however, that also other mechanisms contribute to the downregulation of FcεRI surface expression. It has been shown that an important level of regulation of FcεRI surface expression is the stability of the IgE receptor on the cell surface (Furuichi et al., 1985; Turner and Kinet, 1999). It cannot be excluded that glucocorticoids interfere also at this level.

In the system described in this thesis, downregulation of the FcεRIα was seen only after 12-24 h of glucocorticoid treatment, thus correlating with the kinetics of inhibition of surface expressed FcεRI. Because only fully assembled receptors can be expressed on cell surface (Blank et al., 1989; Ra et al., 1989) negative regulation of expression of the alpha chain could account for decreased surface expression of FcεRI on mast cells. Several observations support the notion that the alpha chain would be the best candidate among the receptor subunits to constitute the limiting step in specifying the levels of FcεRI expression. The alpha chain is the only subunit which is at the same time specific for the FcεRI and absolutely required for its expression. In fact, the gamma chain is used to form also the receptors for IgG (Ernst et al., 1993) and IgA (Pfefferkorn and Yeaman, 1994) while the beta chain is not always required for expression of functional IgE receptors, as in human mast cells the trimeric receptor $\alpha\gamma_2$ is equally functional as the tetrameric $\alpha\beta\gamma_2$. Until present, the alpha chain is the only subunit described to be subjected to specific transcriptional regulation. IL-4 is able to transcriptionally up-regulate the expression only of the FcεRIα gene in human mast cells, and this correlates with increased surface expression of the whole FcεRI (Toru et al., 1996). Conversely, targeted disruption of the alpha chain in mice resulted in failed expression of FcεRI on mast cells (Dombrowicz et al., 1993).

The accomplishment of downregulation of the alpha chain requires 12-16 h of glucocorticoid treatment and *de novo* protein synthesis, as shown in experiments in which simultaneous treatment of RBL-2H3 mast cells with the synthetic glucocorticoid Dexamethasone and the protein synthesis inhibitor cycloheximide abolished glucocorticoid-mediated down-regulation of FcεRIα mRNA levels. Hence, this down-regulation is not likely to be a direct action of glucocorticoids,

rather might imply the mediation of a secondary factor. It has been shown that protein-RNA interaction is in some cases needed to mediate mRNA destabilisation (Garcia-Gras et al., 2000). However, from the analysis of FcεRIβ mRNA decay after block of transcription in presence or absence of glucocorticoids no clear evidence for destabilisation of FcεRIβ mRNA was observed. Instead, it could be shown that glucocorticoids repress FcεRIβ promoter activity and that regulatory elements in the promoter proximal region were involved in the regulation promoter activity by the GR. These elements constitute binding sites for the transcription factors GATA-1, Elf-1, PU.1 and YY1, which were shown to be involved in the regulation of expression of the FcεRIβ gene specifically in FcεRIβ producing cells (Nishiyama et al., 1999; Nishiyama et al., 2002). Mutations targeted to these sites in the FcεRIβ promoter sequence abrogated glucocorticoid-mediated repression of promoter activity, possibly indicating that glucocorticoids mediate downregulation of the FcεRIβ gene expression by affecting expression and/or activity of one or more of these transcription factors. Interestingly, all these transcription factors, with the exception of YY1 which is ubiquitously expressed, are expressed in a cell-type specific manner. GATA-1 is expressed in cells of the myeloid lineage (mast cells, basophils, eosinophils and megakaryocytes (Evans and Felsenfeld, 1989; Martin et al., 1990). PU.1 is expressed in mast cells, B cells, macrophages and neutrophils (Lloberas et al., 1999). Also Elf-1 is exclusively expressed in mast cells, T cells, B cells, megakaryocytes and macrophages (Nishiyama et al., 1999; Nishiyama et al., 2000). Since the FcεRI itself is selectively expressed only on limited number of cell populations (mainly mast cells and basophils, but also megakaryocytes/platelets, eosiniphils Langerhans cells and monocytes), it has been suggested that the cell-type specifity of the FcεRIβ is determined by cell-specific transcription factors (Nishiyama et al., 2002). In turn, by modulating the activity of these transcription factors, glucocorticoids might target only certain cell populations.

One of the possibility the GR has to interfere with transcription factors' activity is a direct protein-protein interaction which results in impeding the other

transcription factor's functionality. GATA-1 was shown to directly interact with the GR (Chang et al., 1993). Also PU.1 and GR were shown to negatively modulate each others' activity, although it couldn't be ruled out whether the interaction is direct or mediated (Gauthier et al., 1993). Glucocorticoids might interfere with these transcription factors also through other mechanisms, such as competition for overlapping binding sites, or competition for co-activators, or *via* nGREs. Additionally, since it has been shown that the complex formed by GATA-1 and its coactivator FOG-1 controls the expression of distinct sets of gene in megakaryocytes and erythroid cells (Wang et al., 2002), one could speculate that glucocorticoids might act on this coactivator in order to regulate GATA-1 function. Alternatively, glucocorticoids might control the expression of the transcription factors themselves. Which of these mechanisms exist(s) in the system described in this work, remains to be ruled out.

4.4 Glucocorticoids promote Lyn inactivation by increased phosphorylation on Lyn's regulatory tyrosine

An additional regulatory mechanism brought about by glucocorticoids in mast cells is the increased phosphorylation of the inactivating tyrosine residue on the lck/yes-related novel (Lyn) Src-like tyrosine kinase. Lyn is the first kinase that is activated in response to receptor aggregation and is found in asociation with the beta subunit of the receptor. Cross-linking of FcεRI in Lyn(-/-) mast cells failed to induce protein-tyrosine phosphorylation of Lyn substrates and provoked a delayed and slow Ca^{2+} mobilisation (Nishizumi and Yamamoto, 1997), indicating the importance of this molecule in mast cell signalling. Glucocorticoid-mediated increased phosphorylation of Lyn correlates with the inhibition of FcεRI surface expression, and it is therefore a late effect of glucocorticoids.

Activity of Lyn is regulated by phosphorylation on regulatory tyrosine (507). This phosphotyrosine interacts with the SH2 domain at the N terminus of the same protein to prevent the catalytic site of the kinase from accessing the external kinase substrates. However, the mechanism through which glucocorticoids increase phosphorylation on Lyn at Tyr(507) remains to be clarified. In non-

activated cells, phosphorylation of Tyr (507), through the action of the c-src tyrosine kinase Csk, keeps Lyn in an inactive state (Ohtake et al., 2002; Okada et al., 1991). Csk was shown to be implicated in the initiation and termination of the FcεRI-mediated Lyn activation; when overexpressed in RBL-2H3 cell line, Csk decreased the specific activity of Lyn at basal states and delayed FcεRI-mediated Lyn activation (Honda et al., 1997). In this study, however, levels of Csk did not vary upon glucocorticoid treatment (data not shown). Glucocorticoids might as well affect the activity of the kinase Csk but this has not yet been confirmed. The phosphatase CD45 is responsible of dephosphorylation of Lyn's regulatory tyrosine upon cell activation (Trowbridge and Thomas, 1994; Thomas et al., 1999). RBL cell lines expressing trace amounts of CD45 showed a reduced degranulation when stimulated with antigen and failed to enhance the protein tyrosine kinase activity associated with FcεRI following receptor aggregation, suggesting that CD45 is critical to activate the receptor associated kinase Lyn (Murakami et al., 2000). However, in the study presented here no downregulation of CD45 surface expression by glucocorticoids was observed (data not shown). The observed glucocorticoid-mediated promotion of Lyn inactivation cannot therefore depend on decreased availability of the phosphatase CD45 and another yet unknown mechanism must account for it.

4.5 Glucocorticoids decrease levels of activated Ras

Another effect of glucocorticoids was observed on the the small guanosine triphosphatase (GTPase) Ras. Following glucocorticoid treatment, Ras activity was clearly dowregulated. It is likely that impaired activation of Ras would affect in turn the activation of Raf-1, and this would then constitute an alternative mechanism of Raf-1 regulation by glucocorticoids in addition to the proposed glucocorticoid-mediated disruption of the Raf-1/Hsp90 complex which prevents Raf-1 from associating to Ras (Cissel and Beaven, 1999). The downregulation of levels of activated Ras does not seem to depend on impaired signallig from the receptor, because it is already observable after 24 h of glucocorticoid treatment, a

time at which downregulation of surface receptor is still minimal and increased inactivation of Lyn is not apparent.

4.6 Summary

The mechanisms of action of glucocorticoids in the regulation of FcεRI signalling in mast cells have been analysed. The data presented here, together with the existing literature, unravel the multifaceted action of glucocorticoids on mast cells. It appears that these hormones act at different levels: downregulation of surface expressed FcεRI, inactivation of Lyn kinase, repression of Ras activity and up-regulation of the MAPK phosphatase MKP-1 (summarised in Fig. 4.1). All these events might finally contribute to the repression of allergic and inflammatory response. The relative contribution of all these mechanisms to blockade of ERK1/2 activation is difficult to dissect. At the moment, it can be speculated that after 24 h of treatment, repression of ERK1/2 depends on the action of the phosphatase MKP-1 and to this effect also reduced levels of activated Ras contribute. At later time points, the glucocorticoid-mediated reduction of FcεRI levels and the inactivation of Lyn further contribute to attenuation of receptor signalling. With longer glucocorticoids incubation periods, all these effects might cumulate. By having more than one “check-point”, glucocorticoids probably ensure an effective repression of ERK1/2. Importantly, it also emerges from this study that certain effects of glucocorticoids are cell type-specific. This notion may be of importance for the optimisation of glucocorticoid-based therapy.

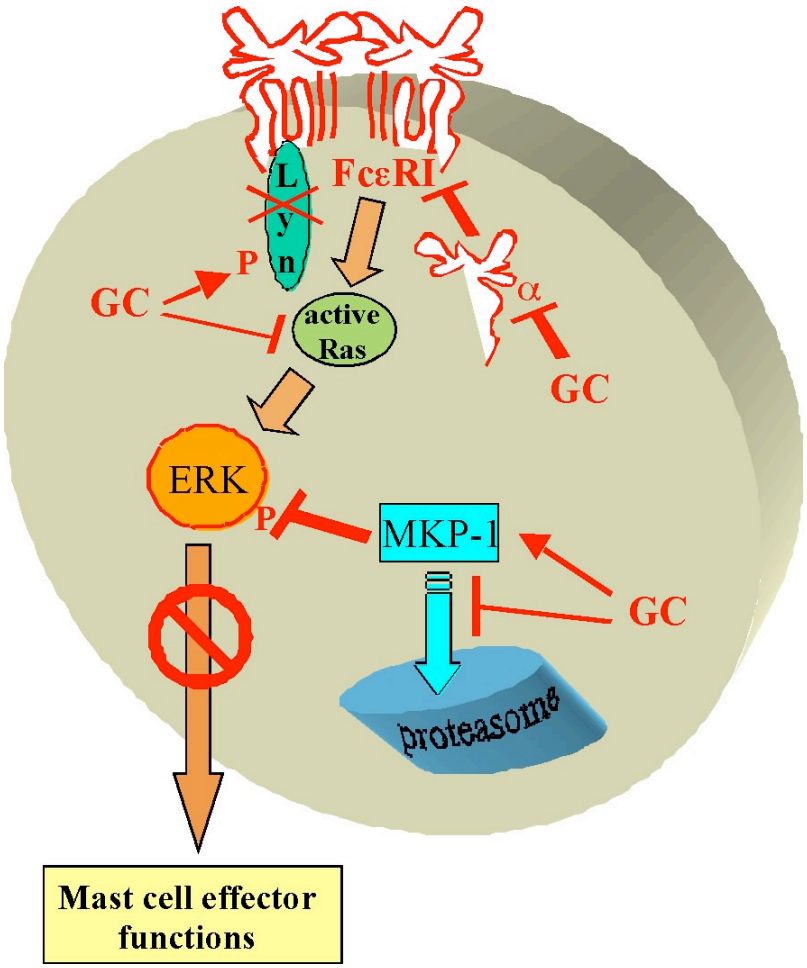

Figure 4.1 Glucocorticoids affect the expression or the functionality of several molecules involved in signal transduction in mast cells. In mast cells, glucocorticoids (GC) inhibit the expression of the gene for the alpha chain of the receptor with high affinity for IgE, by affecting its promoter activity. The downregulation of the alpha chain gene expression is correlated to a reduced surface expression of the whole receptor for IgE. GC treatment promotes inactivation of Lyn, the tyrosine kinase responsible of phosphorylation and activation of the Fc RI, *via* enhanced phosphorylation of the regulatory tyrosine residue of Lyn. Levels of activated Ras are reduced upon GC treatment. In addition, GC repress ERK1/2 function by a dual mechanism, that involves both enhancement of expression of the dual specificity tyrosine phosphatase MKP-1, responsible of dephosphorylation of ERK1/2 in mast cells, and attenuation of MKP-1 protein degradation via the ubiquitin/proteasome pathway. GC-mediated repression of ERK1/2 could account for the described repression of mast cell effector functions by GC.

REFERENCES

Abma EM, Blanken R, De Heide LJ**.** *Cushing's syndrome caused by topical steroid therapy for psoriasis*. Neth J Med. **2002** Apr;60(3):148-50.

Adcock IM, Caramori G. *Cross-talk between pro-inflammatory transcription factors and glucocorticoids*. Immunol Cell Biol. **2001** Aug;79(4):376-84.

Alessi DR, Smythe C, Keyse SM. *The human CL100 gene encodes a Tyr/Thr-protein phosphatase which potently and specifically inactivates MAP kinase and suppresses its activation by oncogenic ras in Xenopus oocyte extracts*. Oncogene. **1993** Jul;8(7):2015-20.

Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. *Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis*. Science. **1995** Oct 13;270(5234):286-90.

Ausubel FM, Brent R, Kingston RE, Moore DD. (eds) *Current protocols in Molecular Biology*. Greene publishing Associates and Wiley-Interscience, **1989**.

Azzolina A, Guarneri P, Lampiasi N. INVOLVEMENT OF p38 AND JNK MAPKs PATHWAYS IN SUBSTANCE P-INDUCED PRODUCTION OF TNF-alpha BY PERITONEAL MAST CELLS. Cytokine. **2002** Apr 21;18(2):72-80.

Barnes PJ, Adcock IM. *Transcription factors and asthma*. Eur Respir J. **1998** Jul;12(1):221-34.

Beato M. *Gene regulation by steroid hormones*. Cell. **1989** Feb 10;56(3):335-44.

Beato M, Chalepakis G, Schauer M, Slater EP. *DNA regulatory elements for steroid hormones*. J Steroid Biochem. **1989** May;32(5):737-47.

Beato M, Herrlich P, Schutz G. *Steroid hormone receptors: many actors in search of a plot*. Cell. **1995** Dec 15;83(6):851-7.

Berger SA, Mak TW, Paige CJ. *Leukocyte common antigen (CD45) is required for immunoglobulin E-mediated degranulation of mast cells*. J Exp Med. **1994** Aug 1;180(2):471-6.

Bhattacharyya A, Pathak S, Datta S, Chattopadhayay S, Basu J, Kundu M. *Mitogen-activated protein kinases and NF-kappaB regulate Helicobacter pylori-mediated interleukin-8 release from macrophages*. Biochem J. **2002** Aug 1 (ahead of print)

Blank U, Ra C, Miller L, White K, Metzger H, Kinet JP. *Complete structure and expression in transfected cells of high affinity IgE receptor*. Nature. **1989** Jan 12;337(6203):187-9.

Blank JL, Gerwins P, Elliott EM, Sather S, Johnson GL. *Molecular cloning of mitogen-activated protein/ERK kinase kinases (MEKK) 2 and 3. Regulation of sequential phosphorylation pathways involving mitogen-activated protein kinase and c-Jun kinase*. J Biol Chem. **1996** Mar 8;271(10):5361-8.

Bochtler M, Ditzel L, Groll M, Hartmann C, Huber R. *The proteasome*. Annu Rev Biophys Biomol Struct. **1999**;28:295-317.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD. *ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF*. Cell. **1991** May 17;65(4):663-75.

Broide DH. *Molecular and cellular mechanisms of allergic disease*. J Allergy Clin Immunol. **2001** Aug;108(2 Suppl):S65-71.

Brondello JM, Pouyssegur J, McKenzie FR. *Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation*. Science. **1999** Dec 24;286(5449):2514-7.

Caelles C, Gonzalez-Sancho JM, Munoz A. *Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway*. Genes Dev. **1997** Dec 15;11(24):3351-64.

Cambier JC. *New nomenclature for the Reth motif (or ARH1/TAM/ARAM/YXXL)* Immunol Today. **1995a** Feb;16(2):110;

Cambier JC. *Antigen and Fc receptor signaling. The awesome power of the immunoreceptor tyrosine-based activationmotif (ITAM)*. J Immunol. **1995b** Oct 1;155(7):3281-5.

Camps M, Nichols A, Arkinstall S. *Dual specificity phosphatases: a gene family for control of MAP kinase function*. FASEB J. **2000** Jan;14(1):6-16.

Cano E, Mahadevan LC. *Parallel signal processing among mammalian MAPKs*. Trends Biochem Sci. **1995** Mar;20(3):117-22.

Capewell S, Reynolds S, Shuttleworth D, Edwards C, Finlay AY. *Purpura and dermal thinning associated with high dose inhaled corticosteroids*. BMJ. **1990** Jun 16;300(6739):1548-51.

Carlstedt-Duke J, Stromstedt PE, Wrange O, Bergman T, Gustafsson JA, Jornvall H. *Domain structure of the glucocorticoid receptor protein*. Proc Natl Acad Sci U S A. **1987** Jul;84(13):4437-40.

Cato AC, Ponta H, Herrlich P. *Regulation of gene expression by steroid hormones*. Prog Nucleic Acid Res Mol Biol. **1992**;43:1-36.

Chang TJ, Scher BM, Waxman S, Scher W. *Inhibition of mouse GATA-1 function by the glucocorticoid receptor: possible mechanism of steroid inhibition of erythroleukemia cell differentiation*. Mol Endocrinol. **1993** Apr;7(4):528-42.

Cissel DS, Beaven MA. *Disruption of Raf-1/heat shock protein 90 complex and Raf signaling by dexamethasone in mast cells*. J Biol Chem. **2000** Mar 10;275(10):7066-70.

Clark J.K. et al., **1992**, *Mechanism of steroid hormones* In: J.D. Wilson and D.W. Foster (eds) Williams Textbook of Endocrinology pp 35-90, WB Saunders Philadelphia).

Cui CH, Adachi T, Oyamada H, Kamada Y, Kuwasaki T, Yamada Y, Saito N, Kayaba H, Chihara J. *The role of mitogen-activated protein kinases in eotaxin-induced cytokine production from bronchial epithelial cells*. Am J Respir Cell Mol Biol**. 2002** Sep;27(3):329-35.

Daeron M, Sterk AR, Hirata F, Ishizaka T. *Biochemical analysis of glucocorticoid-induced inhibition of IgE-mediated histamine release from mouse mast cells*. J Immunol. **1982** Sep;129(3):1212-8.

Dendukuri N, Blais L, LeLorier J. *Inhaled corticosteroids and the risk of diabetes among the elderly*. Br J Clin Pharmacol. **2002** Jul;54(1):59-64.

Dombrowicz D, Flamand V, Brigman KK, Koller BH, Kinet JP. *Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor alpha chain gene*. Cell. **1993** Dec 3;75(5):969-76.

Dorfman K, Carrasco D, Gruda M, Ryan C, Lira SA, Bravo R. *Disruption of the erp/mkp-1 gene does not affect mouse development: normal MAP kinase activity in ERP/MKP-1-deficient fibroblasts*. Oncogene. **1996** Sep 5;13(5):925-31.

Eiseman E, Bolen JB. *Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases*. Nature. **1992** Jan 2;355(6355):78-80.

Eklund KK, Humphries DE, Xia Z, Ghildyal N, Friend DS, Gross V, Stevens RL. *Glucocorticoids inhibit the cytokine-induced proliferation of mast cells, the high affinity IgE receptor-mediated expression of TNF-alpha, and the IL-10-induced expression of chymases*. J Immunol. **1997** May 1;158(9):4373-80.

Ernst LK, Duchemin AM, Anderson CL. *Association of the high-affinity receptor for IgG (Fc gamma RI) with the gamma subunit of the IgE receptor*. Proc Natl Acad Sci U S A. **1993** Jul 1;90(13):6023-7.

Evans T, Felsenfeld G. *The erythroid-specific transcription factor Eryf1: a new finger protein*. Cell. **1989** Sep 8;58(5):877-85.

Fung-Leung WP, De Sousa-Hitzler J, Ishaque A, Zhou L, Pang J, Ngo K, Panakos JA, Chourmouzis E, Liu FT, Lau CY. *Transgenic mice expressing the human high-affinity immunoglobulin (Ig) E receptor alpha chain respond to human IgE in mast cell degranulation and in allergic reactions*. J Exp Med. **1996** Jan 1;183(1):49-56.

Furuichi K, Rivera J, Isersky C. *The receptor for immunoglobulin E on rat basophilic leukemia cells: effect of ligand binding on receptor expression*. Proc Natl Acad Sci U S A. **1985** Mar;82(5):1522-5

Galli SJ, Gordon JR, Wershil BK. *Cytokine production by mast cells and basophils*. Curr Opin Immunol. **1991** Dec;3(6):865-72.

Garcia-Gras EA, Chi P, Thompson EA. *Glucocorticoid-mediated destabilization of cyclin D3 mRNA involves RNA-protein interactions in the 3'-untranslated region of the mRNA*. J Biol Chem. **2000** Jul 21;275(29):22001-8.

Garrington TP, Ishizuka T, Papst PJ, Chayama K, Webb S, Yujiri T, Sun W, Sather S, Russell DM, Gibson SB, Keller G, Gelfand EW, Johnson GL. *MEKK2 gene disruption causes loss of cytokine production in response to IgE and c-Kit ligand stimulation of ES cell-derived mast cells*. EMBO J. **2000** Oct 16;19(20):5387-95.

Gauthier JM, Bourachot B, Doucas V, Yaniv M, Moreau-Gachelin F. *Functional interference between the Spi-1/PU.1 oncoprotein and steroid hormone or vitamin receptors*. EMBO J. **1993** Dec 15;12(13):5089-96.

Gessani S, McCandless S, Baglioni C. *The glucocorticoid dexamethasone inhibits synthesis of interferon by decreasing the level of its mRNA*. J Biol Chem. **1988** Jun 5;263(16):7454-7.

Geyer M, Wittinghofer A. *GEFs, GAPs, GDIs and effectors: taking a closer (3D) look at the regulation of Ras-related GTP-binding proteins*. Curr Opin Struct Biol. **1997** Dec;7(6):786-92.

Gibbs BF, Plath KE, Wolff HH, Grabbe J. *Regulation of mediator secretion in human basophils by p38 mitogen-activated protein kinase: phosphorylation is sensitive to the effects of*

phosphatidylinositol 3-kinase inhibitors and calcium mobilization. J Leukoc Biol. **2002** Aug;72(2):391-400.

Goldfeld AE, Doyle C, Maniatis T. *Human tumor necrosis factor alpha gene regulation by virus and lipopolysaccharide*. Proc Natl Acad Sci U S A. **1990** Dec;87(24):9769-73.

Goldstein JL, Brown MS. *Regulation of the mevalonate pathway*. Nature. **1990** Feb 1;343(6257):425-30.

Gordon JR, Galli SJ. *Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses*. J Exp Med. **1991** Jul 1;174(1):103-7.

Graham TE, Pfeiffer JR, Lee RJ, Kusewitt DF, Martinez AM, Foutz T, Wilson BS, Oliver JM. *MEK and ERK activation in ras-disabled RBL-2H3 mast cells and novel roles for geranylgeranylated and farnesylated proteins in Fc epsilonRI-mediated signaling*. J Immunol. **1998** Dec 15;161(12):6733-44.

Groom LA, Sneddon AA, Alessi DR, Dowd S, Keyse SM. *Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase*. J. **1996** Jul 15;15(14):3621-32.

Heck S, Kullmann M, Gast A, Ponta H, Rahmsdorf HJ, Herrlich P, Cato AC. *A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of thetranscription factor AP-1*. EMBO J. **1994** Sep 1;13(17):4087-95.

Heck S, Bender K, Kullmann M, Gottlicher M, Herrlich P, Cato AC. *I kappaB alpha-independent downregulation of NF-kappaB activity by glucocorticoid receptor*. EMBO J. **1997** Aug 1;16(15):4698-707

Hench PS Kendall EC, Slocumb CH and Polley HF *The efffect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone: compound E) and of pituitary adrenocorticotrophic hormone on rheumatoid arthritis: preliminary report*. Proc. Staff. meet. Mayo Clin. **1949** 24: 181-197.

Herrlich P. *Cross-talk between glucocorticoid receptor and AP-1*. Oncogene. **2001** Apr 30;20(19):2465-75.

Herrlich P, Göttlicher M. *The anti-inflammatory action of glucocorticoids*. **2002** In *Recent advances of glucocorticoid receptor action* eds. Cato, Schaeke, Asadullah; Springer Verlag.

Hirasawa N, Santini F, Beaven MA. *Activation of the mitogen-activated protein kinase/cytosolic phospholipase A2 pathway in a rat mast cell line. Indications of different pathways for release of arachidonic acid and secretory granules*. J Immunol. **1995** May 15;154(10):5391-402.

Hirasawa N, Sato Y, Fujita Y, Mue S, Ohuchi K. *Inhibition by dexamethasone of antigen-induced c-Jun N-terminal kinase activation in rat basophilic leukemia cells*. J Immunol. **1998** Nov 1;161(9):4939-43.

Hirasawa N, Sato Y, Fujita Y, Ohuchi K. *Involvement of a phosphatidylinositol 3-kinase-p38 mitogen activated protein kinase pathway in antigen-induced IL-4 production in mast cells*. Biochim Biophys Acta. **2000** Jan 3;1456(1):45-55.

Hirasawa N, Murakami A, Ohuchi K. *Expression of 74-kDa histidine decarboxylase protein in a macrophage-like cell line RAW 264.7 and inhibition by dexamethasone*. Eur J Pharmacol. **2001** Apr 20;418(1-2):23-8.

Hoffmeyer A, Grosse-Wilde A, Flory E, Neufeld B, Kunz M, Rapp UR, Ludwig S. *Different mitogen-activated protein kinase signaling pathways cooperate to regulate tumor necrosis factor alpha gene expression in T lymphocytes*. J Biol Chem. **1999** Feb 12;274(7):4319-27.

Honda Z, Suzuki T, Hirose N, Aihara M, Shimizu T, Nada S, Okada M, Ra C, Morita Y, Ito K. *Roles of C-terminal Src kinase in the initiation and the termination of the high affinity IgE receptor-mediated signaling*. J Biol Chem. **1997** Oct 10;272(41):25753-60.

Hundley TR, Prasad AR, Beaven MA. *Elevated levels of cyclooxygenase-2 in antigen-stimulated mast cells is associated with minimal activation of p38 mitogen-activated protein kinase*. J Immunol. **2001** Aug 1;167(3):1629-36.

Imasato A, Desbois-Mouthon C, Han J, Kai H, Cato AC, Akira S, Li JD. *Inhibition of p38 MAPK by glucocorticoids via induction of MAP kinase phosphatase-1 enhance nontypeable Haemophilus influenzae-induced expression of toll-like receptor 2*. J Biol Chem. **2002** Sep 27 [ahead of print]

Ishizaka T, Ishizaka K. *Activation of mast cells for mediator release through IgE receptors*. Prog Allergy. **1984**;34:188-235.

Ishizaka T, Conrad DH, Schulman ES, Sterk AR, Ko CG, Ishizaka K. *IgE-mediated triggering signals for mediator release from human mast cells and basophils*. Fed Proc. **1984** Oct;43(13):2840-5.

Ishizuka T, Oshiba A, Sakata N, Terada N, Johnson GL, Gelfand EW. *Aggregation of the FcepsilonRI on mast cells stimulates c-Jun amino-terminal kinase activity. A response inhibited by wortmannin*. J Biol Chem. **1996** May 31;271(22):12762-6.

Ishizuka T, Terada N, Gerwins P, Hamelmann E, Oshiba A, Fanger GR, Johnson GL, Gelfand EW. *Mast cell tumor necrosis factor alpha production is regulated by MEK kinases*. Proc Natl Acad Sci U S A. **1997** Jun 10;94(12):6358-63.

Ishizuka T, Chayama K, Takeda K, Hamelmann E, Terada N, Keller GM, Johnson GL, Gelfand EW. *Mitogen-activated protein kinase activation through Fc epsilon receptor I and stem cell factor receptor is differentially regulated by phosphatidylinositol 3-kinase and calcineurin in mouse bone marrow-derived mast cells*. J Immunol. **1999** Feb 15;162(4):2087-94.

Ismail K, Wessely S. *Psychiatric complications of corticosteroid therapy* Br J Hosp Med **1995** May 17-Jun 6;53(10):495-9.

Jabril-Cuenod B, Zhang C, Scharenberg AM, Paolini R, Numerof R, Beaven MA, Kinet JP. *Syk-dependent phosphorylation of Shc. A potential link between FcepsilonRI and the Ras/mitogen-activated protein kinase signaling pathway through SOS and Grb2*. J Biol Chem. **1996** Jul 5;271(27):16268-72.

Jonat C, Rahmsdorf HJ, Park KK, Cato AC, Gebel S, Ponta H, Herrlich P. *Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone*. Cell. **1990** Sep 21;62(6):1189-204.

Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Gloss B, Lin SC, Heyman RA, Rose DW, Glass CK, Rosenfeld MG. *A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors*. Cell. **1996** May 3;85(3):403-14.

Kang SM, Beverly B, Tran AC, Brorson K, Schwartz RH, Lenardo MJ. *Transactivation by AP-1 is a molecular target of T cell clonal anergy.* Science. **1992** Aug 21;257(5073):1134-8.

Kaspar F, Klocker H, Denninger A, Cato AC. *A mutant androgen receptor from patients with Reifenstein syndrome: identification of the function of a conserved alanine residue in the D box of steroid receptors*. Mol Cell Biol. **1993** Dec;13(12):7850-8.

Kassel O, Sancono A, Kratzschmar J, Kreft B, Stassen M, Cato AC. *Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1*. EMBO J. **2001** Dec 17;20(24):7108-16.

Kassel O. and Cato A.C.B., *Mast cells as targets for glucocorticoids in the treatment of allergic disorders*. **2002** In *Recent advances of glucocorticoid receptor action* eds. Cato A.C.B. Schaeke Asadullah; Springer Verlag.

Keyse SM. *Protein phosphatases and the regulation of mitogen-activated protein kinase signalling*. Curr Opin Cell Biol. **2000** Apr;12(2):186-92.

Kimata M, Inagaki N, Kato T, Miura T, Serizawa I, Nagai H. *Roles of mitogen-activated protein kinase pathways for mediator release from human cultured mast cells*. Biochem Pharmacol. 2000 Aug 15;60(4):589-94.

Kobayashi H, Ishizuka T, Okayama Y. *Human mast cells and basophils as sources of cytokines*. Clin Exp Allergy. **2000** Sep;30(9):1205-12.

König H, Ponta H, Rahmsdorf HJ, Herrlich P. *Interference between pathway-specific transcription factors: glucocorticoids antagonize phorbol ester-induced AP-1 activity without altering AP-1 site occupation in vivo*. EMBO J. **1992** Jun;11(6):2241-6.

Konishi H, Tsutsui H, Murakami T, Yumikura-Futatsugi S, Yamanaka KI, Tanaka M, Iwakura Y, Suzuki N, Takeda K, Akira S, Nakanishi K, Mizutani H. *IL-18 contributes to the spontaneous development of atopic dermatitis-like inflammatory skin lesion independently of IgE/stat6 under specific pathogen-free conditions*. Proc Natl Acad Sci U S A. **2002** Aug 20;99(17):11340-11345.

Kopp F, Kristensen P, Hendil KB, Johnsen A, Sobek A, Dahlmann B. *The human proteasome subunit HsN3 is located in the inner rings of the complex dimer*. J Mol Biol. **1995** Apr 28;248(2):264-72.

Lasa M, Brook M, Saklatvala J, Clark AR. *Dexamethasone destabilizes cyclooxygenase 2 mRNA by inhibiting mitogen-activated protein kinase p38*. Mol Cell Biol. **2001** Feb;21(3):771-80.

Lasa M, Abraham SM, Boucheron C, Saklatvala J, Clark AR. *Dexamethasone Causes Sustained Expression of Mitogen-Activated Protein Kinase (MAPK) Phosphatase 1 and Phosphatase-Mediated Inhibition of MAPK p38*. Mol Cell Biol. **2002** Nov;22(22):7802-11.

Lazaar AL, Plotnick MI, Kucich U, Crichton I, Lotfi S, Das SK, Kane S, Rosenbloom J, Panettieri RA Jr, Schechter NM, Pure E. *Mast cell chymase modifies cell-matrix interactions and inhibits mitogen-induced proliferation of human airway smooth muscle cells*. J Immunol. **2002** Jul 15;169(2):1014-20.

Lee SW, Tsou AP, Chan H, Thomas J, Petrie K, Eugui EM, Allison AC. *Glucocorticoids selectively inhibit the transcription of the interleukin 1 beta gene and decrease the stability of interleukin 1 beta mRNA*. Proc Natl Acad Sci U S A. **1988** Feb;85(4):1204-8.

Libermann TA, Baltimore D. *Activation of interleukin-6 gene expression through the NF-kappa B transcription factor*. Mol Cell Biol. **1990** May;10(5):2327-34.

Lloberas J, Soler C, Celada A. *The key role of PU.1/SPI-1 in B cells, myeloid cells and macrophages*. Immunol Today. **1999** Apr;20(4):184-9.

Macara IG, Lounsbury KM, Richards SA, McKiernan C, Bar-Sagi D. *The Ras superfamily of GTPases.* FASEB J. **1996** Apr;10(5):625-30.

Maniatis T, Fritsch EF and Smabrook J (eds.) *Molecular Cloning*. Cold Spring Harbor, New York **1989**.

Martin DI, Zon LI, Mutter G, Orkin SH. *Expression of an erythroid transcription factor in megakaryocytic and mast cell lineages*. Nature. **1990** Mar 29;344(6265):444-7.

Masini E, Di Bello MG, Bani D, Bigazzi M, Bani Sacchi T, Mannaioni PF. *Relaxin inhibits histamine release from mast cells: involvement of nitric oxide production*. Inflamm Res. **1995** Apr;44 Suppl 1:S12-3.

McKay LI, Cidlowski JA. *Cross-talk between nuclear factor-kappa B and the steroid hormone receptors: mechanisms of mutual antagonism.* Mol Endocrinol. **1998** Jan;12(1):45-56.

McLaughlin F, Mackintosh J, Hayes BP, McLaren A, Uings IJ, Salmon P, Humphreys J, Meldrum E, Farrow SN. *Glucocorticoid-induced osteopenia in the mouse as assessed by histomorphometry, microcomputed tomography, and biochemical markers*. Bone **2002** Jun;30(6):924-30.

Metzger H. *Transmembrane signaling: the joy of aggregation.* J Immunol. **1992** Sep 1;149(5):1477-87.

Morrison DK, Cutler RE. *The complexity of Raf-1 regulation*. Curr Opin Cell Biol. **1997** Apr;9(2):174-9.

Moynihan JA, Callahan TA, Kelley SP, Campbell LM. *Adrenal hormone modulation of type 1 and type 2 cytokine production by spleen cells: dexamethasone and dehydroepiandrosterone suppress interleukin-2, interleukin-4, and interferon-gamma production in vitro*. Cell Immunol. **1998** Feb 25;184(1):58-64.

Muda M, Boschert U, Dickinson R, Martinou JC, Martinou I, Camps M, Schlegel W, Arkinstall S. *MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase*. J Biol Chem. **1996a** Feb 23;271(8):4319-26.

Muda M, Theodosiou A, Rodrigues N, Boschert U, Camps M, Gillieron C, Davies K, Ashworth A, Arkinstall S. *The dual specificity phosphatases M3/6 and MKP-3 are highly selective for inactivation of distinct mitogen-activated protein kinases*. J Biol Chem. **1996b** Nov 1;271(44):27205-8.

Munck A, Guyre PM, Holbrook NJ. *Physiological functions of glucocorticoids in stress and their relation to pharmacological actions*. Endocr Rev. **1984** Winter;5(1):25-44.

Murakami K, Sato S, Nagasawa S, Yamashita T. *Regulation of mast cell signaling through high-affinity IgE receptor by CD45 protein tyrosine phosphatase*. Int Immunol. **2000** Feb;12(2):169-76.

Nishiyama C, Yokota T, Okumura K, Ra C. *The transcription factors Elf-1 and GATA-1 bind to cell-specific enhancer elements of human high-affinity IgE receptor alpha-chain gene*. J Immunol. **1999** Jul 15;163(2):623-30.

Nishiyama C, Takahashi K, Nishiyama M, Okumura K, Ra C, Ohtake Y, Yokota T. *Splice isoforms of transcription factor Elf-1 affecting its regulatory function in transcription-molecular cloning of rat Elf-1*. Biosci Biotechnol Biochem. **2000** Dec;64(12):2601-7.

Nishiyama C, Hasegawa M, Nishiyama M, Takahashi K, Akizawa Y, Yokota T, Okumura K, Ogawa H, Ra C. *Regulation of human Fc epsilon RI alpha-chain gene expression by multiple transcription factors*. J Immunol. **2002** May 1;168(9):4546-52.

Nishizumi H, Yamamoto T. *Impaired tyrosine phosphorylation and Ca2+ mobilization, but not degranulation, in lyn-deficient bone marrow-derived mast cells*. J Immunol. **1997** Mar 1;158(5):2350-5.

Nissen RM, Yamamoto KR. *The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain*. Genes Dev. **2000** Sep 15;14(18):2314-29.

Noguchi T, Metz R, Chen L, Mattei MG, Carrasco D, Bravo R. *Structure, mapping, and expression of erp, a growth factor-inducible gene encoding a nontransmembrane protein tyrosine phosphatase, and effect of ERP on cell growth*. Mol Cell Biol. **1993** Sep;13(9):5195-205.

Nolte H. *The role of mast cells and basophils in immunoregulation*. Allergy Asthma Proc. **1996** Jan-Feb;17(1):17-21.

Ohtake H, Ichikawa N, Okada M, Yamashita T. Cutting Edge: *Transmembrane phosphoprotein Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains as a negative feedback regulator of mast cell signaling through the FcepsilonRI.* J Immunol. **2002** Mar 1;168(5):2087-90.

Okabe T, Hide M, Koro O, Yamamoto S. *Substance P induces tumor necrosis factor-alpha release from human skin via mitogen-activated protein kinase*. Eur J Pharmacol. **2000** Jun 16;398(2):309-15.

Okada M, Nada S, Yamanashi Y, Yamamoto T, Nakagawa H. *CSK: a protein-tyrosine kinase involved in regulation of src family kinases*. J Biol Chem. **1991** Dec 25;266(36):24249-52.

Paolini R, Jouvin MH, Kinet JP. *Phosphorylation and dephosphorylation of the high-affinity receptor for immunoglobulin E immediately after receptor engagement and disengagement*. Nature. **1991** Oct 31;353(6347):855-8.

Parks WP, Scolnick EM, Kozikowski EH *Dexamethasone stimulation of murine mammary tumor virus expression: a tissue culture source of virus*. Science **1974** 184: 158-160.

Pfefferkorn LC, Yeaman GR. *Association of IgA-Fc receptors (Fc alpha R) with Fc epsilon RI gamma 2 subunits in U937 cells. Aggregation induces the tyrosine phosphorylation of gamma 2*. J Immunol. **1994** Oct 1;153(7):3228-36.

Plas DR, Johnson R, Pingel JT, Matthews RJ, Dalton M, Roy G, Chan AC, Thomas ML. *Direct regulation of ZAP-70 by SHP-1 in T cell antigen receptor signaling*. Science. **1996** May 24;272(5265):1173-6.

Plaut M, Pierce JH, Watson CJ, Hanley-Hyde J, Nordan RP, Paul WE. *Mast cell lines produce lymphokines in response to cross-linkage of Fc epsilon RI or to calcium ionophores*. Nature. **1989** May 4;339(6219):64-7.

Pratt WB. *The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor*. J Biol Chem. **1993** Oct 15;268(29):21455-8.

Pribluda VS, Pribluda C, Metzger H. *Transphosphorylation as the mechanism by which the high-affinity receptor for IgE is phosphorylated upon aggregation*. Proc Natl Acad Sci U S A. **1994** Nov 8;91(23):11246-50.

Quarto R, Metzger H. The receptor for immunoglobulin E: examination for kinase activity and as a substrate for kinases. Mol Immunol. **1986** Nov;23(11):1215-23.

Ra C, Jouvin MH, Kinet JP. *Complete structure of the mouse mast cell receptor for IgE (Fc epsilon RI) and surface expression of chimeric receptors (rat-mouse-human) on transfected cells*. Biol Chem. **1989** Sep 15;264(26):15323-7.

Ravetch JV, Kinet JP. *Fc receptors*. Annu Rev Immunol. **1991**;9:457-92.

Reichstein T and Shoppee CW, *Hormones of the adrenal cortex*. Vitam. Horm. **1943** 1, 345-413.

Rider LG, Hirasawa N, Santini F, Beaven MA. *Activation of the mitogen-activated protein kinase cascade is suppressed by low concentrations of dexamethasone in mast cells*. J Immunol. **1996** Sep 15;157(6):2374-80.

Ringold GM, Yamamoto KR, Tomkins GM, Bishop M, Varmus HE *Dexamethasone-mediated induction of mouse mammary tumor virus RNA: a system for studying glucocorticoid action*. Cell. **1975** Nov;6(3):299-305.

Robinson MJ, Cobb MH. *Mitogen-activated protein kinase pathways*. Curr Opin Cell Biol**. 1997** Apr;9(2):180-6.

Sakai DD, Helms S, Carlstedt-Duke J, Gustafsson JA, Rottman FM, Yamamoto KR. *Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene*. Genes Dev. **1988** Sep;2(9):1144-54.

Saxena M, Williams S, Brockdorff J, Gilman J, Mustelin T. *Inhibition of T cell signaling by mitogen-activated protein kinase-targeted hematopoietic tyrosine phosphatase (HePTP)*. J Biol Chem. **1999** Apr 23;274(17):11693-700.

Scheidereit C, Geisse S, Westphal HM, Beato M. *The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumour virus*. Nature. **1983** Aug 25-31;304(5928):749-52.

Scheinman RI, Gualberto A, Jewell CM, Cidlowski JA, Baldwin AS Jr. *Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors*. Mol Cell Biol. **1995** Feb;15(2):943-53.

Schneider H, Cohen-Dayag A, Pecht I. *Tyrosine phosphorylation of phospholipase C gamma 1 couples the Fc epsilon receptor mediated signal to mast cells secretion*. Int Immunol. **1992** Apr;4(4):447-53).

Schreck R, Baeuerle PA. *NF-kappa B as inducible transcriptional activator of the granulocyte-macrophage colony-stimulating factor gene*. Mol Cell Biol. **1990** Mar;10(3):1281-6.

Sewell WA, Scurr LL, Orphanides H, Kinder S, Ludowyke RI. *Induction of interleukin-4 and interleukin-5 expression in mast cells is inhibited by glucocorticoids*. Clin Diagn Lab Immunol. **1998** Jan;5(1):18-23.

Song JS, Haleem-Smith H, Arudchandran R, Gomez J, Scott PM, Mill JF, Tan TH, Rivera J. *Tyrosine phosphorylation of Vav stimulates IL-6 production in mast cells by a Rac/c-Jun N-terminal kinase-dependent pathway*. J Immunol. **1999** Jul 15;163(2):802-10.

Stein B, Yang MX. *Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta*. Mol Cell Biol. **1995** Sep;15(9):4971-9.

Stevens RL, Austen KF. *Recent advances in the cellular and molecular biology of mast cells*. Immunol Today. **1989** Nov;10(11):381-6.

Stromstedt PE, Poellinger L, Gustafsson JA, Carlstedt-Duke J. *The glucocorticoid receptor binds to a sequence overlapping the TATA box of the human osteocalcin promoter: a potential mechanism for negative regulation*. Mol Cell Biol. **1991** Jun;11(6):3379-83.

Subramaniam N, Cairns W, and Okret S. *Studies on the mechanism of glucocorticoid-mediated repression from a negative glucocorticoid response element from the bovine prolactin gene*. DNA Cell Biol. **1997** Feb; 16(2):153-63.

Sun H, Charles CH, Lau LF, Tonks NK. *MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo*. Cell. **1993** Nov 5;75(3):487-93.

Swantek JL, Cobb MH, Geppert TD. *Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK*. Mol Cell Biol. **1997** Nov;17(11):6274-82.

Tamura T, Shimbara N, Aki M, Ishida N, Bey F, Scherrer K, Tanaka K, Ichihara A. *Molecular cloning of cDNAs for rat proteasomes: deduced primary structures of four other subunits*. J Biochem (Tokyo). **1992** Oct;112(4):530-4.

Tanaka,K. Direct Submission Rat mRNA for proteasome subunit RCX, complete cds gi|639870|dbj|D45247.1|RATPSRCX[639870] Submitted (23-JAN-**1995**) Keiji Tanaka, The Tokyo Metropolitan Institute of Medical Science, Cancer Therapeatics; 18-22 Honkomagome 3-chome Bunkyo-ku, Tokyo 113, Japan

Thomas ML, Brown EJ. *Positive and negative regulation of Src-family membrane kinases by CD45*. Immunol Today. **1999** Sep;20(9):406-11.

Tobler A, Meier R, Seitz M, Dewald B, Baggiolini M, Fey MF. *Glucocorticoids downregulate gene expression of GM-CSF, NAP-1/IL-8, and IL-6, but not of M-CSF in human fibroblasts*. Blood. **1992** Jan 1;79(1):45-51.

Toru H, Ra C, Nonoyama S, Suzuki K, Yata J, Nakahata T. *Induction of the high-affinity IgE receptor (Fc epsilon RI) on human mast cells by IL-4*. Int Immunol. **1996** Sep;8(9):1367-73.

Trowbridge IS, Thomas ML. *CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development*. Annu Rev Immunol. **1994**;12:85-116.

Turner H, Kinet JP. *Signalling through the high-affinity IgE receptor Fc epsilonRI*. Nature. **1999** Nov 25;402(6760 Suppl):B24-30.

Voges D, Zwickl P, Baumeister W. *The 26S proteasome: a molecular machine designed for controlled proteolysis*. Annu Rev Biochem. **1999**;68:1015-68.

Wang X, Crispino JD, Letting DL, Nakazawa M, Poncz M, Blobel GA. *Control of megakaryocyte-specific gene expression by GATA-1 and FOG-1: role of Ets transcription factors*. EMBO J. **2002** Oct 1;21(19):5225-34.

Wershil BK, Tsai M, Geissler EN, Zsebo KM, Galli SJ. *The rat c-kit ligand, stem cell factor, induces c-kit receptor-dependent mouse mast cell activation in vivo. Evidence that*

signaling through the c-kit receptor can induce expression of cellular function. J Exp Med. **1992** Jan 1;175(1):245-55.

Wershil BK, Furuta GT, Lavigne JA, Choudhury AR, Wang ZS, Galli SJ. *Dexamethasone or cyclosporin A suppress mast cell-leukocyte cytokine cascades. Multiple mechanisms of inhibition of IgE- and mast cell-dependent cutaneous inflammation in the mouse*. J Immunol. **1995** Feb 1;154(3):1391-8.

Williams NG, Roberts TM. *Signal transduction pathways involving the Raf proto-oncogene*. Cancer Metastasis Rev. **1994** Mar;13(1):105-16.

Wodnar-Filipowicz A, Heusser CH, Moroni C. *Production of the haemopoietic growth factors GM-CSF and interleukin-3 by mast cells in response to IgE receptor-mediated activation*. Nature. **1989** May 11;339(6220):150-2.

Wofsy C, Vonakis BM, Metzger H, Goldstein B. *One lyn molecule is sufficient to initiate phosphorylation of aggregated high-affinity IgE receptors*. Proc Natl Acad Sci U S A. **1999** Jul 20;96(15):8615-20).

Wrange O, Gustafsson JA. *Separation of the hormone- and DNA-binding sites of the hepatic glucocorticoid receptor by means of proteolysis*. J Biol Chem**. 1978** Feb 10;253(3):856-65.

Yamaguchi M, Lantz CS, Oettgen HC, Katona IM, Fleming T, Miyajima I, Kinet JP, Galli SJ. *IgE enhances mouse mast cell Fc(epsilon)RI expression in vitro and in vivo: evidence for a novel amplification mechanism in IgE-dependent reactions*. J Exp Med. **1997** Feb 17;185(4):663-72.

Yamaguchi M, Sayama K, Yano K, Lantz CS, Noben-Trauth N, Ra C, Costa JJ, Galli SJ. *IgE enhances Fc epsilon receptor I expression and IgE-dependent release of histamine and lipid mediators from human umbilical cord blood-derived mast cells: synergistic effect of IL-4 and IgE on human mast cell Fc epsilon receptor I expression and mediator release*. J Immunol. **1999** May 1;162(9):5455-65.

Yamaguchi M, Hirai K, Komiya A, Miyamasu M, Furumoto Y, Teshima R, Ohta K, Morita Y, Galli SJ, Ra C, Yamamoto K. *Regulation of mouse mast cell surface Fc epsilon RI expression by dexamethasone*. Int Immunol. **2001** Jul;13(7):843-51.

Yamashita T, Mao SY, Metzger H. *Aggregation of the high-affinity IgE receptor and enhanced activity of p53/56lyn protein-tyrosine kinase*. Proc Natl Acad Sci U S A. **1994** Nov 8;91(23):11251-5).

Yanagi S, Shimbara N, Tamura T. *Tissue and cell distribution of a mammalian proteasomal ATPase, MSS1, and its complex formation with the basal transcription factors*. Biochem Biophys Res Commun. **2000** Dec 20;279(2):568-73.

Yang-Yen HF, Chambard JC, Sun YL, Smeal T, Schmidt TJ, Drouin J, Karin M. *Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction*. Cell. **1990** Sep 21;62(6):1205-15.

Yano K, Yamaguchi M, de Mora F, Lantz CS, Butterfield JH, Costa JJ, Galli SJ. *Production of macrophage inflammatory protein-1alpha by human mast cells: increased anti-IgE-dependent secretion after IgE-dependent enhancement of mast cell IgE-binding ability*. Lab Invest. **1997** Aug;77(2):185-93.

Yi TL, Bolen JB, Ihle JN. *Hematopoietic cells express two forms of lyn kinase differing by 21 amino acids in the amino terminus*. Mol Cell Biol. **1991** May;11(5):2391-8.

Zhang C, Baumgartner RA, Yamada K, Beaven MA. *Mitogen-activated protein (MAP) kinase regulates production of tumor necrosis factor-alpha and release of arachidonic acid in mast cells. Indications of communication between p38 and p42 MAP kinases*. J Biol Chem. **1997** May 16;272(20):13397-402.

APPENDIX

Dexamethasone treatment of RBL cells:
16h

geneID	ept. #1 Ratio* (1)	expt. #2 Ratio* (2)	description

Genes that go 2 times up and 0 times down.
==========

geneID	ept. #1 Ratio* (1)	expt. #2 Ratio* (2)	description
U11275mRNA_s	>29	>22	U11275mRNA RNPNMTS1 Rattus norvegicus WKY and SHRSP phenylethanolamine N-methyltransferase (PNMT) gene, exon 1
A16585cds_s_at	19	5,1	A16585cds R.norvegicus mRNA for preprorelaxin
J00780_at	8,4	9,6	rat preprorelaxin mrna /cds=(43,603) /gb=J00780 /gi=206606 /ug=Rn.9830 /len=800
U75404UTR#1_s_at	8,4	4,4	U75404UTR#1 RNU75404 Rattus norvegicus Ssecks 322 mRNA, 3' untranslated region, partial sequence
AF023087_s_at	2,8	8,7	Rattus norvegicus nerve growth factor induced factor A mRNA, partial 3'UTR
S81497_s_at	4,4	6,9	lysosomal acid lipase=intracellular hydrolase [rats, Wolman, liver, mRNA, 3144 nt]
rc_AA894332_at	>2.9	>6.1	EST198135 Rattus norvegicus cDNA, 3' end /clone=RSPAW79/clone_end=3' /gb=AA894332 /gi=3021211 /ug=Rn.17129 /len=503
rc_AI137583_at	4,5	3,5	UI-R-C0-hf-a-03-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-C0-hf-a-03-0-UI /clone_end=3' /gb=AI137583 /ug=Rn.3272 /len=496
rc_AA874784_s_at	>3.2	>4.6	UI-R-E0-bw-e-07-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-E0-bw-e-07-0-UI /clone_end=3' /gb=AA874784 /gi=2979732 /ug=Rn.3307 /len=483
rc_AA894004_at	4,3	3,2	EST197807 Rattus norvegicus cDNA, 3' end /clone=RPLAO48/clone_end=3' /gb=AA894004 /gi=3020883 /ug=Rn.8945 /len=430
AF095576_at	4,8	2,6	Rattus norvegicus APS protein mRNA, complete cds
D12769_g_at	>2.5	>4.8	RATBTEB Rattus norvegicus mRNA for BTE binding protein
rc_AA944422_at	3,9	3,3	EST199921 Rattus norvegicus cDNA, 3' end /clone=REMAJ01/clone_end=3' /gb=AA944422 /gi=3104338 /ug=Rn.871/len=641
rc_AA891194_s_at	3,4	3,4	EST194997 Rattus norvegicus cDNA, 3' end /clone=RHEAQ37 /clone_end=3' /gb=AA891194 /gi=3018073 /ug=Rn.24612 /len=465
X63594cds_g_at	4,5	2,2	X63594cds RRRLIF1 R.rattus RL/IF-1 mRNA
rc_AA799323_at	2,4	4,2	EST188820 Rattus norvegicus cDNA, 3' end /clone=RHEAA31 /clone_end=3' /gb=AA799323 /gi=2862278 /ug=Rn.6178 /len=328
U67888_at	2,5	3,7	RNU67888 Rattus norvegicus mast cell protease 3 (RMCP-3) mRNA, partial cds
M96601_at	2,3	3,9	Rattus norvegicus taurine transporter mRNA, complete cds /cds=(126,1991) /gb=M96601 /gi=207541 /ug=Rn.9968 /len=2476
M91652complete_seq_at	3,1	3	M91652completeSeq Rat glutamine synthetase (glnA) mRNA, complete cds /cds=UNKNOWN /gb=M91652

			/gi=204348 /ug=Rn.2204 /len=2793
rc_AA893267_at	2,9	3	EST197070 Rattus norvegicus cDNA, 3' end /clone= RKIBE29 /clone_end=3' /gb=AA893267 /gi=3020146 /ug=Rn.6937 /len=472
D17809_at	2,6	3,3	Rat mRNA for beta-4N-acetylgalactosaminyltransferase, complete cds /cds=(30,1631) /gb=D17809 /gi=497841 /ug=Rn.10119 /len=2166
U76206_at	2,6	3,3	Rattus norvegicus VTR 15-20 receptor mRNA, complete cds /cds=(238,1155) /gb=U76206 /gi=2459584 /ug=Rn. 16317 /len=1690
M12492mRNA#1 _at	2,3	3,5	M12492mRNA#1 Rat type II cAMP-dependent protein kinase regulatory subunit mRNA, 3' end /cds=UNKNOWN /gb=M12492 /gi=206670 /ug=Rn.4075 /len=3108
rc_AA892284_at	2,7	2,9	EST196087 Rattus norvegicus cDNA, 3' end /clone= RKIAO58/clone_end=3' /gb=AA892284 /gi=3019163 /ug=Rn.22719 /len=572
M13100cds#3_f_ at	>2.1	>3.3	M13100cds#3 RATLIN3A Rat long interspersed repetitive DNA sequence LINE3 (L1Rn)
rc_AA893244_at	2,2	3,1	EST197047 Rattus norvegicus cDNA, 3' end /clone= RKIBE02 /clone_end=3' /gb=AA893244 /gi=3020123 /ug=Rn.3668 /len=433
D12769_at	>1.5	>3.8	RATBTEB Rattus norvegicus mRNA for BTE binding protein
rc_AI236721_r_a t	2,4	2,9	EST233283 Rattus norvegicus cDNA, 3' end /clone= ROVDJ72/clone_end=3' /gb=AI236721 /ug=Rn.2503 /len=345
rc_AA893667_g _at	2,8	2,4	EST197470 Rattus norvegicus cDNA, 3' end /clone=RPLAI 23 /clone_end=3' /gb=AA893667 /gi=3020546 /ug=Rn.4237 /len=485
rc_AA900900_s_ at	2,4	2,7	UI-R-E0-dc-b-04-0-UI.s1 Rattus norvegicus cDNA, 3' end / clone=UI-R-E0-dc-b-04-0-UI /clone_end=3' /gb=AA900900 /gi=3036254 /ug=Rn.7107 /len=462
U82623_at	2,2	2,8	Rattus norvegicus cytocentrin mRNA, complete cds / cds=(119,2200) /gb=U82623 /gi=2697021 /ug=Rn.7107 /len=3602
X62950mRNA_f _at	2,5	2,5	X62950mRNA RNPBUS30 R.norvegicus mRNA (pBUS30) with repetitive elements
rc_AI230572_at	2,1	2,8	EST227267 Rattus norvegicus cDNA, 3' end /clone= REMCY30/clone_end=3' /gb=AI230572 /ug=Rn.3181 /len=317
M91652complete _seq_g_at	2,6	2,3	M91652completeSeq Rat glutamine synthetase (glnA) mRNA, complete cds /cds=UNKNOWN /gb=M91652 / gi=204348 /ug=Rn.2204 /len=2793
rc_AA799421_at	2,2	2,7	EST188918 Rattus norvegicus cDNA, 3' end /clone =RHEAA87 clone_end=3' /gb=AA799421 /gi=2862376 /ug=Rn.19951 /len=570
AB015308_s_at	>2.4	2,2	Rattus norvegicus mRNA for GTP binding protein alpha 15, complete cds
rc_AI232783_s_ at	2,5	2	EST229471 Rattus norvegicus cDNA, 3' end /clone= RKICG50/clone_end=3' /gb=AI232783 /ug=Rn.2204 len=478
rc_AA892647_at	2	2,5	EST196450 Rattus norvegicus cDNA, 3' end /clone =RKIAV30 /clone_end=3' /gb=AA892647 /gi=3019526 /ug=Rn.1659 /len=421
rc_AA859612_f_ at	2,2	2,3	UI-R-E0-bs-f-12-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-E0-bs-f-12-0-UI /clone_end=3' /gb=AA859612/gi=2949132 /ug=Rn.8481 /len=297
rc_AI014169_at	2,5	2	EST207724 Rattus norvegicus cDNA, 3' end /clone= RSPBF88 /clone_end=3' /gb=AI014169 /ug=Rn.2758 /len=553
rc_AA893690_g _at	2,2	2,1	EST197493 Rattus norvegicus cDNA, 3' end /clone =RPLAI47/clone_end=3' /gb=AA893690 /gi=3020569 /ug=Rn.3377/len=492
AB015433_s_at	2	2,1	Rattus norvegicus mRNA for 4F2 heavy chain (4F2hc),

			complete cds
rc_AA800613_at	>1.8	>2.3	EST190110 Rattus norvegicus cDNA, 3' end /clone= RLUAB70 /clone_end=3' /gb=AA800613 /gi=2863568 /ug=Rn.2454 /len=506
U61729_at	2	2,1	Rattus norvegicus proline rich protein mRNA, complete cds/cds=(175,984) /gb=U61729 /gi=1408276 /ug =Rn.10967 /len=1619
rc_AA800303_at	>1.6	>2.4	EST189800 Rattus norvegicus cDNA, 3' end /clone =RHEAN65 /clone_end=3' /gb=AA800303 /gi=2863258 /ug=Rn.22784 /len=569
M15883_g_at	>1.5	2,3	Rat clathrin light chain (LCB2) mRNA, complete cds /cds= (28,717) /gb=M15883 /gi=203358 /ug=Rn.3440 /len=982
rc_AI639453_f_a t	>1.6	2,1	Rat mixed-tissue library Rattus norvegicus cDNA clone rx 00152 3', mRNA sequence [Rattus norvegicus]
L20822_at	>1.2	2,1	Rattus norvegicus syntaxin 5 mRNA, complete cds /cds =(129,1034) /gb=L20822 /gi=349322 /ug=Rn.5782 /len= 1608
rc_AA891037_at	>1.2	>2	EST194840 Rattus norvegicus cDNA, 3' end /clone=RHE AO17 /clone_end=3' /gb=AA891037 /gi=3017916 /ug=Rn. 16548 /len=401
D31873_at	>1.4	>1.8	Rat mRNA for LIMK-1, complete cds /cds=(208,2151) / gb=D31873 /gi=1684611 /ug=Rn.11250 /len=3258
rc_AI639207_at	>1.3	>1.9	Rat mixed-tissue library Rattus norvegicus cDNA clone rx 03980 3', mRNA sequence [Rattus norvegicus]
M28255_s_at	<1.4	<3	RATCYO8A Rat cytochrome c oxidase subunit VIII mRNA, 3' end
S81478_s_at	>6.1	undef	3CH134/CL100 PTPase=oxidative stress-inducible protein tyrosine phosphatase [rats, peritoneal macrophage cDNA library, mRNA, 1912 nt]
rc_AA892128_at	>4.8	undef	EST195931 Rattus norvegicus cDNA, 3' end /clone=RKIA M69 /clone_end=3' /gb=AA892128 /gi=3019007 /ug=Rn. 25177 /len=443
rc_AI639246_at	>4.5	undef	Rat mixed-tissue library Rattus norvegicus cDNA clone rx03959 3', mRNA sequence [Rattus norvegicus]
rc_AA891521_at	>4.4	undef	EST195324 Rattus norvegicus cDNA, 3' end /clone =RHEAZ48 /clone_end=3' /gb=AA891521 /gi=3018400 /ug=Rn.7299 /len=470
rc_AA892332_at	1,6	4,7	EST196135 Rattus norvegicus cDNA, 3' end /clone =RKIAP18 /clone_end=3' /gb=AA892332 /gi=3019211 /ug=Rn.14750 /len=191
X70369_s_at	>3.1	undef	R.norvegicus mRNA for pro alpha 1 collagen type III /cds =(0,1911) /gb=X70369 /gi=57915 /ug=Rn.3247 /len=2183
rc_AI230256_at	4,5	1,6	EST226951 Rattus norvegicus cDNA, 3' end /clone=REM CU23 /clone_end=3' /gb=AI230256 /ug=Rn.3272 /len=499
U41183_g_at	>3	undef	Rattus norvegicus placental pre-progrowth hormone-eleasing hormone (GHRH) mRNA, complete cds /cds =(18,434) /gb=U41183 /gi=1845215 /ug=Rn.10693 /len=496
U42627_at	3,9	1,8	Rattus norvegicus dual-specificity protein tyrosine phosphatase (rVH6) mRNA, complete cds /cds= (360,1505) /gb=U42627 /gi=1185551 /ug=Rn.4313 /len=2104
rc_AA892154_g _at	1,5	4,1	EST195957 Rattus norvegicus cDNA, 3' end /clone= RKIAN02 /clone_end=3' /gb=AA892154 /gi=3019033 /ug=Rn. 3279 /len=386
rc_AA799598_at	3,6	1,8	EST189095 Rattus norvegicus cDNA, 3' end /clone= RHEAC94 /clone_end=3' /gb=AA799598 /gi=2862553 /ug=Rn.3655 /len=506
U90261UTR#1_ g_at	1,7	>3.3	U90261UTR#1 Rattus norvegicus hypertension-regulated vascular factor-1 (HRVF-1) mRNA, 3' UTR sequence
rc_AA893869_g _at	undef	2,5	EST197672 Rattus norvegicus cDNA, 3' end /clone= RPLAM85 /clone_end=3' /gb=AA893869 /gi=3020748

			/ug=Rn.14614 /len=451
rc_AI014091_at	1,3	3,1	EST207646 Rattus norvegicus cDNA, 3' end /clone=RSP BE78 /clone_end=3' /gb=AI014091 /ug=Rn.221 /len=608
rc_AI171085_at	1,6	2,8	EST217033 Rattus norvegicus cDNA, 3' end /clone=RMU BF92 /clone_end=3' /gb=AI171085 /gi=3711125 /ug=Rn. 6172 /len=357
X06483cds_at	undef	>2.2	X06483cds RNRPL32 Rat mRNA for ribosomal protein L32
D90109_at	2,7	1,7	Rat mRNA for long-chain acyl-CoA synthetase (EC 6.2.1.3) /cds=(13,2112) /gb=D90109 /gi=220717 /ug=Rn.6215 /len= 3657
X55660_g_at	1,9	2,5	Rat pcRF104 mRNA for furin /cds=(443,2824) /gb=X55660 /gi=56171 /ug=Rn.3220 /len=4259
rc_AA800719_at	1,5	2,9	EST190216 Rattus norvegicus cDNA, 3' end /clone=RLUAK 63 /clone_end=3' /gb=AA800719 /gi=2863674 /ug=Rn.6624 /len=663
M75153_at	1,5	2,7	R.norvegicus ras p21-like small GTP-binding protein (24KG) mRNA, complete cds /cds=(0,650) /gb=M75153 /gi=206566 /ug=Rn.1016 /len=895
rc_AA799523_g _at	1,7	2,4	EST189020 Rattus norvegicus cDNA, 3' end /clone=RHEA C11 /clone_end=3' /gb=AA799523 /gi=2862478 /ug=Rn.4057 /len=483
AB015432_s_at	2,2	1,9	Rattus norvegicus mRNA for LAT1 (L-type amino acid transporter 1), complete cds
rc_AA892036_at	2,2	1,9	EST195839 Rattus norvegicus cDNA, 3' end /clone =RKIAL05 /clone_end=3' /gb=AA892036 /gi=3018915 /ug=Rn.13453 /len=454
U25746_g_at	1,8	2,3	Rattus norvegicus RNA helicase with arginine-serine-rich domain mRNA, complete cds /cds=(152,3250) /gb= U25746 /gi=897914 /ug=Rn.3436 /len=3531
X54510_g_at	1,7	2,3	R.norvegicus mRNA for coupling factor 6 of mitochondrial ATP synthase complex /cds=(161,487) /gb=X54510 /gi= 14214 /ug=Rn.5790 /len=573
X52840_r_at	1,8	2,1	Rat mRNA for smooth muscle myosin RLC-B /cds= (17,535) /gb=X52840 /gi=56702 /ug=Rn.2967 /len=1113
rc_AI011998_at	1,8	2,1	EST206449 Rattus norvegicus cDNA, 3' end /clone =RPLAR43 /clone_end=3' /gb=AI011998 /ug= Rn.11296 /len=495
rc_AI102044_at	1,7	2,2	EST211333 Rattus norvegicus cDNA, 3' end /clone =RBRBY28 /clone_end=3' /gb=AI102044 /gi=3706879 /ug=Rn.4229 /len=549
rc_AI639338_at	2,1	1,8	Rat mixed-tissue library Rattus norvegicus cDNA clone rx01696 3', mRNA sequence [Rattus norvegicus]
rc_AI044900_s_ at	1,8	2,1	UI-R-C1-kk-c-05-0-UI.s1 Rattus norvegicus cDNA, 3' end clone=UI-R-C1-kk-c-05-0-UI /clone_end=3' /gb=AI044900 ug=Rn.6215 /len=388
rc_AI010480_g_ at	1,6	2,2	EST204931 Rattus norvegicus cDNA, 3' end /clone= RLUBZ96 /clone_end=3' /gb=AI010480 /ug=Rn.1011 /len=590
rc_AI170403_at	1,5	2,3	EST216329 Rattus norvegicus cDNA, 3' end /clone= RLUCH85 /clone_end=3' /gb=AI170403 /gi=3710443 /ug=Rn.1617 /len=670
S79304_s_at	1,3	2,4	cytochrome oxidase subunit I, Ser-tRNA [rats, Sertoli cells, mRNA Mitochondrial, 987 nt]
rc_AA893485_g _at	1,5	2,2	EST197288 Rattus norvegicus cDNA, 3' end /clone =RLIAD06 /clone_end=3' /gb=AA893485 /gi=3020364 /ug=Rn.4088 /len=434
rc_AA892146_f_ at	2,1	1,6	EST195949 Rattus norvegicus cDNA, 3' end /clone= RKIAM90 /clone_end=3' /gb=AA892146 /gi=3019025 /ug=Rn.24928 /len=439
L26268_g_at	1,7	1,8	Rattus norvegicus anti-proliferative factor (BTG1) mRNA, complete cds /cds=(0,515) /gb=L26268 /gi=1167495 /ug=Rn.1000 /len=1464

Genes that go 0 times up and 2 times down.
==========

geneID	ept. #1 Ratio* (1)	expt. #2 Ratio* (2)	sample #2 description
X52815cds_f_at	-2,1	-22	X52815cds RRGAMACT Rat mRNA for cytoplasmic-gamma isoform of actin
E12625cds_at	-3,2	<-17	E12625cds cDNA encoding a rat novel protein which is expressed with nerve injury
M21622_at	-2,4	-14	Rat high-affinity IgE receptor (Fc-epsilon-R-I)mRNA, complete cds, clones R8-2b and R3-3/cds=(176,853) /gb=M21622 /gi=204109 /ug=Rn.9677 /len=1179
X97443_s_at	<-13	<-3	R.norvegicus mRNA for transmembrane protein Tmp21-I /cds=(0,611) /gb=X97443/gi=1360135 /ug=Rn.22674 /len=706
rc_AA924326_s_at	-2,4	<-13	UI-R-A1-dy-h-11-0-UI.s1 Rattus norvegicus cDNA, 3' end / clone=UI-R-A1-dy-h-11-0-UI /clone_end=3' /gb=AA924326 /gi=3071462 /ug=Rn.1774 /len=361
U68562mRNA#2_s_at	-2,2	<-9.4	U68562mRNA#2 RNU68562 Rattus norvegicus chaperonin 60 (Hsp60) and chaperonin 10 (CPN10) genes, nuclear genes encoding mitochondrial proteins, complete cds
X98490cds_at	-2	<-7.4	X98490cds RNRPA2 R.norvegicus mRNA for p32-subunit of replication protein A
M58495mRNA_at	<-1.9	<-7.5	M58495mRNA RATQUINA R.norvegicus NAD(P)H: Quinone reductase mRNA, complete cds
J02722cds_at	-2,8	-6,4	J02722cds RATHOXA Rat heme oxygenase gene, complete cds
M64301_at	<-2.6	<-6.4	RATERK3 Rat extracellular signal-related kinase (ERK3) mRNA, complete cds
U64705cds_i_at	-2,1	<-6.6	U64705cds RNU64705 Rattus norvegicus protein Synthesis initiation factor 4AII gene, partial cds, and E3 small nucleolar RNA gene, complete sequence
L32591mRNA_at	-2,2	<-6.3	L32591mRNA RATGADD45X Rattus norvegicus GADD45 mRNA, complete cds
X74565cds_at	-2	<-6.5	X74565cds RNTBFII R.norvegicus TBFIImRNA for poly pyrimidine tract binding protein
M21476_s_at	-3,3	<-4.9	RAT5MDA Rat iodothyronine 5'-monodeiodinase (5'-MD) mRNA, partial cds
D10926_s_at	-2	<-6.1	RATTFPI Rat mRNA for tissue factor pathway inhibitor, complete cds
L32591mRNA_g_at	-3,3	-4,8	L32591mRNA RATGADD45X Rattus norvegicus GADD45 mRNA, complete cds
M29249cds_at	<-2.4	<-5.5	M29249cds RAT3H3M Rat 3-hydroxy-3-methylglutaryl coenzyme A reductase gene, partial cds
rc_AA892801_g_at	-4,5	<-3.2	EST196604 Rattus norvegicus cDNA, 3' end /clone =RKIAX44 /clone_end=3' /gb=AA892801/gi=3019680 /ug=Rn.3610 /len=528
rc_AI177004_s_at	-2,3	-5,3	EST220611 Rattus norvegicus cDNA, 3' end/clone =ROVBZ64/clone_end=3' /gb=AI177004 /ug=Rn.5106 /len=332
M34097_at	-3,8	-3,7	Rat natural killer (NK) cell protease 1 (RNKP-1) mRNA, complete cds /cds=(108,854) /gb=M34097 /gi=206689 /ug=Rn.9837 /len=1113
U73174_at	-2,6	<-4.7	RNU73174 Rattus norvegicus glutathione reductase mRNA complete cds
AF035951_at	<-2.2	<-4.7	Rattus norvegicus kinesin-related protein KRP1 (KRP1) mRNA, partial cds
rc_AI008677_s_at	<-4.3	<-2.6	EST203128 Rattus norvegicus cDNA, 3' end/clone=REMB B54 /clone_end=3' /gb=AI008677/ug=Rn.6872 /len=480
AJ222813_s_at	-2,2	<-4.6	RNAJ813 Rattus norvegicus mRNA for precursor interleukin 18 (IL-18), complete cds

X74565cds_g_at	-2,3	<-4.4	X74565cds RNTBFII R.norvegicus TBFII mRNA for poly pyrimidine tract binding protein
rc_AI145680_s_at	<-1.6	<-4.9	UI-R-BT0-qd-b-09-0-UI.s1 Rattus cDNA, 3' end /clone =UI-R-BT0-qd-b-09-0-UI/clone_end=3' /gb=AI145680 /ug=Rn.6085 /len=464
rc_AI231164_s_at	<-1.9	<-4.4	EST227852 Rattus norvegicus cDNA, 3' end /clone =REMDG57 /clone_end=3' /gb=AI231164 /ug=Rn.8538 /len=491
X12535cds_at	<-4	<-2.2	X12535cds RNRASP23 Rat mRNA for ras-related protein p23
X92097_at	-2,4	-3,6	R.norvegicus mRNA for transmembrane protein rnp21.4/cds=(23,628) /gb=X92097 /gi=1213220 /ug =Rn.22775 /len=716
J02780_at	-2	-4	Rat tropmyosin (TM-4) mRNA, complete cds /cds= (26,772) gb=J02780 /gi=207503 /ug=Rn.11115 /len=900
U64705cds_f_at	-2,1	<-3.8	U64705cds RNU64705 Rattus norvegicus protein synthesis initiation factor 4AII gene,partial cds, and E3 small nucleolar RNA gene,complete sequence
L13619_at	-2	-3,9	RATCL6A Rattus rattus insulin-induced growth response protein (CL-6) mRNA,complete cds
U67995_s_at	-2	<-3.8	Rattus norvegicus stearyl-CoA desaturase 2mRNA, partial cds /cds=(0,92) /gb=U67995/gi=1763026 /ug=Rn.10650 /len=315
U67913_s_at	<-2.2	<-3.5	RNU67913 Rattus norvegicus mast cell protease 10 (RMCP-10) mRNA, partial cds
U22297_at	-2,2	<-3.4	Rattus norvegicus casein kinase 1 gamma 2isoform mRNA,complete cds /cds=(192,1436)/gb=U22297 / gi=854734 /ug Rn.10083 /len=1572
rc_AI231547_at	-2	-3,5	EST228235 Rattus norvegicus cDNA, 3' end/clone =REMDL77 /clone_end=3' /gb=AI231547 /ug =Rn.23741 /len=542
AJ011608_at	-2,2	<-3.2	RNO011608 Rattus norvegicus mRNA for DNA Polymerase alpha subunit IV (primase),partial
U73174_g_at	-2,7	<-2.6	RNU73174 Rattus norvegicus glutathione reductase mRNA, complete cds
U75920_at	-2,2	<-2.9	RNAPCBP1 Rattus norvegicus APC binding protein EB1 mRNA, complete cds
D42148_at	-2,1	<-2.9	RATGPF Rat mRNA for growth potentiating factor, complete cds
AA685903_at	-2	-3	EST108576 Rat PC-12 cells, NGF-treated (9 days) Rattus sp. cDNA clone RPNAC215' end similar to Glucose Regulated protein, 94 kDa (GRP94), mRNA sequence [Rattus sp.]
rc_AI136977_at	<-2.4	<-2.5	UI-R-C2p-nz-f-10-0-UI.s1 Rattus norvegicus cDNA, 3' end/clone=UI-R-C2p-nz-f-10-0-UI /clone_end=3' /gb=AI136977 /ug=Rn.23741/len=376
M57728_at	<-1.6	<-3.2	Rat general mitochondrial matrix processing protease (MPP)mRNA, 3' end /cds=(0,1574)/gb=M57728 /gi =205516 /ug=Rn.11175 /len=1712
U13895_s_at	-2,7	<-2.1	RNU13895 Rattus norvegicus MSS1 protein (MSS1) mRNA, partial cds
rc_AI639255_g_at	-2	<-2.7	Rat mixed-tissue library Rattus norvegicuscDNA clone rx01039 3', mRNA sequence[Rattus norvegicus]
M13979_at	-2,1	<-2.6	Rat brain glucose-transporter protein mRNA, complete cds/cds=(208,1686) /gb=M13979 /gi=204413 /ug= Rn.3205 /len=2571
rc_AA891829_at	<-1.6	<-3	EST195632 Rattus norvegicus cDNA, 3' end /clone =RKIAH34 /clone_end=3' /gb=AA891829 /gi=3018708 /ug=Rn.3498 /len=667
S67722_s_at	-3,1	<-1.5	cyclooxygenase isoform COX-2 [rats, Sprague-Dawley, lipopolysaccharide-stimulated peritoneal macrophages, mRNA, 4154 nt]
X78949_at	-2,2	-2,3	R.norvegicus mRNA for prolyl 4-hydroxylasealpha subunit

			/cds=(69,1673) /gb=X78949 /gi=474939 /ug=Rn.8531 /len=1838
rc_AA875126_at	<-1.6	<-2.9	UI-R-E0-bu-d-03-0-UI.s2 Rattus norvegicus cDNA, 3' end clone=UI-R-E0-bu-d-03-0-UI/clone_end=3' /gb=AA875126 /gi=2980074/ug=Rn.2799 /len=552
rc_AI070295_g_at	-2,1	-2,3	UI-R-Y0-lt-d-01-0-UI.s1 Rattus norvegicus cDNA, 3' end clone=UI-R-Y0-lt-d-01-0-UI /clone_end=3' /gb=AI070295 /ug=Rn.10250/len=545
U63923_at	-2,2	<-2.1	RRU63923 Rattus rattus thioredoxin reductase mRNA, complete cds
M31178_at	<-2.4	<-1.9	Rat calbindin D28 mRNA, complete cds /cds= (285,1070) gb=M31178 /gi=203234 /ug=Rn.3908 /len=2280
L11319_at	<-1.7	<-2.5	Rat signal peptidase mRNA, complete cds /cds= (74,613) /gb=L11319 /gi=206977 /ug=Rn.24875 /len=643
rc_AA859990_s_at	<-2.2	<-2	UI-R-E0-ca-a-08-0-UI.s1 Rattus norvegicuscDNA, 3' end clone=UI-R-E0-ca-a-08-0-UI /clone_end=3' /gb=AA859990 /gi=2949510 /ug=Rn.861 /len=441
S74572_g_at	<-1.6	<-2.5	Mg2+ dependent protein phosphatase betaisoform {alternatively spliced} [rats, brain, mRNA, 1503 nt]
X62323_at	-2,4	<-1.6	R.norvegicus Pan-1 mRNA /cds=(0,1917) /gb=X62323 gi=35277 /ug=Rn.10290 /len=2001
AF048828_at	-2,1	<-1.8	Rattus norvegicus voltage dependent anionchannel (RVDAC1) mRNA, complete cds
X55286_at	<-1.4	<-2.5	R.norvegicus mRNA for HMG-CoA reductase/cds= (0,734/gb=X55286 /gi=296924 /ug=Rn.10469 /len=1159
M64300_at	<-1.4	<-2.5	Rat extracellular signal-related kinase (ERK2) mRNA, completecds /cds=(171,1247) /gb=M64300 /gi=204055 /ug=Rn.13651/len=1467
X99723cds_at	<-1.6	<-2.1	X99723cds RNBRAHMA R.norvegicus mRNA for homologueof brahma protein, C terminal
AF048828_g_at	<-1.9	<-1.8	Rattus norvegicus voltage dependent anionchannel (RVDAC1) mRNA, complete cds
rc_AA997614_s_at	-2,1	<-1.4	UI-R-C0-hy-g-09-0-UI.s1 Rattus norvegicuscDNA, 3' end clone=UI-R-C0-hy-g-09-0-UI/clone_end=3' /gb= AA997614 /ug=Rn.6150 /len=348
M28255_s_at	<1.4	<3	RATCYO8A Rat cytochrome c oxidase subunit VIII mRNA, 3' end
U20643mRNA#2_f_at	>-2.1	>-3.4	U20643mRNA#2 RNU20643 Rattus norvegicus aldolase A gene, 5' region
L17127_at	>-2	>-16	RATRN3 Rattus norvegicus proteasome RN3 subunit mRNA, complete cds
X62146cds_at	-1,1	>-4.8	X62146cds RRRPL11 R.rattus mRNA for ribosomal protein L11
rc_AI234604_s_at	-1,3	>-23	EST231166 Rattus norvegicus cDNA, 3' end /clone= RLUDC11 /clone_end=3' /gb=AI234604 /ug=Rn.3672 /len=454
M89945mRNA_at	-1,7	<-19	M89945mRNA RATFARDIPH Rat farnesyl diphosphate synthase gene, exons 1-8
U77918_at	-15	-1,2	Rattus norvegicus spermatogenic cell/sperm-associated Tat-binding protein homolog Sata mRNA, complete cds /cds=(228,1556) /gb=U77918 /gi=1710983 /ug =Rn.11173/len=1627
D10706cds#2_s_at	-1,8	-14	D10706cds#2 RATODCB Rat mRNA for ornithine decarboxylase antizyme, complete cds
rc_AA892776_at	-11	-1,5	EST196579 Rattus norvegicus cDNA, 3' end /clone =RKIAX19 /clone_end=3' /gb=AA892776 /gi=3019655 /ug=Rn.3606 /len=588
J03481mRNA_at	-1,6	<-8.6	J03481mRNA RATDTR Rat dihydropteridine reductase mRNA, complete cds
D45247_at	-1,4	<-7.6	RATPSRCX Rat mRNA for proteasome subunit RCX, complete cds

E03358cds_at	-1,7	<-7	E03358cds cDNA encoding rat polyfunctional protease component C3
AF052042_s_at	-1,5	<-6.8	Rattus norvegicus zinc finger protein Y1 (RLZF-Y) mRNA, complete cds
L12380_at	-1,7	<-6.2	Rattus norvegicus ADP-ribosylation factor 1 mRNA, complete cds /cds=(74,619) /gb=L12380 /gi=438861 /ug=Rn.1391 /len=900
X14210cds_at	-1,3	-6,6	X14210cds RNRPS4 Rat mRNA for ribosomal protein S4
M93297cds_at	-1,3	<-6.4	M93297cds RATROAT04 Rattus norvegicus ornithine aminotransferase (rOAT) gene, exon 7
X87885_at	-1,5	-6,1	R.norvegicus mRNA for mammalian fuscaprotein /cds= (0,1415) /gb=X87885 /gi=871527 /ug=Rn.16873 /len=1416
S57478cds_s_at	-1,7	-5,6	S57478cds S57440S13 lipocortin I [rats, Genomic, 361 nt, segment 13 of 13]
X53363cds_s_at	-1,7	-5,5	X53363cds RNCALRET Rat mRNA for calreticulin
E12286cds_at	undef	<-3.6	E12286cds cDNA encoding rat GM2 activator protein
D89514_at	-1,7	<-5.4	Rattus norvegicus mRNA for 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase,complete cds /cds=(55,1833) /gb =D89514 /gi=2541905/ug=Rn.11052 /len=1928
rc_AI177503_at	-1,8	-5,3	EST221135 Rattus norvegicus cDNA, 3' end /clone =RPLCA81 /clone_end=3' /gb=AI177503 /ug =Rn.11066 /len=575
Y15068_at	-1,5	-5,6	RNRNAHOP Rattus norvegicus mRNA for Hsp70/Hsp90 organizing protein
M22924_g_at	-1,5	<-5.3	Rat IgE Fc receptor deleted beta-subunit mRNA, completecds /cds=(54,392) /gb=M22924 /gi=204118 /ug=Rn.10922/len=648
X74401_at	-1,5	<-5.3	R.norvegicus rab GDI beta mRNA /cds=(30,1367) /gb=X74401 /gi=396432 /ug=Rn.11011 /len=1395
D10754_at	-1,7	-5,1	RATPRORD Rat mRNA for proteasome subunit R-DELTA, complete cds
D63411_s_at	-1,6	<-5.1	RATMPR Rat mRNA for mitochondrial precursor receptor, complete cds
U94340_at	-1,5	<-4.7	RNU94340 Rattus norvegicus poly(ADP-ribose) polymerase mRNA, complete cds
M24604_g_at	-1,4	-4,8	Rat proliferating cell nuclear antigen (PCNA/cyclin) MRNA complete cds /cds=(62,847)/gb=M24604 /gi=206047 /ug=Rn.223 /len=1160
rc_AI059508_s_at	-1,5	-4,6	UI-R-C1-kw-a-06-0-UI.s1 Rattus norvegicus cDNA, 3' end/clone=UI-R-C1-kw-a-06-0-UI /clone_end=3' /gb =AI059508 /ug=Rn.5950 /len=346
D13127_at	-1,8	<-4.3	RATOSCP Rattus norvegicus mRNA for oligomycin sensitivity conferring protein, complete cds
rc_AA799650_at	-1,6	<-4.2	EST189147 Rattus norvegicus cDNA, 3' end /clone= RHEAD59 /clone_end=3' /gb=AA799650 /gi=2862605 /ug=Rn.2011 /len=593
Y09332cds_s_at	-1,5	<-4.3	Y09332cds RNCPPIACT R.norvegicus mRNA for cytosolicperoxisome proliferator-induced acyl-CoA thioesterase
L11930_at	-1,6	<-4	Rattus norvegicus cyclase-associated protein homologue (MCH1) mRNA, complete cds /cds= (21,1445) /gb=L11930 /gi=310173 /ug=Rn.21389 /len=1460
rc_AI235585_s_at	-1,7	-3,9	EST232147 Rattus norvegicus cDNA, 3' end /clone =ROVCT80 /clone_end=3' /gb=AI235585 /ug=Rn. 11085/len=384
AF083269_at	-1,7	-3,8	Rattus norvegicus p41-Arc mRNA, complete cds
rc_AI031019_at	-1,5	<-3.9	UI-R-C0-je-h-10-0-UI.s1 Rattus norvegicus cDNA, 3' end clone=UI-R-C0-je-h-10-0-UI /clone_end=3' /gb= AI031019 /ug=Rn.9181 /len=510

L17127_g_at	-1,7	-3,5	RATRN3 Rattus norvegicus proteasome RN3 subunit mRNA, complete cds
M19936_at	-3,2	-1,9	Rat sulfated glycoprotein 1 (SGP-1) mRNA, complete cds /cds=(27,1691) /gb=M19936 /gi=206904 /ug Rn.877 /len=2175
rc_AI044341_s_at	-1,8	<-3.3	UI-R-C0-is-a-11-0-UI.s1 Rattus norvegicus cDNA, 3' end/clone=UI-R-C0-is-a-11-0-UI /clone_end=3' /gb =AI044341/ug=Rn.3820 /len=409
M55015cds_s_at	-1,5	-3,6	M55015cds RATNUCIA1 Rat nucleolin gene
D13907_at	-1,5	-3,5	Rat mRNA for mitochondrial processing protease P52, partial sequence /cds=(0,1463) /gb=D13907 /gi=397698 /ug=Rn.841 /len=1570
M38759_at	-1,8	-3,2	Rat androgen binding protein (ABP) mRNA,complete cds /cds=(12,2660) /gb=M38759 /gi=202629 /ug= Rn.705 /len=2960
AF025308_f_at	-1,6	<-3.3	Rattus norvegicus MHC class Ib antigen (RT1.Cl) gene, complete cds /cds=(0,1133) /gb=AF025308 /gi=2570820 /ug=Rn.11244 /len=1134
J02585_at	-1,6	-3,2	Rat liver stearyl-CoA desaturase mRNA, complete cds cds=(102,1178) /gb=J02585/gi=206859 /ug=Rn.10982 /len=4689
AF036335_at	-1,5	-3,3	Rattus norvegicus NonO/p54nrb homolog mRNA, partial cds /cds=(0,506) /gb=AF036335 /gi=2674208 /ug =Rn.1926 /len=1020
D13962_g_at	-3,1	-1,6	RATGLUT3 Rat mRNA for neuron glucose transporter
M21622_g_at	-1,8	-2,9	Rat high-affinity IgE receptor (Fc-epsilon-R-I) mRNA, complete cds, clones R8-2b and R3-3 /cds=(176,853) gb=M21622 /gi=204109/ug=Rn.9677 /len=1179
AB010119_at	-1,5	<-3.2	Rattus norvegicus mRNA for Tctex-1, complete cds
U35774_at	-1,6	<-2.9	Rattus norvegicus cytosolic branch chain amino transferase mRNA, complete cds /cds=(62,1297) /gb=U35774 /gi=1173633 /ug=Rn.8273 /len=1370
Y12635_at	-1,7	-2,7	R.norvegicus mRNA for vacuolar adenosine triphosphatase subunit B /cds=(14,1549) /gb= Y12635 /gi=2058353 /ug=Rn.13436 /len=1614
AF039085_at	-1,6	-2,8	Rattus norvegicus cellugyrin mRNA, complete cds /cds=(153,857) /gb=AF039085 /gi=2773063 /ug=Rn.8682 /len=1108
Y08355cds#2_at	<-2.2	undef	Y08355cds#2 RNPKCZIP R.norvegicus mRNA for PKC-zeta-interacting protein
D10756_at	-1,5	<-2.8	RATPRORZ Rat mRNA for proteasome subunit R-ZETA, complete cds
AJ011606_at	-1,7	<-2.6	RNO011606 Rattus norvegicus mRNA for DNA polymerase alpha subunit II, partial
D84477_at	-1,7	-2,5	Rattus norvegicus mRNA for RhoA, partial cds
rc_AA944397_at	-1,9	<-2.3	EST199896 Rattus norvegicus cDNA, 3' end /clone REMAG54 /clone_end=3' /gb=AA944397 /gi=3104313 /ug=Rn.5916 /len=542
rc_AA799663_g_at	-2,2	-1,9	EST189160 Rattus norvegicus cDNA,3' end /clone =RHEAD74/clone_end=3' /gb=AA799663 /gi=2862618 /ug=Rn.6216/len=478
rc_AA799819_at	-1,5	-2,5	EST189316 Rattus norvegicus cDNA, 3' end /clone =RHEAF74 /clone_end=3' /gb=AA799819 /gi=2862774 /ug=Rn.22779 /len=356
AF009330_at	-2,1	-1,9	Rattus norvegicus enhancer-of-split and hairy-related protein 2 (SHARP-2) mRNA, complete cds /cds= (319,1554) /gb=AF009330 /gi=2267588 /ug =Rn.10785/len=2388
rc_AA858607_at	-1,8	-2,1	UI-R-E0-bq-a-08-0-UI.s1 Rattus norvegicus cDNA, 3' end clone=UI-R-E0-bq-a-08-0-UI /clone_end=3' /gb= AA858607/gi=2948947/ug=Rn.3532 /len=487
M94043_at	-2,1	-1,7	Rat rab-related GTP-binding protein mRNA, complete cds cds=(49,684) /gb=M94043 /gi=206542 /ug=Rn. 9824 /len=1405

M24324_f_at	-1,5	<-2.2	Rat MHC class I RT1 (RTS) mRNA (u haplotype), 3' end cds=(0,281) /gb=M24324 /gi=205459 /ug=Rn.3577 /len=521

Dexamethasone treatment of RBL cells: 24h

geneID	ept. #1 Ratio* (1)	expt. #2 Ratio* (2)	description

Genes that go 2 times up and 0 times down.
==========

geneID	ept. #1 Ratio* (1)	expt. #2 Ratio* (2)	description
A16585cds_s_at	19	30	A16585cds R.norvegicus mRNA for preprorelaxin
U11275mRNA_s_at	>19	>18	U11275mRNA RNPNMTS1 Rattus norvegicus WKY and SHRSP phenylethanolamine N-methyltransferase (PNMT) gene, exon 1
S81478_s_at	>13	>4.6	3CH134/CL100 PTPase=oxidative stress-inducible protein tyrosine phosphatase [rats, peritoneal macrophage cDNA library, mRNA, 1912 nt]
J00780_at	6,9	4,4	rat preprorelaxin mrna /cds=(43,603) /gb=J00780 /gi=206606 /ug=Rn.9830 /len=800
X53501cds_at	6,8	3,4	X53501cds RRTPH2 Rat mRNA for tryptophan hydroxylase (EC 1.14.16.4)
rc_AI137583_at	4,1	4,4	UI-R-C0-hf-a-03-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-C0-hf-a-03-0-UI /clone_end=3' /gb=AI137583 /ug=Rn.3272 /len=496
U75404UTR#1_s_at	5,1	3	U75404UTR#1 RNU75404 Rattus norvegicus Ssecks 322 mRNA, 3' untranslated region, partial sequence
rc_AI230256_at	3,5	4,1	EST226951 Rattus norvegicus cDNA, 3' end /clone=REMCU23 /clone_end=3' /gb=AI230256 /ug=Rn.3272 /len=499
rc_AA894004_at	3,7	3,8	EST197807 Rattus norvegicus cDNA, 3' end /clone=RPLAO48 /clone_end=3' /gb=AA894004 /gi=3020883 /ug=Rn.8945 /len=430
rc_AI235758_s_at	2,4	4,8	EST232320 Rattus norvegicus cDNA, 3' end /clone=ROVCW69/clone_end=3' /gb=AI235758 /ug=Rn.4075 /len=460
D12769_g_at	>3.8	>3.2	RATBTEB Rattus norvegicus mRNA for BTE binding protein
rc_AA894332_at	>2.5	>4.3	EST198135 Rattus norvegicus cDNA, 3' end /clone=RSPAW79 /clone_end=3' /gb=AA894332 /gi=3021211 /ug=Rn.17129 /len=503
rc_AA900505_at	4	2,3	UI-R-E0-dl-b-07-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-E0-dl-b-07-0-UI /clone_end=3' /gb=AA900505 /gi=3035859 /ug=Rn.2042 /len=595
rc_AI639338_at	2,5	3,8	Rat mixed-tissue library Rattus norvegicus cDNA clone rx01696 3', mRNA sequence [Rattus norvegicus]
rc_AA874784_s_at	>4.4	>1.8	UI-R-E0-bw-e-07-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-E0-bw-e-07-0-UI /clone_end=3' /gb=AA874784 /gi=2979732 /ug=Rn.3307 /len=483
rc_AI014169_at	3,5	2,6	EST207724 Rattus norvegicus cDNA, 3' end /clone=RSPBF88 /clone_end=3' /gb=AI014169 /ug=Rn.2758 /len=553
AF023087_s_at	2,5	3,2	Rattus norvegicus nerve growth factor induced factor A mRNA, partial 3'UTR
Z12158cds_at	2,5	3,2	Z12158cds RRPDHYE1A R.rattus pyruvate

			dehydrogenase E1 alpha form 1 subunit
U42627_at	3,3	2,4	Rattus norvegicus dual-specificity protein tyrosine phosphatase(rVH6) mRNA, complete cds /cds=(360, 1505) /gb=U42627 /gi=1185551 /ug=Rn.4313 /len=2104
AF095576_at	3,1	2,5	Rattus norvegicus APS protein mRNA, complete cds
L14463_at	3,5	2,1	RATESP2A Rattus rattus R-esp2 mRNA, complete cds
X70369_s_at	>3.3	>2.3	R.norvegicus mRNA for pro alpha 1 collagen type III /cds (0,1911) /gb=X70369 /gi=57915 /ug=Rn.3247 /len=2183
rc_AA799323_at	2,9	2,5	EST188820 Rattus norvegicus cDNA, 3' end /clone =RHEAA31 /clone_end=3' /gb=AA799323 /gi=2862278 /ug=Rn.6178 /len=328
U76206_at	2,3	3	Rattus norvegicus VTR 15-20 receptor mRNA, complete cds /cds=(238,1155) /gb=U76206 /gi=2459584 /ug= Rn.16317 /len=1690
AB015308_s_at	>2.2	3	Rattus norvegicus mRNA for GTP binding protein alpha 15, complete cds
rc_AI011998_at	2,8	2,4	EST206449 Rattus norvegicus cDNA, 3' end /clone =RPLAR43 /clone_end=3' /gb=AI011998 /ug=Rn.11296 /len=495
D17809_at	2,3	2,9	Rat mRNA for beta-4N-acetylgalactosaminyltransferase, complete cds /cds=(30,1631) /gb=D17809 /gi=497841 /ug =Rn.10119 /len=2166
rc_AA944422_at	2,8	2,3	EST199921 Rattus norvegicus cDNA, 3' end /clone=REMA J01 /clone end=3' /gb=AA944422 /gi=3104338 /ug=Rn.871 /len=641
L20681_at	2,6	2,4	Rat proto-oncogene (Ets-1) mRNA, complete cds /cds=(294,1619) /gb=L20681 /gi=404781 /ug=Rn.7142 /len=4991
AF058791_at	2,6	2,3	Rattus norvegicus G10 protein homolog (edg2) mRNA, complete cds/cds=(184,618) /gb=AF058791 /gi=3064069 /ug=Rn.8172 /len=816
rc_AA893267_at	2,4	2,5	EST197070 Rattus norvegicus cDNA, 3' end /clone =RKIBE29 /clone_end=3' /gb=AA893267 /gi=3020146 /ug=Rn.6937 /len=472
X63594cds_at	>3.3	>1.5	X63594cds RRRLIF1 R.rattus RL/IF-1 mRNA
M32062_at	2,2	2,5	Rat Fc-gamma receptor mRNA, complete cds /cds= (49,852) /gb=M32062 /gi=204114 /ug=Rn.6050 /len=1341
D12769_at	>2.4	>1.9	RATBTEB Rattus norvegicus mRNA for BTE binding protein
M91652complete_seq_g_at	2,3	2	M91652completeSeq Rat glutamine synthetase (glnA) mRNA, complete cds /cds=UNKNOWN /gb=M91652 /gi=204348 /ug=Rn.2204 /len=2793
AB000717exons#1-8_s_at	2,1	2,1	AB000717exons#1-8 AB000716S2 Rattus rattus gene for non-hepatic-type S-adenosylmethionine synthetase, exon 2 and complete cds
L26268_g_at	2	2,2	Rattus norvegicus anti-proliferative factor (BTG1) mRNA, complete cds /cds=(0,515) /gb=L26268 /gi=1167495 /ug=Rn.1000 /len=1464
rc_AI070124_at	>2.3	>1.8	UI-R-Y0-lu-d-08-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-Y0-lu-d-08-0-UI /clone_end=3' /gb=AI070124 /ug=Rn.11286 /len=527
U24652_at	>1.7	>2.3	Rattus norvegicus Lnk1 mRNA, complete cds /cds= (75,953) /gb=U24652 /gi=1109773 /ug=Rn.11228 /len=3285
rc_AA799396_g_at	>1.6	>2.3	EST188893 Rattus norvegicus cDNA, 3' end /clone= RHEAA74 /clone_end=3' /gb=AA799396 /gi=2862351 /ug=Rn.263 /len=637
U66723_s_at	undef	>4	RNU66723 Rattus norvegicus purine-selective sodium/ nucleoside cotransporter (rCNT2) mRNA, complete cds
rc_AA925887_at	undef	>3	UI-R-A1-eo-h-06-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-A1-eo-h-06-0-UI /clone_end=3' /gb=AA925887 /gi=3073023 /ug=Rn.8368 /len=417
S81497_i_at	3,9	1,6	lysosomal acid lipase=intracellular hydrolase [rats,

			Wolman, liver, mRNA, 3144 nt]
M32062_g_at	1,7	3,2	Rat Fc-gamma receptor mRNA, complete cds /cds =(49,852) /gb=M32062 /gi=204114 /ug=Rn.6050 /len=1341
U61729_at	1,8	2,9	Rattus norvegicus proline rich protein mRNA, complete cds /cds=(175,984) /gb=U61729 /gi=1408276 /ug =Rn.10967 /len=1619
M91652complete_seq_at	2,6	1,9	M91652completeSeq Rat glutamine synthetase (glnA) mRNA, complete cds /cds=UNKNOWN /gb=M91652 /gi=204348 /ug=Rn.2204 /len=2793
D90109_at	2,6	1,8	Rat mRNA for long-chain acyl-CoA synthetase (EC 6.2.1.3) /cds=(13,2112) /gb=D90109 /gi=220717 /ug=Rn.6215 /len=3657
rc_AI232783_s_at	1,9	2,5	EST229471 Rattus norvegicus cDNA, 3' end /clone= RKICG50 /clone_end=3' /gb=AI232783 /ug=Rn.2204 /len=478
rc_AA892154_g_at	1,8	2,4	EST195957 Rattus norvegicus cDNA, 3' end /clone= RKIAN02 /clone_end=3' /gb=AA892154 /gi=3019033 /ug=Rn.3279 /len=386
X62951mRNA_s_at	2,2	1,9	X62951mRNA RNPBUS19 R.norvegicus mRNA (pBUS19) with repetitive elements
M12492mRNA#1_at	2,2	1,9	M12492mRNA#1 Rat type II cAMP-dependent protein kinase regulatory subunit mRNA, 3' end /cds =UNKNOWN /gb=M12492 /gi=206670 /ug=Rn.4075 /len=3108
AB015432_s_at	2,2	1,8	Rattus norvegicus mRNA for LAT1 (L-type amino acid transporter 1), complete cds
rc_AA875620_at	2,1	1,9	UI-R-E0-cv-d-12-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-E0-cv-d-12-0-UI /clone_end=3' /gb= AA875620 /gi=2980568 /ug=Rn.2978 /len=387
rc_AA799396_at	>2	undef	EST188893 Rattus norvegicus cDNA, 3' end /clone= RHEAA74 /clone_end=3' /gb=AA799396 /gi=2862351 /ug=Rn.263 /len=637
rc_AA818593_at	2	1,9	UI-R-A0-bc-g-01-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-A0-bc-g-01-0-UI /clone_end=3' /gb=AA818593 /gi=2889332 /ug=Rn.1944 /len=475
M12492mRNA#1_g_at	1,9	2	M12492mRNA#1 Rat type II cAMP-dependent protein kinase regulatory subunit mRNA, 3' end /cds= UNKNOWN /gb=M12492 /gi=206670 /ug=Rn.4075 /len=3108
X62950mRNA_f_at	2	1,7	X62950mRNA RNPBUS30 R.norvegicus mRNA (pBUS30) with repetitive elements
rc_AA892146_f_at	2	1,5	EST195949 Rattus norvegicus cDNA, 3' end /clone =RKIAM90 /clone_end=3' /gb=AA892146 /gi=3019025 /ug=Rn.24928 /len=439

Genes that go 0 times up and 2 times down.

==========

geneID	ept. #1 Ratio* (1)	expt. #2 Ratio* (2)	description
M34097_at	-4	-5,2	Rat natural killer (NK) cell protease 1 (RNKP-1) mRNA, complete cds /cds=(108,854) /gb=M34097 /gi=206689 /ug=Rn.9837 /len=1113
S67722_s_at	-2,8	<-4.6	cyclooxygenase isoform COX-2 [rats, Sprague-Dawley, lipopolysaccharide-stimulated peritoneal macrophages, mRNA, 4154 nt]
M38759_at	-2,9	-3,1	Rat androgen binding protein (ABP) mRNA, complete cds /cds=(12,2660) /gb=M38759 /gi=202629 /ug= Rn.705 /len=2960

rc_AI175935_at	<-3.3	<-2.5	EST219508 Rattus norvegicus cDNA, 3' end /clone =ROVBH40 /clone_end=3' /gb=AI175935 /ug=Rn.8737 /len=448
M21622_at	-2,3	-3,3	Rat high-affinity IgE receptor (Fc-epsilon-R-I) mRNA, complete cds, clones R8-2b and R3-3 /cds=(176,853) /gb=M21622 /gi=204109 /ug=Rn.9677 /len=1179
rc_AA859990_s_at	<-2.7	-2,1	UI-R-E0-ca-a-08-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-E0-ca-a-08-0-UI /clone_end=3' /gb= AA859990 /gi=2949510 /ug=Rn.861 /len=441
rc_AA799663_g_at	-2,4	-2,3	EST189160 Rattus norvegicus cDNA, 3' end /clone= RHEAD74 /clone_end=3' /gb=AA799663 /gi=2862618 /ug=Rn.6216 /len=478
rc_AA800735_g_at	-2	-2,6	EST190232 Rattus norvegicus cDNA, 3' end /clone= RLUAK81 /clone_end=3' /gb=AA800735 /gi=2863690 /ug=Rn.6627 /len=552
D42148_at	-2,3	-2,2	RATGPF Rat mRNA for growth potentiating factor, c omplete cds
M94287_at	<-1.8	<-2.6	Rattus norvegicus nucleolar phosphoprotein of 140kD, Nopp140 mRNA, complete cds /cds=(52,2166) /gb= M94287 /gi=205749 /ug=Rn.9517 /len=3609
X96426_at	-2,1	-2,1	R.norvegicus mRNA for skeletal muscle elongation factor-2 kinase /cds=(290,2464) /gb=X96426 /gi= 1495778 /ug=Rn.10958 /len=4782
rc_AA892855_at	<-2.5	<-1.6	EST196658 Rattus norvegicus cDNA, 3' end /clone =RKIAY14 /clone_end=3' /gb=AA892855 /gi=3019734 /ug=Rn.14796 /len=532
rc_AI639255_at	-2,1	-2	Rat mixed-tissue library Rattus norvegicus cDNA clone rx01039 3', mRNA sequence [Rattus norvegicus]
rc_AA800735_at	<-1.6	-2,4	EST190232 Rattus norvegicus cDNA, 3' end /clone= RLUAK81 /clone_end=3' /gb=AA800735 /gi=2863690 /ug=Rn.6627 /len=552
M21622_g_at	-1,9	-2,9	Rat high-affinity IgE receptor (Fc-epsilon-R-I) mRNA, complete cds, clones R8-2b and R3-3 /cds=(176,853) /gb=M21622 /gi=204109 /ug=Rn.9677 /len=1179
X66693_f_at	-1,6	-3	R.norvegicus mRNA for granzyme-like protein I /cds= (70,816) /gb=X66693 /gi=296177 /ug=Rn.10459 /len=925
rc_AI639255_g_at	-2,4	-1,7	Rat mixed-tissue library Rattus norvegicus cDNA clone rx01039 3', mRNA sequence [Rattus norvegicus]
rc_AA799995_g_at	-1,5	-2,5	EST189492 Rattus norvegicus cDNA, 3' end /clone= RHEAI05 /clone_end=3' /gb=AA799995 /gi=2862950 / ug=Rn.11511 /len=556
rc_AI045440_at	-1,8	-2,2	UI-R-C1-jz-a-12-0-UI.s2 Rattus norvegicus cDNA, 3' end /clone=UI-R-C1-jz-a-12-0-UI /clone_end=3' /gb =AI045440 /ug=Rn.11144 /len=396
rc_AA859966_s_at	-1,9	-2	UI-R-E0-ca-g-03-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-E0-ca-g-03-0-UI /clone_end=3' /gb= AA859966 /gi=2949486 /ug=Rn.861 /len=392
X66870_at	-2	-1,7	R.norvegicus mRNA for lamin A /cds=(165,2087) /gb =X66870 /gi=56550 /ug=Rn.90 /len=3069
M64301_at	-2,5	-1,7	RATERK3 Rat extracellular signal-related kinase (ERK3) mRNA, complete cds

*Ratio is measure of fold induction.

ABBREVIATIONS

A	Adenine
A	Ampere
Ag	Antigen
APS	Ammonium persulfate
ATP	Adenosine tri-phosphate
BMMC	Bone marrow-derived mast cells
bp	Base pairs
Bq	Bequerel
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
Ci	Curie
Cpm	Counts per minute
Da	Dalton
DBD	DNA-binding domain
ddNTP	Di-deoxy nucleoside tri-phosphate
ddH_2O	Double-distilled water
DMEM	Dulbecco´s Modified Eagles Medium
DMSO	Dimethylsulfoxide
DNA	Deoxy-ribo nucleic acid
DNP	Dinitrophenyl
dNTP	Deoxynucleotide tri-phosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylendiamine-N, N-tetracetate
EST	Expressed sequence tag
FACS	Fluorescence activated cell sorter
Fc□RI	Receptor with high affinity for IgE
FCS	Foetal calf serum
Fig.	Figure
FITC	Fluorescein isothocianate
g	Gram
g	Gravity (unit of relative centrifugal force)
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GTP	Guanosine triphosphate
h	Hour
HBD	Hormone-binding domain
HEPES	N-2-hydroxyethilpiperasine-N´-2 ethansulfonic acid
HRP	Horseradish peroxidase
Hsp/HSP	Heat shock protein
IgE	Immunoglobulin E
IL-	Interleukin-
IMDM	Iscove´s Modified Dulbecco´s Medium
k	Kilo
l	Liter

Luc	Luciferase
m	Meter
m	Milli
M	Molar
min	Minute
MMTV	Mouse mammary tumour virus
MOPS	4-morpholinepropanesulfonic acid
mRNA	Messenger RNA
µ	Micro
n	Nano
nGRE	Negative glucocorticoid response element
NP-40	Nonidet P- 40
OD	Optical density
p	Pico
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl fluoride
RIPA	Radioimmunoprecipitation assay (buffer)
RNA	Rybonucleic acid
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute (culture medium)
RT	Room temperature
RT-PCR	Reverse transcription PCR
SCF	Stem cell factor
s	Second
SD	Standard deviation
SDS	Sodium-Dodecyl- Sulfate
SSC	Standard saline citrate
T	Thymine
TAE	Tri/acetate/EDTA electrophoresis buffer
TBE	Tris-borate EDTA
TE	Tris-EDTA
TEMED	N, N, N´N´-tetramethylethylendiamine
T_m	Melting temperature
TPA	12-*O*-tetradecanoyl phorbol 13-acetate
TRIS	Tris-(hydroxymethyl)-aminomethane
U	Unit
UV	Ultraviolet
V	Volt
Vol	Volume
v/v	Volume on volume
W	Watt
wt	Wild type
w/v	Weight on volume
Y	Tyrosine

Acknowledgements

This work was performed at the Institut für Toxikologie und Genetik (ITG), Forschungszentrum Karlsruhe and financially supported by the Forschungszentrum Karlsruhe.

I would like to thank Prof. Dr. Peter Herrlich for giving me the opportunity to do my PhD at ITG.

I am especially grateful to Prof. Dr. Andrew Cato for giving me the opportunity to work in his group and for his patience with a "student with principles", many of which I meanwhile betrayed, as most of scientists do.

I would like to express my gratitude to Prof. Dr. Margot Zöller for accepting to review this thesis.

I would like to thank Dr. C. Hesslinger and Dr. M. Stassen who generously helped me with mast cell culture and Schering AG, Berlin for performing the Affymetrix gene expression analysis.

I am thankful to all the members of Lab. 114, the ones who are still there and the ones who left, for their encouragement, advice and practical help. Special thanks to Elke and Sigrun for their endless support, especially in positively claiming (against any evidence) that "there is no such thing as a stupid question", to Olivier for constant help and advice in the course of my work, to Alex for introducing me to the mysteries of RNA preparation, to Andy for reading this manuscript. Ljuba has been a helpful companion throughout this PhD (dis)adventure, I wish her all the best for the future.

Many people I would like to thank but I am afraid it would be too long a list; in all these years nobody has ever been greedy of help, knowledge, advice... and protocols and plasmids and gel chambers and TRIS pH 8.8 and car lifts and coffee coins... Thank you to all the people of ITG. Thanks to Giusy and Andrea for bringing a ray of sunshine in my days with the melodious sound of our motherrRRtongue and to the pirate crew. Special thanks to the people of the Animal House.

Love annuls distances but unfortunately not telephone bills, nobody knows it better than my parents, whom I will never thank enough for supporting me with comprehension and affection and for respecting my choices, however inscrutable they might have seemed.

To Marc very special thanks for having been always near to my heart, wiping away tears and working out solutions… and of course for *Gilberto*, without which all this would not have been possible.

Grazie. (Punto)!!!