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Studies on the role of Nur77 nuclear orphan receptors and Rel/NF-κB transcription factors in T cell receptor-mediated apoptosis*

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Untersuchungen zur Rolle der Nur77 Orphan-Receptoren und der Rel/NF-κB Transcriptionsfactoren bei der T-Zellreceptor-vermittelten Apoptose.

Zusammenfassung

Beim sogenannten Aktivierungs-induzierten Zelltod (AICD) induziert der T-Zellrezeptor (TZR) Apoptose in aktivierten T-Zellen des peripheren lymphoiden Systems. AICD eliminiert so die potenziel schadenden selbst-reaktiven T-Zellen und verhindert eine unlimitierte Expansion der T-Zellen während einer Immunantwort. AICD wirkt in einer klonal-spezifischen Weise in der nur die TZR aktivierten T-Zellen sterben. Ein defekter AICD ist eine der möglichen Ursachen von Autoimmunerkrankungen. Ein Verständnis der molekularen Mechanismen der TZR-vermittelten Apoptose ist daher von großer medizinischer Bedeutung. Das Nur77 Protein, ein Mitglied der Steroidhormonrezeptorfamilie für das bisher noch kein Ligand identifiziert wurde, und die Rel/NF-κB Proteine sind Transkriptionsfaktoren, für die eine Rolle beim TZR-vermittelten programmierten Zelltod gezeigt werden konnte. Da die genauen molekularen Mechanismen allerdings noch weitgehend unbekannt sind, wurde in dieser Arbeit die Funktion von Nur77 und NF-κB beim AICD in einem Zellkulturmodell genauer untersucht.

Es war vorher veröffentlicht, daß Nur77 eine positive Rolle bei AICD spielt. Zusätzlich, wurde es gezeigt, daß in Prostata-Krebs-Zelllinie Nur77 die Apoptose direkt durch Bindung an Mitochondrien induzieren kann.

In dieser Dissertation konnte es gezeigt werden, daß die Aktivierung des TZR in T-Zellhybridomen eine starke und anhaltende Expression von Nur77 bewirkt, was gut mit der Induktion von Apoptose korrelierte. Die Stimulierung des TZR führte zu einer quantitativen Translokation von Nur77 in den Zellkern und der TZR-vermittelte Zelltod konnte nicht durch das anti-apoptotische Bcl-2 Protein blockiert werden. Diese Daten legen daher nahe, daß Nur77 die Expression von Genen reguliert, die an der TZR-vermittelten Apoptose beteiligt sind, auch wenn die Überexpression von Nur77 alleine nicht ausreichte, um in diesem System Apoptose auszulösen.

Um mögliche Nur77 Zielgene zu identifizieren wurde ein System etabliert, in dem Nur77 spezifisch aktiviert werden konnte. Von insgesamt 8000 untersuchten Genen zeigten lediglich fünf eine reproduzierbare durch Nur77 vermittelte Induktion. Für zwei dieser Gene, Enolase-α und vasoactive intestinal peptide (VIP), war bereits eine Rolle bei der Regulation von Apoptose bzw. bei der T-Zellentwicklung beschrieben worden. Mit diesem experimentellen Ansatz können demnach potentielle Nur77 Zielgene isoliert werden, was zu einem besseren Verständnis der Nur77 Funktion beitragen sollte.

Abhängig vom Modell-System zeigten die vorher publizierten Ergebnisse entweder eine anti- oder pro-apoptotische Funktion der Rel/NF-κB Proteine in AICD.
In dieser Dissertation führte die Aktivierung des TZR in dem T-Zellhybridom Modell des AICD zu einer starken NF-κB Induktion. Gleichzeitig resultierte die Blockierung von NF-κB in einer erhöhten Sensitivität der Zellen gegenüber AICD was mit einer defekten Expression des anti-apoptotischen NF-κB Zielgenes *A20* koreliierte. Interessanterweise wurde der Zelltod in NF-κB-defizienten Zellen durch den Tumor-Nekrose-Faktor (TNF) und nicht durch den FasL/Fas Signalweg vermittelt. Es hat sich auch herausgestellt, daß TNF in der Abwesenheit von NF-κB die unstimulierten T-Zellen *in trans* tötet und deshalb die physiologische Regulation der AICD blockiert. Durch Rekonstitutionsexperimente konnte schließlich nachgewiesen werden, daß A20 hinreichend ist, um den TZR-vermittelten Zelltod in NF-κB-defizienten Zellen wieder auf das normale Maß zu reduzieren. Ektopisch exprimierte A20 konnte der TNF-Signalweg blockieren und gleichzeitig auch den Fas-abhängigen Zelltod in NF-κB-defizienten Zellen wiederherstellen. Diese Befunde zeigen, daß NF-κB in diesem Modell des AICD sowohl anti- als auch pro-apoptotische Funktionen ausübt. Demnach kontrolliert NF-κB durch die Expression seines Zielgenes *A20* die Balance zwischen Fas- und TNF-vermitteltem programmiertem Zelltod, was zur Erhaltung der klonalen Spezifität des AICD beiträgt.

In Zusammenfassung die in dieser Dissertation erhaltene Ergebnissen führen zu einem besseren Verständnis der molekularen Mechanismen der TZR-vermitteltem programmiertem Zelltodes.

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Summary

The Activation Induced Cell Death (AICD) is a process in which T cell receptor (TCR) induces apoptosis in activated T cells in the peripheral lymphoid system. AICD serves to remove potentially harmful self-reactive T cells and to limit T cell expansion during an immune response. AICD operates in a strictly clone specific fashion where cells stimulated through TCR, but not bystander cells, undergo apoptosis. Defective AICD is a cause of autoimmune syndromes and the understanding of the molecular mechanisms of TCR-mediated apoptosis is therefore of great medical importance. The Nur77 nuclear orphan receptor and Rel/NF-κB proteins are two families of transcription factors, which are involved in TCR-mediated cell death. Molecular mechanisms, by which these proteins contribute to AICD remain, however, obscure. In the work presented here the function of Nur77 nuclear orphan receptors and Rel/NF-κB proteins in TCR-mediated apoptosis was studied by the application of *in vitro* models of AICD.

It has been previously reported in the literature that Nur77 promotes AICD. In addition, published experiments with prostate cancer cells revealed that Nur77 translocates to mitochondria to induce apoptosis.

In this work it was shown that in T cell hybridoma Nur77 induction by TCR was prolonged and correlated with apoptosis. However, Nur77 overexpression alone did not result in apoptosis. Furthermore, Nur77 was found in the nucleus of TCR stimulated T cell hybridoma cells and TCR-mediated apoptosis was insensitive to the action of anti-apoptotic protein Bcl2 arguing against the involvement of mitochondria in this cell system. Both findings suggest that in T cells Nur77 executes its pro-apoptotic activities through gene regulation.

Conditional expression of Nur77 in Jurkat T cells was therefore used to identify genes, which respond to Nur77 activation. Out of 8000 genes tested only 5 shown reproducible up-regulation by Nur77. Among those were α-enolase and VIP genes known to regulate apoptosis and T cell development, respectively. These data validate

the suitability of gain-of-function approaches to characterise Nur77 target genes and pave the way to a better understanding of Nur77 function.

Previous studies on NF-κB function in TCR-induced apoptosis revealed either anti- or pro-apoptotic role for NF-κB depending on the AICD model used.

In the T cell hybridoma model of AICD TCR strongly induced NF-κB and inhibition of NF-κB resulted in increased sensitivity to TCR-mediated apoptosis. This correlated with a lack of induction of the anti-apoptotic A20 cytoplasmic zinc finger protein, a well-known inhibitor of TNF-induced apoptosis. Strikingly, NF-κB-deficient hybridoma cells showed TNF-dependent death rather than normal Fas-dependent AICD. In addition, in the absence of NF-κB, TNF induced bystander apoptosis demonstrating that AICD lost its clonal specificity. Introduction of an A20 transgene into NF-κB-negative T cell hybridoma cells completely reversed the phenotype. These data indicate that NF-κB plays both anti- and pro-apoptotic roles during AICD via the expression of A20. In addition, NF-κB enables cells to maintain clonal specificity of AICD by controlling the balance between Fas and TNF cell death pathways.

Together the data presented in this work increase the understanding of the molecular mechanisms underlying the process of T cell receptor induced cell death.

TABLE OF CONTENTS

ABBREVIATIONS

Ab(s)	Antibody (dies)
Ag	Antigen
ACAD	Activated T cell Autonomous Death
AICD	Activation Induced Cell Death
bp	base pair
^{o}C	degrees Celsius
cDNA	complementary cDNA
Ci	Curie
cpm	Counts pre minute
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylosulfooxide
DNA	deoxyribonucleic acid
d.p.c	days *post coitus*
DP	double-positive thymocyte
DTT	Dithiothreitol
E	Embryonic day
EBD	Estrogen binding domain
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine-N,N-tetraacetate
EGTA	Ethyleneguanidyne-N,N-tetraacetate
FCS	Fetal calf serum
g	gram
GBD	glucocorticoid binding domain
GR	glucocorticoid receptor
hr	hour (s)
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	interleukin
I□B	Inhibitor of NF-□B
IKK	I□B kinase

IP	Immunoprecipitation
IF	Immunofluorescence
kD	Kilodalton
LBD	Ligand binding domain
LN	Lymph Node
MEF	Mouse embryonic fibroblast
M	Molar
m	milli (10^{-3})
mAbs	monoclonal Antibodies
mg	milligram
MHC	Major histocompatibility complex
min	minute (s)
ml	millilitre
mM	millimolar
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger RNA
μ	micro (10^{-6})
μg	microgram
μl	microliter
μM	micromolar
n	nano (10^{-9})
NEMO	NF-κB essential modulator (IKKγ)
NES	nuclear export signal
NF-κB	nuclear factor kappa B
ng	nanogram
NLS	nuclear localisation signal
OD	optical density
O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidenedifluoride

RHD	Rel homology domain
RNA	ribonucleic acid
RT	room temperature
rpm	rotations per minute
RT-PCR	Reverse transcriptase PCR
SDS	Sodium lauryl sulphate
TBE	Tris-boric acid-EDTA
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethylene-diamine
tg	transgenic
TNF	tumor necrosis factor
TNFR	TNF receptor
TRAF	TNFR associated factor
U	Unit (s)
UV	Ultra-violet light
V	volt
v/v	volume per volume
WB	Western blot
WCE	whole cell extract
wt	wild-type
w/v	weight per volume

1 INTRODUCTION

1.1 How the adaptive immune system avoids the self-reactivity.

Specific antigen recognition by the immune system is achieved by the random generation of antigen-specific B and T cell receptors (BCRs and TCRs, respectively). Only a small number of genes encoding BCRs and TCRs are used to generate the enormously complex set of cell surface receptors. Eventually, this gives rise to a very large repertoire of B and T cells where each individual cell expresses single receptor type recognising a particular antigen and initiate a properly directed immune response. However, due to the random process of receptor generation, a fraction of TCRs and BCRs is able to recognise epitopes presented by the host. This process is detrimental to the host and proper mechanisms evolved to eliminate T cells and B cells bearing self-antigen recognising receptors. TCRs on the surface of maturing T cells in the thymus are subject to two major tests: positive and negative selection. Positive selection allows the survival of T cells that successfully rearranged a functional TCR that can interact with major histocompatibility complex (MHC) molecules. During negative selection, T cells bearing TCRs that recognize self-peptides bound to MHC with high affinity undergo apoptosis. This constitutes a mechanism of so-called "central tolerance" and this clonal deletion of auto-reactive T cells in the thymus is crucial for the establishment of the ability to discriminate between self- and non-self (Robey and Fowlkes, 1994)(Nossal, 1994).

Central tolerance is however incomplete. The reasons for this are two-fold. Firstly a prerequisite for thymic deletion is the presence of antigen in the thymus. There are examples of proteins, which are expressed in a tissue-specific fashion and therefore may not be present at the sites of negative selection. Secondly, a too stringent deletion would result in a very narrow T cell repertoire in the periphery. Indeed it was observed that high affinity T cells are predominantly eliminated by central tolerance (Bouneaud *et al.*, 2000). As a consequence healthy individuals harbour self-reactive T cells in the periphery (Lohmann *et al.*, 1996) though these cells are more likely to bear low affinity TCRs for self-antigens (Bouneaud *et al.*, 2000). Since autoimmunity is a

rather rare phenomenon mechanism must exist to restrict expansion of such self-reactive T cells. These mechanisms are jointly called "peripheral tolerance" and involve either induction of anergy or apoptosis in self-reactive T cells. Anergy is a state of functional inactivation, which is a result of TCR engagement by peptide loaded MHC complex concomitantly with signalling through inhibitory coreceptors (e.g., CTLA-4) (Perez *et al.*, 1997). Anergy operates in parallel with apoptosis to control self-reactive T cells in the periphery. Peripheral deletion is also not complete and may serve simply to reduce the precursor frequency to a level at which anergy can effectively operate (Walker and Abbas, 2002).

Peripheral deletion by induction of apoptosis is a major mechanism of controlling self-reactive T cells in the periphery. Two distinct molecular pathways are now recognised, which result in the induction of apoptosis in lymphocytes destined to die. Activation induced cell death (AICD) is an antigen-dependent (clonotype-specific, cell-autonomous) process, which involves TCR-mediated sensitisation to apoptosis followed by death receptor dependent cell death (Combadiere *et al.*, 1998). The death receptors involved are either Fas or TNFR, which are transmembrane proteins belonging to TNFR superfamily and capable of inducing apoptosis. AICD was initially described in transformed T cells (Ucker *et al.*, 1989). Later primary pre-activated T cells were also shown to die in response to TCR engagement via similar death receptor dependent mechanisms. AICD is insensitive to action of anti-apoptotic Bcl2 family proteins and does not rely on cytochrome c released from mitochondria. For many years it was assumed that AICD is a major mechanism of peripheral T cell deletion. Surprisingly, however, activated T cells can be eliminated in animals lacking death receptors (Van Parijs *et al.*, 1998).

The existence of AICD independent T cell deletion mechanism became apparent from experiments with triple mutant mice lacking Fas, TNFR1 and TNFR2. Injection of staphylococcal enterotoxin B superantigen (SEB) into normal mice results in initial expansion, followed by elimination of V□8 bearing (SEB specific) T cells. Since deletion of SEB-specific T cells is normal in triple mutant mice it was postulated that another mechanism of peripheral tolerance existed (Hildeman *et al.*, 2002a). This second pathway of peripheral T cell deletion has recently been named ACAD

(activated T-cell autonomous cell death). ACAD is equivalent to so-called "cell death due to cytokine withdrawal" and proceeds with the involvement of mitochondria. In contrast to AICD, ACAD can be blocked by Bcl2 and is promoted by its pro-apoptotic homologue Bim (Bouillet *et al.*, 1999)(Hildeman *et al.*, 2002a). The importance of the Fas system and Bim in the elimination of activated lymphocytes is underscored by the phenotypes of mice bearing inactivating mutations in any of these genes. These animals suffer from T cell dependent autoimmunity and with age accumulate proliferating T cells in the periphery (Bouillet *et al.*, 1999)(Watanabe-Fukunaga *et al.*, 1992).

T cell responses may have both tolerogenic (T cells specific for an antigen are deleted after an immune response so that no secondary response can occur - tolerance against the antigen is induced) as well as immunogenic (T cell memory is established) character. On the basis of the analysis of Fas and Bim mutant animals it was suggested that during the tolerogenic type of response AICD is predominantly responsible for the removal of self-antigen recognising mature T cells. Immunogenic responses, on the other hand, use ACAD as a mechanism to delete T cells (Hildeman *et al.*, 2002b).

1.2 The phenomenon of apoptosis

Central and peripheral tolerance mechanisms induce apoptosis in these cells that have to be deleted from the T cell repertoire. Apoptosis is a form of physiological cell death frequently also called programmed cell death (PCD). Apoptosis is required for normal development and maintenance of tissue homeostasis (Vaux and Korsmeyer, 1999). Cells undergoing apoptosis show characteristic morphological changes including chromatin condensation, shrinkage of cytoplasm and plasma membrane blebbing (Nagata, 1997). Apoptosis, as opposed to necrosis, does not result in plasma membrane rupture and the release of cytoplasmic material. Apoptotic cells are phagocytosed by neighbouring cells or specialised macrophages in the tissue so that, for example, organ remodelling can occur without mobilisation of potentially dangerous immune responses.

Molecular changes that occur during apoptosis include DNA cleavage, randomisation of the distribution of phosphatidyl serine between the inner and outer leaflets of the plasma membrane and the release of cytochrome c and other proteins from mitochondria. These common morphological and molecular events are elicited by a broad range of physiological or experimentally applied death stimuli and are observed in diverse cells, tissue types and species. This is because those independent signalling pathways converge on a common, evolutionary conserved, death effector machinery (Vaux and Strasser, 1996). There are now two major apoptotic pathways distinguished: the extrinsic pathway initiated by "death receptors" and the intrinsic pathway initiated by Bcl2 proteins at the level of mitochondria (Figure IA).

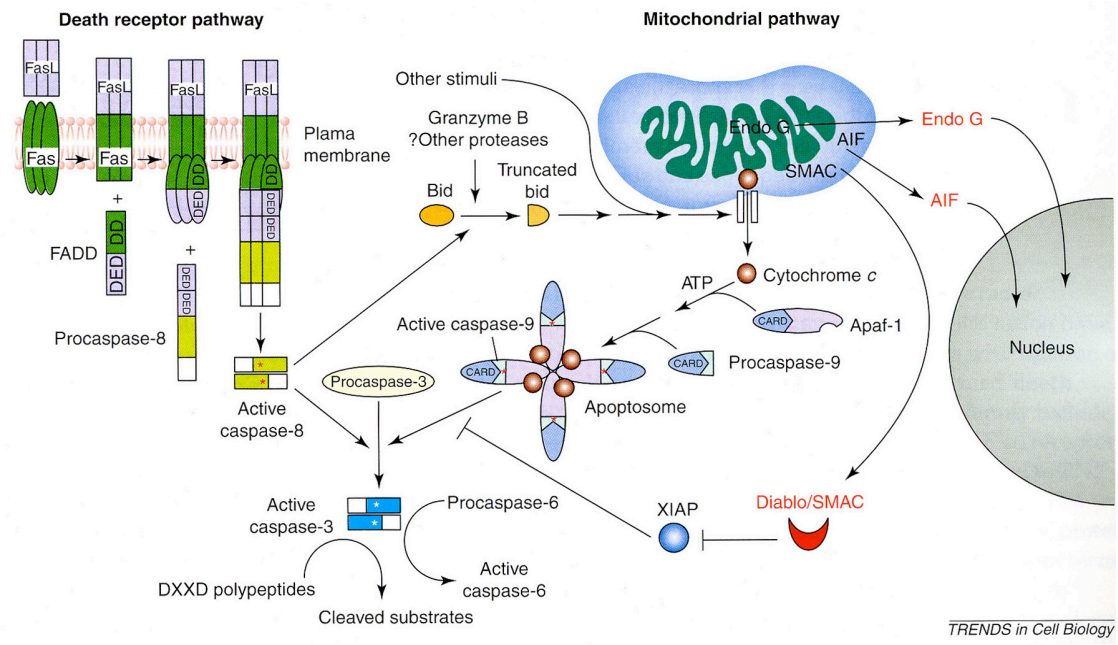


Figure IA. Comparison of deduced mechanisms of extrinsic (Death receptor pathway) and intrinsic (Mitochondrial pathway) apoptotic pathways (adapted from (Kaufmann and Hengartner, 2001)). Engagement of death receptor (here Fas/FasL) leads to FADD dependent caspase 8 processing, which in turn either directly activates effector caspase 3 or cleaves tBID molecule enabling it to cause cytochrome c from mitochondria. Mitochondrial apoptotic pathway starts from cytochrome c release, which in turn activates caspase 9 in Apaf-1 dependent fashion. Activated caspase 9 cleaves procaspase 3 causing its initiation. XIAP is an inhibitor of caspases and Diablo/SMAC proteins promote apoptosis by blocking XIAP activity. AIF and EndoG are propapototic proteins, which promote chromatin condensation or DNA degradation during apoptosis in caspase-independent fashion, respectively.

Recent advances have lead to the identification of major functional groups of proteins involved in triggering and executing the apoptotic process. These include "death

receptors" from the TNF receptor superfamily (extrinsic pathway), caspases (proteases responsible for destroying cell content), adaptor proteins linking apoptotic signals to caspase activation and Bcl2 family members proteins acting at the level of mitochondria (intrinsic pathway).

The extrinsic pathway of apoptosis begins with the ligation of specialised cell-surface receptors termed "death receptors" (DRs). Members of the DR family include Fas/CD95, TNF receptor 1 and DR4/DR5 (receptors for TRAIL ligand). The common feature of these type I transmembrane proteins is the presence of a conserved cytoplasmic motif termed "death domain", which is capable of binding to homologous domains of cytoplasmic adaptor molecules upon ligand mediated receptor aggregation. For example, ligation of Fas by Fas ligand (FasL) or by agonistic anti-Fas antibodies (Abs) leads to the recruitment of FADD adaptor protein to Fas via homotypic death domain-death domain interaction (Hengartner, 2000). FADD then recruits procaspase 8, which interacts with FADD through homotypic docking of death effector domains (DEDs) of those proteins. Procaspase 8 is then cleaved and this leads to its activation. Caspase 8 is considered an initiator caspase and serves to activate caspase 3 (effector caspase), which then executes the apoptotic program ((Ashkenazi and Dixit, 1999) and Figure IA).

The initiation of the cell intrinsic apoptotic pathway starts at the mitochondria. One of the first events is the release of certain regulatory proteins from the intermembrane compartment of the mitochondria. The most prominent factor is cytochrome c, a protein normally constituting part of the mitochondrial respiratory chain (Desagher and Martinou, 2000). Once in the cytoplasm, cytochrome c binds the Apaf-1 adapter protein causing its oligomerisation and the resulting molecular complex triggers caspase 9 activation. Active caspase 9 then cleaves procaspase 3 and the degradation phase of apoptosis follows.

In some cells, in which the levels of processed caspase 8 are low and insufficient to directly induce caspase 3 activity, the Bcl2 homologue BID is the first target of caspase 8. The resulting polypeptide (tBID) translocates then to mitochondria where it causes cytochrome c release. This leads to an amplification of the activities resulting

in caspase 3 activation (Budihardjo *et al.*, 1999). Thus, tBID is a molecule linking two prototypical apoptotic pathways (Figure IA).

1.3 The Bcl2 family of regulators of apoptosis.

Bcl2 is the founding member of a growing family of proteins that regulate apoptosis at the level of mitochondria. Bcl2 was originally cloned as an oncogene overexpressed in patients with B cell lymphoma (Korsmeyer, 1992). Later on it was demonstrated that enforced Bcl2 overexpression inhibits multiple forms of apoptosis (Hockenbery *et al.*, 1990). The Bcl2 family includes at least 15 members in mammals, some of which are anti-apoptotic (e.g., Bcl2, Bcl-X_L, A1) whereas others promote apoptosis (e.g., Bax, Bak, Bad, Bim) (Strasser *et al.*, 2000).

The mechanism by which Bcl2 and Bcl-X_L proteins work is not very well characterised but in cells overexpressing these proteins apoptotic stimuli fail to cause permeabilisation of the mitochondrial outer membrane, which is an obligatory event before cytochrome c (and other proteins) can be released. One of the proposed models of Bcl2 action relates to the apparent ability of this protein to form ion channels in synthetic membranes by which Bcl2 family could function as mitochondrial outer membrane channel controlling efflux of molecules that promote apoptosis (Minn *et al.*, 1997). In this scenario pro-apoptotic Bcl2-like proteins simply counteract the function of anti-apoptotic Bcl2 family members. This is possible due to the direct binding between Bcl2 proteins. Apoptosis or rescue from apoptosis may depend on the molar ratio between the levels of pro- and anti-apoptotic proteins.

1.4 TCR-mediated apoptosis

The T cell receptor constitutes a sophisticated molecular complex, which enables T cells to respond to signals provided by antigen-presenting cells (APCs) in a clone-specific fashion. TCR activation can result in either proliferation (activation) or apoptosis. Immature $CD4^+CD8^+$ double-positive thymocytes undergo apoptosis upon TCR triggering (Shi *et al.*, 1991). Mature naïve T cells become activated, enter cell cycle and after several cell divisions reach the so-called “blast” stage. The transition

from the naïve to the blast stage occurs in the presence of secreted IL-2 and is associated with down regulation of the anti-apoptotic protein c-FLIP. T cell blasts die in response to TCR signalling (Figure IB). Thus, engagement of the TCR leads to distinct outcomes depending on the T cell differentiation status and is connected to T cell function at a particular developmental stage. One of the central issues in molecular immunology is the mechanism by which TCR induces apoptosis. In order to study the molecular events during TCR-mediated apoptosis several model systems have been employed.

TCR stimulation induces apoptosis in transformed T cell hybridomas (Ucker *et al.*, 1989). T cell hybridoma cells activated with surface bound anti-CD3 antibodies (mimicking TCR engagement) constitute a convenient model to study the biochemistry of TCR-mediated apoptosis. In this cell system, TCR signalling triggers upregulation of FasL at the cell surface. This leads to engagement of Fas, followed by cell-autonomous PCD. It is now believed that hybridomas model peripheral deletion of T cells rather than negative selection in the thymus (Lenardo *et al.*, 1999).

The primary T cells can be primed *in vitro* to undergo AICD. The advantage of using primary cells to study mechanism of TCR-mediated apoptosis is the possibility to use certain mouse mutant strains. This enables to test the involvement of individual gene products in regulation of AICD *in vitro*. Studies with primary cells obtained from TCR transgenic mice have shown that TCR triggering, apart from induction of FasL expression, is required for the induction of susceptibility to Fas- (and TNF-) mediated apoptosis (Combadiere *et al.*, 1998). Thus, the TCR serves a dual role in AICD - it induces death receptor ligands and by sensitising cells to death signals the TCR ensures maintenance of clonotypic specificity of AICD. The molecular nature of this so-called "susceptibility to die" signal is presently not known. It is, however, expected to be some kind of posttranslational effect since it can be delivered by the TCR in the presence of cycloheximide (Wong *et al.*, 1997).

Injection of anti-CD3 (or anti-TCR) antibodies into mice results in massive apoptosis of $CD4^+CD8^+$ immature thymocytes whereas mature thymocytes are spared. This effect has been used to address the function of certain genes in TCR-mediated apoptosis. For instance thymocyte apoptosis induced by the injection of anti-CD3

antibodies does not rely on the action of Fas and instead needs the Bim protein (Bouillet *et al.*, 2002). Thus, by the use of such an assay it has been possible to conclude that TCR-induced apoptosis of immature T cells in the thymus (site of central tolerance) proceeds through different mechanism than apoptosis of peripheral mature T cells despite the fact that both are initiated by the same cell surface receptor.

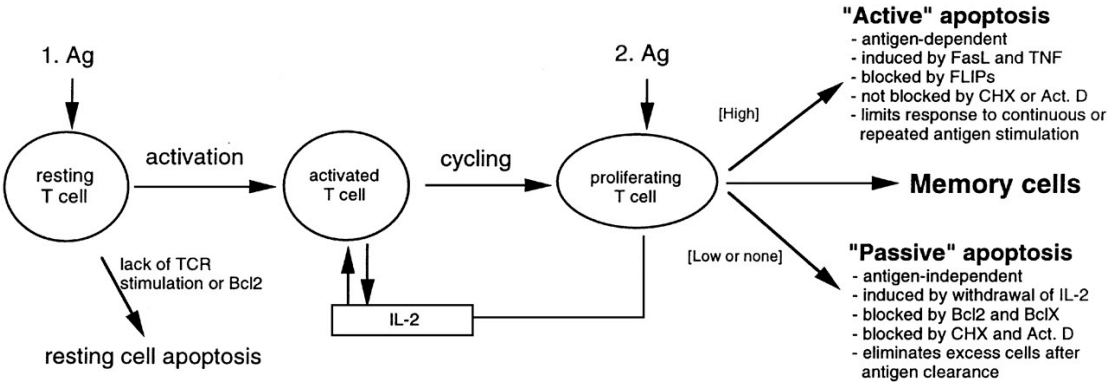


Figure IB. Schematic representation of the fates of T cells during an immune response (adapted from (Lenardo *et al.*, 1999)). The "active" apoptosis is equivalent to AICD. The "passive" apoptosis is identical to ACAD. For other details see text.

Several mouse models have been exploited to study the molecular requirements for TCR-mediated apoptosis. A common disadvantage of such systems is the "black-box" nature of a complex organism, making it difficult to dissociate direct from indirect effects or to discriminate between structural and functional requirements for a particular gene's function. These models typically rely on the use of TCR-transgenic mice. Such mice can be immunised with TCR-specific antigens and the fate (deletion/lack of deletion/uncontrolled expansion etc.) of T cells in thymus and periphery can be monitored (Van Parijs *et al.*, 1998)(Hildeman *et al.*, 2002a). Although clearly valuable such systems are not very well suited to study biochemical events during TCR-mediated apoptosis. They are, however, the model systems of choice for verification studies once candidate genes mediating TCR-induced apoptosis have been identified.

The early events of TCR-mediated apoptosis are well understood and involve mobilisation of second messengers and MAP kinases followed by the activation of

several types of transcription factors (Zhang *et al.*, 1999). Similarly, late processes including death receptor-mediated apoptosis and the caspase cascade have been relatively well characterised. Transcription factors that have been reported to participate in the regulation of TCR-mediated apoptosis include NFAT, EGR (Rengarajan *et al.*, 2000), Nur77 (Zhou *et al.*, 1996) and Rel/NF-κB families (Hettmann *et al.*, 1999). While the function of transcription factors from NFAT and EGR families has been connected to the regulation of the TCR-mediated FasL and Nur77 expression (Rengarajan *et al.*, 2000), the major gap in current knowledge of molecular aspects of TCR-induced apoptosis is the mechanism of action of Nur77 and Rel/NF-κB proteins. Therefore the role of Nur77 and Rel/NF-κB families in TCR-mediated apoptosis deserves a special interest and has been a subject of studies performed in this thesis.

1.4.1 The Nur77 family of orphan nuclear receptors.

Nur77 (called TR3 in humans) is an inducible orphan nuclear receptor initially identified as a serum-induced immediate-early gene (Ryseck *et al.*, 1989), and later as a gene induced by TCR triggering in T cells (Woronicz *et al.*, 1994)(Liu *et al.*, 1994). Besides serum and TCR signalling, Nur77 is also induced by a wide variety of external stimuli including phorbol esters, BCR and nerve growth factor (NGF) (Zhang *et al.*, 1999).

Similar to other steroid receptor superfamily members, Nur77 consists of an N-terminal AF1 transactivation domain, a DNA-binding domain with two zinc fingers, and a C-terminal putative ligand-binding domain (Figure IC). Two *nur77* paralogous genes have been identified: *nor-1* and *nurr1* (Ohkura *et al.*, 1994), (Saucedo-Cardenas *et al.*, 1997). They are approx. 90% identical at the amino acid level to Nur77 in their DNA binding domains, approx. 60% identical in their putative ligand binding domains, but in the N-terminus they are diverged (approx. 20% identity). All three Nur77 family members bind as monomers to a consensus NBRE sequence. This is an artificial DNA sequence selected from random oligomer library on the basis of the ability to bind Nur77 (Wilson *et al.*, 1991). On the contrary the NuRE palindromic

DNA motif binds as homo- and heterodimers of Nur77 family members. NuRE has been initially found in the promoter of POMC gene (Philips *et al.*, 1997a). Expression of Nur77, Nor-1 or Nurr1 is sufficient to activate NBRE or NuRE-directed transcription, suggesting that the Nur77 family members are "constitutive" orphan steroid receptors that do not require ligands for activation. Nur77 can also act as modulator of transcription of other steroid receptors. Nur77 and Nurr1, but not Nor-1, (Zetterstrom *et al.*, 1996) can heterodimerise with RXR in the presence of retinoids and modulate the activities of a subclass of retinoid response elements (Forman *et al.*, 1995).

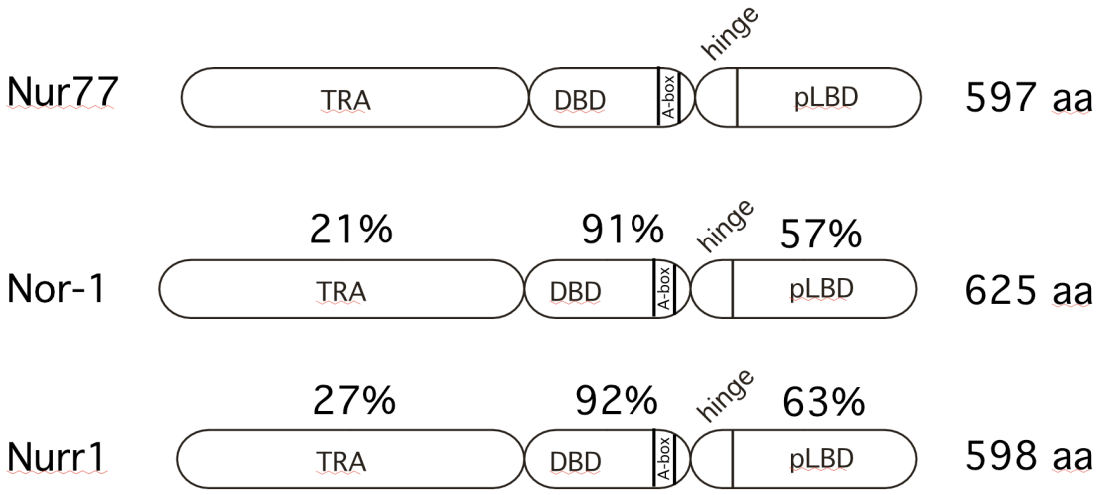


Figure IC. Schematic representation of the Nur77 family. TRA - AF1 bearing domain responsible for transactivation ; DBD - DNA binding domain ; pLBD - putative ligand binding domain ; "A-box" is a stretch of amino acids outside of zinc finger domain, which are necessary for DNA binding ; hinge is a conserved region connecting DBA and pLBD ; (%) - relates to identity at the amino acid level between Nur77 and the indicated homologue.

1.4.1.1 Regulation of Nur77 expression.

Transcriptional induction of *nur77* mRNA is regulated by a complex network of transcription factors and accessory proteins. In promoter deletion experiments analysis, two MEF2 binding sites were identified as TCR-regulated response elements in the *nur77* promoter region (Woronicz *et al.*, 1995). Recently, several repressors and stimulators were found to associate with MEF2D, which is the dominant MEF2

family member in T cells. In resting cells, Cabin-1, a calcineurin-interacting repressor protein, was found to associate with MEF2D (Youn and Liu, 2000)(Youn *et al.*, 1999). MEF2D was also capable of binding histone deacetylase HDAC4 (Miska *et al.*, 1999) and the HDAC-like protein MITR (Sparrow *et al.*, 1999), which may have overlapping functions to inhibit Nur77 induction under resting conditions. MEF2D itself is a weak transcription factor, and dissociation of various repressors from MEF2D is not sufficient to activate *nur77* transcription. NF-ATc1, through a DNA-binding independent mechanism, can co-activate MEF2D on MEF2-specific DNA elements (Youn *et al.*, 2000). In addition, ERK5, a kinase with an unique C-terminal transcriptional activation domain, can also associate with MEF2D and deliver a powerful transcriptional activity (Kasler *et al.*, 2000).

Thus, coordinated regulation through release of repressors and recruitment of co-activators constitutes a mechanism by which T cells maintain very low basal levels of *nur77* mRNA but can activate Nur77 expression to high level in a relatively short time (Figure ID).

1.4.1.2 Phosphorylation of Nur77.

Nur77 is a phosphoprotein that typically migrates between 65 and 75 kDa in denaturing SDS-PAGE gels. Phosphorylation of Nur77 results in its predominant localisation in the cytoplasm (Katagiri *et al.*, 2000). The phosphorylation of Nur77 can be carried out by several kinases *in vitro* including members of the MAP kinase family and the protein kinase Akt. Akt phosphorylates Nur77 at its DNA binding domain (Ser350), resulting in reduced Nur77 DNA binding. Overexpression of Akt inhibits AICD in the T cell hybridoma system and partially protects against Nur77 induced apoptosis in Rat1a fibroblasts (Masuyama *et al.*, 2001).

In PC12 cells, phosphorylation of Nur77 at its N-terminal region (Ser105) by MAP kinases regulates the ability of Nur77 to be exported to the cytoplasm in response to NGF. Interestingly, RXR is also transported to the cytoplasm along with Nur77 through heterodimerization. Thus, NGF treatment results in Nur77 dependent alteration of retinoid signalling in this cell system (Katagiri *et al.*, 2000). Together,

these results demonstrate that posttranslational modifications can have profound influence on Nur77 function.

1.4.1.3 Nur77 function in TCR mediated apoptosis.

Nur77 is readily detectable in thymocytes stimulated with phorbol ester and ionomycin (Woronicz *et al.*, 1995), two agents used to mimic TCR signalling. Maximal induction of Nur77 in double positive (DP) thymocytes *in vitro* requires in addition to a TCR signal a co-stimulation through the CD28 receptor (Amsen *et al.*, 1999). Efficient apoptosis of thymocytes *in vitro* also requires co-stimulation (Kishimoto and Sprent, 1999). These data suggested a potential involvement of Nur77 in regulation of TCR-mediated apoptosis. Consistent with these observations overexpression of dominant-negative Nur77 (DN-Nur77) inhibits negative selection in the thymus (Calnan *et al.*, 1995). DN-Nur77 protein, however, has been shown to inhibit the activities of all Nur77-related molecules (Cheng *et al.*, 1997). Since at least Nor-1 is also a TCR-inducible protein, functional redundancy between Nur77 family members was postulated in order to explain the lack of a clear phenotype in *nur77*$^{-/-}$ animals (Lee *et al.*, 1995). Nur77 overexpression is sufficient to induce apoptosis in transgenic immature thymocytes *in vivo* (Weih *et al.*, 1996) and the same is true for Nor-1 (Cheng *et al.*, 1997). Several transgenic lines were created which overexpress different Nur77 deletion mutants in immature thymocytes. In those transgenic mice, Nur77 transcriptional activity correlates with the mutant's ability to induce apoptosis (Kuang *et al.*, 1999). It is therefore likely, but not proven, that Nur77 induces apoptosis in immature thymocytes by controlling transcription of yet uncharacterised target genes.

Nur77 was also found necessary for AICD of T cell hybridomas (Woronicz *et al.*, 1994)(Liu *et al.*, 1994), an *in vitro* model for T cell peripheral deletion. In these cells antisense *nur77* cDNA or DN-Nur77 inhibits TCR-mediated apoptosis. TCR-induced secretion of IL-2, however, was not affected, indicating that Nur77 is responsible for specifically promoting apoptosis signalling downstream of TCR. The structural requirements and subcellular localisation of Nur77 were not addressed in these

reports. The mechanism of Nur77 contribution to TCR-induced apoptosis in this cell system remains elusive.

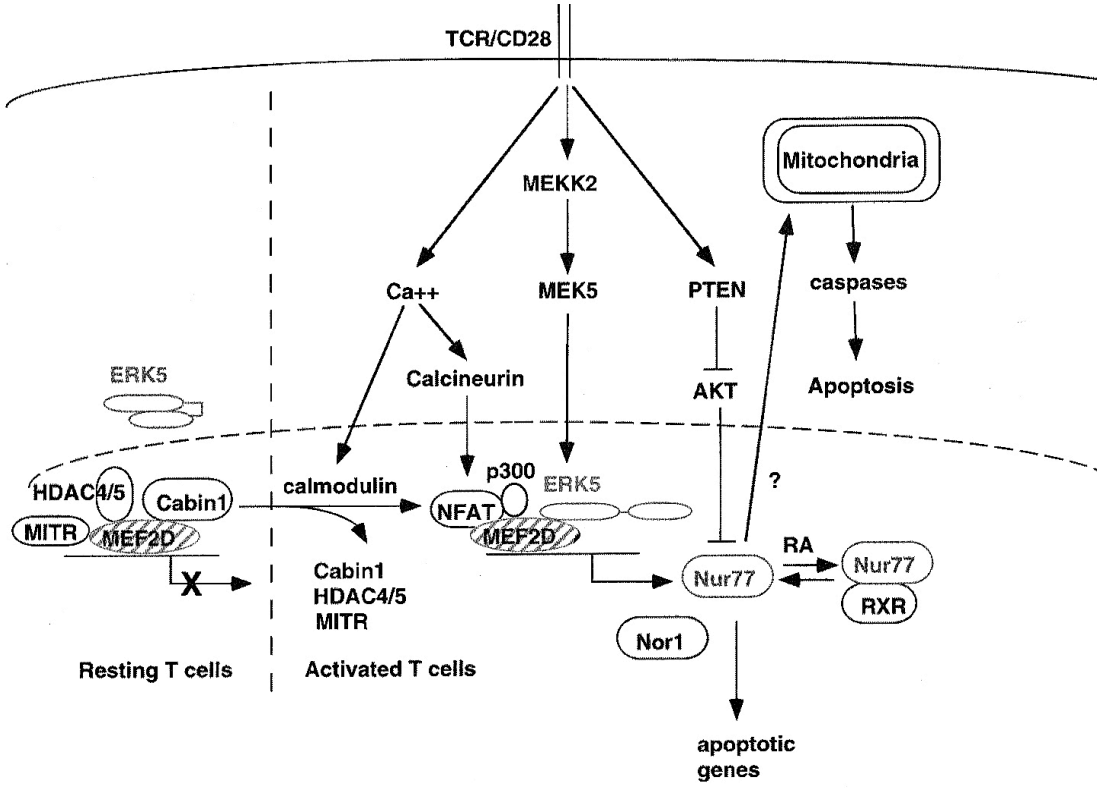


Figure ID. Nur77 regulation and function in TCR mediated apoptosis. (adapted from (Winoto and Littman, 2002)). MEKK2/MEK5 are MAP kinases activated by TCR, PTEN is a tumor suppressor protein negatively regulating the activity of Akt kinase, for other details see text.

Glucocorticoids are potent inhibitors of AICD (Iwata *et al.*, 1991). Glucocorticoids are also produced by thymic epithelial cells and were suggested to influence the effectiveness of negative selection by setting a threshold on the TCR signal sufficient to induce apoptosis (Vacchio *et al.*, 1994). Nur77, on the other hand, has been reported to inhibit transcriptional activity of the glucocorticoid receptor (Philips *et al.*, 1997b). Conversly, TCR signalling can also reduce glucocorticoid-induced cell death in T cell hybridomas. The mechanism seems to be the formation of transcriptionaly inactive heterodimers between Nur77 and GR mediated by the DNA-binding domains. These observations built a framework for the so-called "mutual antagonism"

model of T cell selection in which the fate of T cells is dictated by the outcome of competition between TCR and GR activities (Vacchio and Ashwell, 1994).

Recently, Nur77 has been reported to initiate apoptosis by translocating to mitochondria, followed by the release of cytochrome c into the cytoplasm (Dawson *et al.*, 2001), (Li *et al.*, 2000). The latter event causes activation of Apaf-1 and caspase-9, followed by activation of effector caspases and apoptosis (Li *et al.*, 1997). These experiments, performed in prostate and lung cancer cell lines, showed that the Nur77 DNA-binding domain is dispensable for this process. These data suggest that Nur77-mediated transcription is not required for apoptosis under these conditions.

Thus, the Nur77 family constitutes a group of protein capable of influencing apoptosis by distinct mechanisms: either as "conventional" transcription factors or by initiating certain non-genomic functions. The undisputable demonstration for Nur77 family members to be transcriptional activators, however, would be the characterisation of direct target genes, a pressing issue in the field of nuclear orphan receptors.

1.4.2 Rel/NF-κB family of transcription factors.

NF-κB was first discovered as a constitutive nuclear transcription factor in mature B cells. NF-κB was bound to a DNA element in the kappa immunoglobulin light-chain enhancer, hence the name "nuclear factor κB". “Classical” NF-κB consists of two subunits with molecular weights of 50 kDa (p50) and 65 kDa (p65) and is present not only in B cells but in most cell types in an inactive cytoplasmic form bound to an inhibitory protein called IκB (Perkins, 2000). NF-κB can be activated by a large variety of inducers which include bacteria or bacterial products, viruses, inflammatory cytokines, stress conditions, mitogens, growth factors and hormones, environmental hazards and many different chemical agents. When a cell receives any one of these extracellular signals, NF-κB is released from IκB inhibition, rapidly translocates to the nucleus and activates gene expression. The active NF-κB transcription factor promotes the expression of over 150 target genes the majority of which participate in the host immune response. These include genes for cytokines and chemokines, for immunoreceptors such as mouse histocompatibility antigen

molecules (MHC) involved in antigen presentation, for cell adhesion molecules, acute phase proteins, stress response genes, cell surface receptors, regulators of apoptosis, growth factors and their modulators, early response genes, transcription factors and for many others (Pahl, 1999).

The isolation of the gene encoding the p50 subunit (p105/p50) showed that the DNA binding and dimerisation domain was highly homologous to the viral oncoprotein v-Rel, its cellular counterpart c-Rel and to the *Drosophila melanogaster* developmental protein Dorsal. Subsequent isolation cDNAs for RelA and for two other highly homologous proteins, p100 (p52) and RelB, established the presence of the multigene Rel/NF-κB family of transcription factors which is conserved from *Drosophila* to humans (Gilmore, 1999; Perkins, 2000) and (Figure IE)**.** All members of this family contain an approximately 300 amino acid N-terminal Rel homology domain (RHD) that is required for DNA binding, homo- and heterodimerisation, and interaction with inhibitory IκB proteins. It also contains a nuclear localization signal (NLS) within this region (Figure IE). The p50 and p52 subunits, which lack transactivation domains, are produced by processing of precursor molecules of 105 kDa and 100 kDa, respectively while c-Rel, RelB and RelA (p65) do not require proteolytic processing to generate their active forms and contain non-homologous transactivation domains in their C termini (Perkins, 2000)(Hatada *et al.*, 2000).

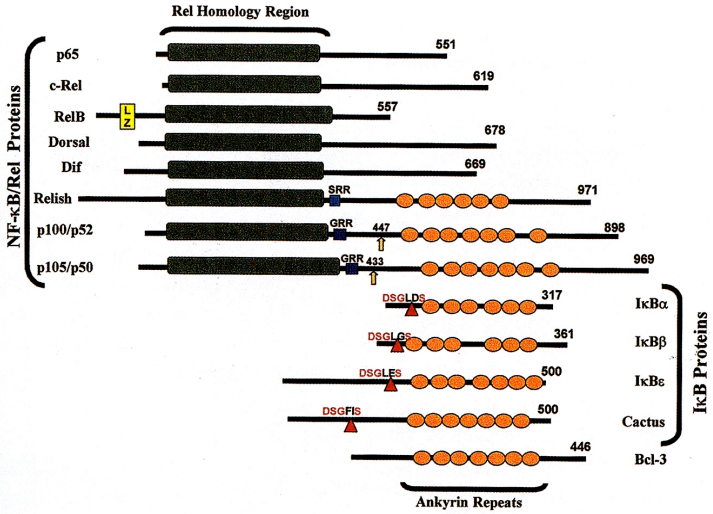


FIGURE IE. Family of Rel/NF-κB and IκB proteins. Mammalian members of Rel/NFκB and IκB proteins together with *Drosophila* homologues (Dorsal and Dif, Cactus) are schematically represented. RHD is indicated as black box. Proteolytic cleavage (arrows) of p100 and p105 gives rise to p52 and p50, respectively. Ankyrin repeats are indicated with orange circles and conserved residues located at N-termini of IκB proteins required for inducible degradation are red in colour. LZ: leucine zipper, SRR: serine rich region, GRR: glycine rich region; adapted from (Karin and Ben-Neriah, 2000).

1.4.2.1 The NF-κB Activation Pathway.

The activity of NF-κB is tightly regulated by interaction with the inhibitory IκB protein. Similar to the Rel/NF-κB family, IκB is a family of molecules, which consists of IκBα, IκBβ, IκBε, IκBγ and Bcl-3 proteins. They are distinguished by different affinities for individual Rel/NF-κB complexes, are distinctly regulated and expressed in a tissue specific manner. All IκBs have either six or seven ankyrin repeats at their C-termini (Figure IE). These repeats bind to the RHD and mask the NLS of NF-κB, causing cytoplasmic retention. Ankyrin repeats are also found in the C-terminal sequences of p105 and p100 and also sequester NF-κB in the cytoplasm prior to their proteolytic processing. The classical NF-κB activation cascade, initiated by inducers such as TNF or IL-1, leads to the rapid degradation of IκBs. For IκBα, these inducible degradation steps are well characterized. The cascade is initiated by phosphorylation of serines 32 and 36 of IκBα by a IκB kinase (IKK). Phosphorylation leads to polyubiquitinylation of IκBα at lysines 21 and 22 by ubiquitin ligase. Upon this modification, the protein is rapidly degraded by the 26S

proteasome. The so exposed NF-κB nuclear localisation signal (NLS), causes the rapid translocation of NF-κB to the nucleus (Karin and Ben-Neriah, 2000) and (Figure IF) where NF-κB binds DNA response elements in the promoters of genes it regulates to initiate their transcription. The specificity and selectivity of this process is achieved by the differential activation of distinct NF-κB complexes, which can have different DNA-binding specificities, targeting them to different genes. The different NF-κB subunits seem to have distinct non-overlapping roles *in vivo* since inactivation of each individual Rel family member in knockout mice produced a distinct phenotype (Gerondakis *et al.*, 1999).

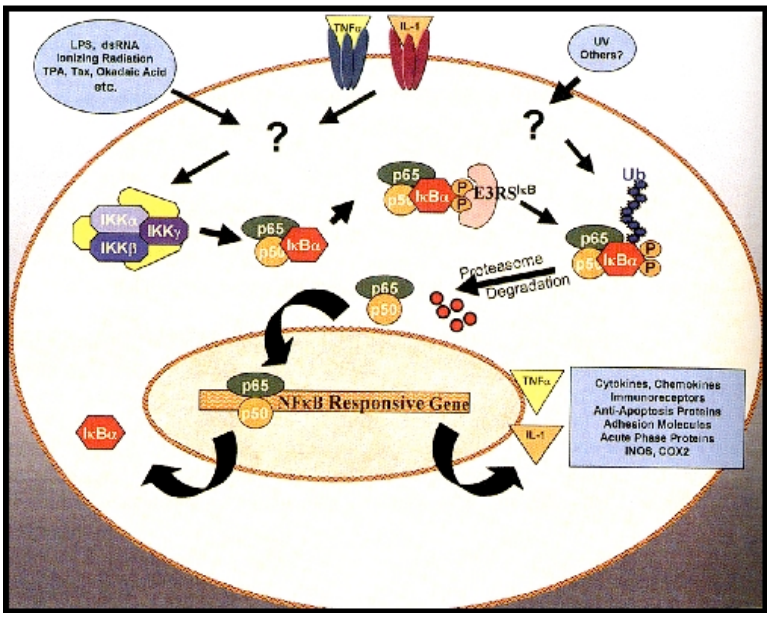


FIGURE IF. Schematic representation of the consensus NF-κB activation pathway. Different stimuli activate the IKK complex which phosphorylates IκBα followed by polyubiquitination by $E3RS^{IκB}$ resulting in rapid degradation by the 26S proteasome. This is followed by nuclear translocation of NF-κB and transcriptional regulation of its target genes including IκBα which terminates this activity in an autoregulatory loop. Adapted from (Karin and Ben-Neriah, 2000).

1.4.2.2 NF-κB function in apoptosis.

The initial evidence indicating that Rel/NF-κB transcription factors might be involved in apoptosis came from the study of retroviral oncoprotein v-Rel. v-Rel is encoded by the avian Rev-T retrovirus that causes fatal lymphoid tumors in birds. In

1991, Neiman et al. showed that v-Rel-transformed chicken tumor cells were resistant to several apoptosis-inducing stimuli (Neiman *et al.*, 1991). Within the last 11 years, abundant evidence has implicated cellular Rel/NF-κB transcription factors in the control of apoptosis in many systems. Taken together these studies indicate that Rel/NF-κB can have both negative and positive influence on apoptosis depending on the cell death-inducing stimulus and cell type (reviewed in (Barkett and Gilmore, 1999).

1.4.2.3 NF-κB function in TCR-mediated apoptosis.

Most of the published reports document an anti-apoptotic role for NF-κB in TCR-mediated apoptosis, but in some experimental systems NF-κB also promotes TCR-induced PCD. This is in contrast to TNF-mediated cell death where NF-κB is exclusively anti-apoptotic (see below).

Lenardo's team reported increased sensitivity to TCR-mediated apoptosis in 2B4 hybridomas transduced with NF-κB inhibitory peptides. Conversely, cells overexpressing p50 and p65 Rel proteins showed reduced TCR-mediated apoptosis. Similarly, experiments with mature T cells from transgenic mice overexpressing a non-degradable mutant IκBα molecule showed comparable results (Dudley *et al.*, 1999). In Jurkat T cells, mutation of the IKKγ subunit of the IKK complex results in lack of NF-κB mobilisation upon PMA/ionomycin treatment (Rivera-Walsh *et al.*, 2000). These cells showed also increased apoptosis in response to PMA/ionomycin.

Some other groups reported an apparent positive role in NF-κB in TCR-mediated apoptosis. Thymocytes from transgenic mice overexpressing a non-degradable mIκBα molecule are protected from anti-CD3 induced apoptosis *in vivo* (Hettmann *et al.*, 1999). Treatment of A1.1 hybridoma cells with NF-κB inhibitory peptides resulted in defective FasL expression and rescue from AICD (Kasibhatla *et al.*, 1999). In none of those reports the mechanisms underlying the observed phenotypes have been further explored creating an interesting field for future studies.

1.4.2.4 NF-κB function in TNF-mediated apoptosis.

One of the first indications that suppression of TNF induce apoptosis is an important NF-κB function was the phenotype of *relA*$^{-/-}$ mice, which die at embryonic day 15 as a result of extensive liver apoptosis (Beg *et al.*, 1995). This phenotype was caused by TNF since *relA*$^{-/-}$ mice survived until birth when crossed on TNF knockout background (Doi *et al.*, 1999). Independently, it was established that NF-κB is necessary for protection from TNF-mediated cell death in mouse embryonic fibroblast (Beg and Baltimore, 1996) and later in many other cell types (Karin and Lin, 2002). Since it was known that TNF induces apoptosis in normal cells when *de novo* protein synthesis is blocked it was hypothesised that NF-κB protects cells from TNF by induction of anti-apoptotic target genes. Indeed it is know well documented that NF-κB downstream of TNFR1 is able to induce multiple anti-apoptotic proteins, which suppress apoptosis (Figure IG).

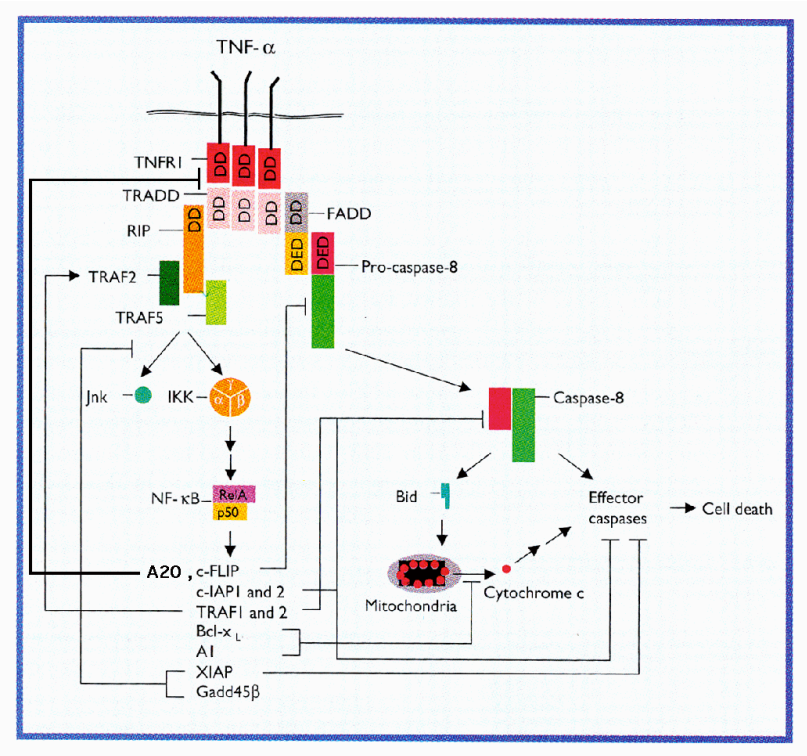


Figure IG. Mechanism of NF-κB mediated rescue from TNF mediated apoptosis (adapted from (Karin and Lin, 2002)). TNF induces apoptosis through TNFR1-FADD-caspase-8 dependent pathway, which in some cell types couples to mitochondria through the action of tBID protein (for more details see the mechanisms of apoptosis in this chapter). TNFR1 activates NF-κB through RIP1/TRAF/IKK dependent pathway. NF-κB is responsible for induction of anti-apoptotic genes. A20 is a zinc finger cytoplasmic protein blocking early events in TNFR1 signalling,, c-IAP1/2 and XIAP are proteins inhibiting the activity of effector caspases, TRAF1/2 are adaptor proteins interfering with TRAF5 recruitment to TNFR1. Bcl-X_L and A1 are anti-apoptotic Bcl2 homologues and block cytochrome c release from mitochondria. GADD45β is most likely an inhibitor of pro-apoptotic signalling elicited by JNK kinase.

1.5 GOALS OF THE PROJECT

This study focuses on the function of Nur77 nuclear orphan receptors and Rel/NF-κB proteins in TCR-mediated apoptosis. The involvement of Nur77 and NF-κB transcription factors in TCR-mediated cell death has been previously documented. Molecular mechanisms, by which these transcription factors participate in this process, however, remain obscure.

The first part of the work focuses on the Nur77 family. Nur77 regulation during apoptosis will be studied and the question of whether Nur77 suffices to induce apoptosis will be addressed by transient overexpression of Nur77 in T cell hybridomas. Since Nur77 has been recently reported to induce apoptosis in prostate cancer cells directly at the level of mitochondria, Nur77 subcellular localisation will be determined in TCR-stimulated Do.11.10 hybridoma cells. To characterise Nur77 target genes both loss-of-function (use of dominant-negative Nur77 mutant) and gain-of-function approaches will be attempted. The latter approach will rely on the *in vitro* and *in vivo* conditional expression of Nur77.

The second part of the work concentrates on the role of NF-κB in the regulation of AICD in T-cell hybridoma system. In the initial phase of the work the consequences of NF-κB inhibition in TCR-stimulated Do.11.10 hybridoma cells will be determined with the emphasis on the death receptor involvement. Then the regulation of the known NF-κB target genes in response to TCR will be characterised and functional experiments will be performed to link the phenotype resulting from NF-κB inhibition to the transcription of certain NF-κB targets.

Transformed T cells (mouse hybridomas or human Jurkat leukaemic cells) have been chosen as a model system in most experiments because of the ease of performing biochemical experiments and genetic manipulations.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

All general chemicals were supplied from Merck (Darmstadt), Carl Roth GmbH & Co (Karlsruhe) and Sigma Chemie GmbH (Deisenhofen) and with highest purity grade.

2.1.2 Radiochemicals

[^{32}□-P]-dCTP; [^{33}□□P]-dATP (Amersham Life Science).

2.1.3 Primers

All the primers were synthesized by MWG Biotech GmbH and were of HPSF purified grade.

Primers for construction of Nur77/EBD/GBD fusion proteins

For pEBD plasmid construction

ECOSFIKPN	AATTCGGCCAAAAAGGCCGGTAC
KPNSFIECO	CGGCCTTTTTGGCCG
SFIGLOTASPE	GGCCGCGGCCAAAAAGGCCTGTGTTCACTAGCAACC TCAAACAGACACCATGGCTAGCATGACTGGTGGACA GCAAATGGGTA
SPETAGLOSFI	CTAGTACCCATTTGCTGTCCACCAGTCATGCTAGCCA TGGTGTCTGTTTGAGGTTGCTAGTGAACACAGGCCTTT TTGGCCGC

GBD primers (5'BamHI, 3'EcoRI)

GBD5END	AAAAGGATCCGTACGGGCCACTACAGGAGTC
GBD3END	AAAAGAATTCTCACTTTTGATGAAACAGAAG

Primers for *nur77*/*nurr1* cDNA:

5´primer (conatins SpeI site, 21 bp of Nur77 encoding first 7 aa)

NUR775END	AAAAACTAGTCCCTGTATTCAAGCTCAATAT

3´primers (contain BamHI site, 21 bp encoding last 7 aa of respective Nur77 portions 1-400 aa , full length)

NUR77400B	AAAAGGATCCCCCGAAGCGGGGCAGCACCAG
NUR773END	AAAAGGATCCGAAAGACAATGTGTCCATAAA

3´primers (contain XbaI site (blocked by methylation), stop codon, 21 bp encoding last 6 aa of respective Nur77 portions 1-360 aa, 1-400 aa, full length)

NUR77400X	AAAATCTAGATCACCCGAAGCGGGGCAGCACCAG
NUR773ENDX	AAAATCTAGATCAGAAAGACAATGTGTCCAT

5´primer (conatins SpeI site, 21 bp of NURR1 encoding first 7 aa)

NURR5END	AAAAACTAGTCCTTGTGTTCAGGCGCAGTAT

3´primer (contain XbaI site (blocked by methylation), encoding last 6aa and a stop codon of Nurr1)

NURR3ENDX	AAATAGATCTTTAGAAAGGTAAGGTGTCCAGGAA

Primers for RT-PCR

Actin-5	AGAGGTATCCTGACCCTGAAGTACC
Actin-3	CCACCAGACAACACTGTGTTGGCAT
RTNUR7715	AGCACTGCCAAATTGGAC
RTNUR7713	AAGATGAGCTTCCCCTCAC
RTNOR115	CCAGAGACCTTGATTACTCC
RTNOR113	CTTCAGCAGTGTTTGACCGAC
RTNURR115	ACTATTCCAGGTTCCAGGC
RTNURR113	CCCATTGCAAAAGATGAG
492-mEnolase-511	TTTACCGCCACATTGCTGAC
741-mEnolase-722	AATCCACCCTCATCACCCAC
903-hEnolase-920	ACATCTCGCCTGACCAGC
1167-hEnolase-1150	TTGCACGCCTGAAGA
138-mVIP-156	GAAGGAAACAGCCAAGGAG
560-mVIP-542	TGTGAAGACGGCATCAGAG
2882-hWapl-2903	GCATAATCCCGAAAATCAAAGC
3361-hWapl-3340	CTATCATCCCCTTCTCCACTAC

RTFAS15	ATGCACACTCTGCGATGAAG
RTFAS13	TTCAGGGTCATCCTGTCTCC

RTFASL15	CAGCTCTTCCACCTGCAGAAGG
RTFASL13	AGATTCCTCAAAATTGATCAGAGAGAG

Site directed mutagenesis of *nur77* (L354V mutation).

Nur77mut51	GGGCGGCGGGGCCGGGTACCTTCAAAACCCAAG
Nur77mut31	CTTGGGTTTTGAAGGTACCCGGCCCCGCCGCCC

Primers for sequencing (TexasRed labelled)

Maps to pGL3-Basic vector 5‘ to polylinker and is oriented towards polylinker

PGL3PRIMER	GGCTGTCCCCAGTGCAAGTGC

for pET28 vector derivatives from 3`end relative to cDNA insert

T7-term	GCTAGTTATTGCTCAGCGG

for lck-transgenes in pTLC vector (placed in 5'end of lck promoter towards BamHI cloning site):

lck-prom	TGGGCCTCCTGTGAACTTG

for the Nur77/GBD junctions.

GBD1	AACAGTGACACCAGGGTAG

for Nur77/EBD junctions.

EBD2	CCCTCTACACATTTTCCC
EBD3	TCAGCATCCAACAAGGCAC

Primers for expression of Nur77/NOR-1/Nurr1/A20 as His-tagged proteins in *E.coli* (NheI site in 5’ primer, stop+XhoI site in 3‘ primer)

Nur77His5	AAAAGCTAGCCCCTGTATTCAAGCTCAATAT
Nur77His32	AAAACTCGAGTCAGCCAGTGGGAGGACTGAAGGA
Nor1His5	AAAAGCTAGCCCCTGCGTGCAAGCCCAATAT
Nor1His32	AAAACTCGAGTCATGGCGGTGAGGGCTTGAAGAA
Nurr1His5	AAAAGCTAGCCCTTGTGTTCAGGCGCAGTAT
Nurr1His32	AAAACTCGAGTCAGACGTGCAGAGGCCCGTCGAA
A20C3	AAAACTCGAGTCAGCCATACATCTGCTTGAACTG
A20C5	AAAAGCTAGCCCACACTCTTGCCACCGGACT

2.1.4 Plasmids

Commercially available plasmids

pEGFP-N1 (Clontech); pRcCMV (Invitrogen); pGL3-Basic (Promega); pET28(a) (Novagen)

Plasmid gifts (obtained form)

pHFE1/pHFE1 521V, pBKC-CreGRI747T (Dr A.F. Stewart); pCFG5 IEGZ (Dr D. Lindemann); pTLC (Dr R. Bravo lab); pBabepuro-hBcl2 (Dr R. Jäger); pRcCMV-LacZ (Dr C. Günes); pBS34-Nurr1 (Dr O. Conneely); pBSK-Nor-1 (Dr Ohkura); pBSK-Nur77 (clone #19 Dr R. Bravo plasmid database); pBR322-GAPDH (clone #202 Dr R. Bravo plasmid database); pCAGGS-Flag-A20 (Dr R. Beyaert)

Plasmid constructs

Nur77 reporter genes

Tk promoter was released from pBLCAT5 with HindIII and BglII enzymes. Tk promoter was subcloned in HindIII site of pGL3-Basic vector (Promega). Resulting plasmid (called pGL3TKMax) was digested with HindIII/EcoRI enzymes (to delete distal SP1 binding sites), blunted, gel purified and self-ligated. This plasmid was named pGL3TkMin. To multimerise Nur77 response elements (NBRE and NuRE) oligonucleotides (strucure shown below) were anealed, ligated in the presence of PEG and counterselected with BamHI/BglII enzymes. Oligos were then resolved on 2% agarose and desired multimers (3xNBRE, 4xNBRE, 3xNuRE or 4xNuRE) were cut out from gel, purified and subcloned in the BglII site of pGL3Tkmin plasmid. Selected clones were sequenced to verify proper sequence and orientation of the multimer.

NBRE:

	BamHI	BglII
NBRE-up	5'- GATCC TCGTGCGAAAAGGTCAAGCGCT A	-3'
NBRE-lo	3'- G AGCACGCTTTTCCAGTTCGCGA TCTAG	-5'

NuRE:

	BamHI	BglII
NuRE-up	5'-GATCC TAGTGATATTTACCTCCAAATGCCAGG	A -3'
NuRE-lo	3'- G ATCACTATAAATGGAGGTTTACGGTCC	TCTAG -5'

Nur77 reporter gene encoding plasmids were given the following numbers

3xNBRE 455-24; 4xNBRE 472-12; 3xNuRE 460-10; 4xNuRE 474-36

Nur77/EBD and Nur77/GBD fusion proteins

pBluescript KS(+) (pBSK, Stratagene) was digested with NotI/SpeI enzymes and gel purified. An annealed oligos (SFIGLOTASPE/SPETAGLOSFI) containing NotI/SpeI compatible ends (these sites were recreated after cloning of the oligo) and SfiI site, globin initiator sequence and T7 tag was cloned in NotI/SpeI sites of pBSK. Resulting plasmid was digested with KpnI/EcoRI enzymes and annealed oligos (ECOSFIKPN/ KPNSFIECO) containing SfiI site was liagted recreating EcoRI/KpnI sites. BamHI/EcoRI restriction fragment from pHFE1 or pHFE1 521V plasmid encoding EBDs (G400V single =EBD^{V} or G400V G521V double mutant=EBD^{VV}) or BamHI/EcoRI adapted PCR product (template: pBKC-CreGRI747T primers GBD5END/GBD3END) encoding GBD were subcloned in BamHI/EcoRI sites of the resulting plasmid. This lead to creation of plasmid (pEBD/pEBDV or pGBD) in which Nur77 and $Nur77^{400}$ truncation mutants could be subcloned as SpeI/BamHI adapted PCR products in-frame via SpeI/BamHI sites and resulting T7 tagged EBD fusion protein could be released via SfiI digest. Correct in frame fusion was confirmed by sequencing (EBD2/EBD3 primers). Nur77/EDB or nur77/GBD fusion proteins were then released via SfiI digest from pEBD/pEBDV or pGBD plasmids, blunted and subcloned in HindIII blunt site of pRcCMV vector.

Plasmid numbers: pNur77400EBD 553-1; pNur77400EBDV 340-1; pNur77GBD 497-2; pNur77400GBD510-1; pRcCMVNur77400EBD 556-25; pRcCMVNur77400EBDV 354-22; pRcCMVNur77GBD 503-5; pRcCMVNur77400GBD 544-2

Nur77/Nor-1/Nurr1 expression constructs

An oligo containing SfiI site, globin initiator and T7 tag was cloned in XbaI/SpeI site of pBluescript KS(+) vector. In resulting plasmid (denoted pS7 vector) XbaI site was recreated downstream of T7 tag and used for subcloning SpeI/XbaI adapted PCR products (nur77 full length template pBSK-Nur77 ; primers NUR775END and NUR77400X or NUR773ENDX, Nurr1 full length template pBS34-Nurr1 ; primers NURR5END/NURR3ENDX). Plasmid pS7-Nurr77 was used as a template to create Nur77L354V point mutant via site-directed mutagenesis (Quick change (Stratagene) system, primers: Nur77mut51/Nur77mut31). Introduction of L354V point mutation in *nur77* cDNA resulted in creation of KpnI enzyme restriction site. Nur77, Nur77L354V, $Nur77^{400}$ and Nurr1 cDNAs were released from pS7 vector with SfiI/XbaI enzymes, blunted and subcloned in HindIII blunt site of pRcCMV vector. Nor-1 cDNA was released as XbaI/EcoRI fragment from pBSK-Nor1 plasmid, blunted and subcloned in HindIII blunt site of pRcCMV vector.

Plasmid numbers: pS7-Nur77 156-11; pS7-Nur77L354V 156-11mut; pS7-Nur77400 358-24; pS7-Nurr1 154-1; pRcCMVNur77 352-5; pRcCMVNur77L352V 708-16; pRcCMVNur77400 369-34; pRcCMVNurr1 730-3; pRcCMVNor-1 315-4

Nur77 Tet-Off expression construct

Nur77 full length cDNA was released from pTLC-N10 plasmid (#548 in Bravo plasmid database) with BamHI enzyme and subloned in pTRE2pur vector in BamHI site. pTRE2pur-Nur77 plasmid number 710-2.

Nur77/Nor-1/Nurr1 N-terminus and A20 C-terminus encoding bacterial expression plasmids

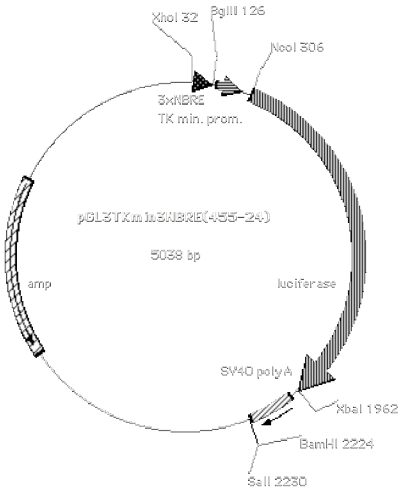
Nur77/Nor1/Nurr1 first N-terminal 200 amino acid portions (templates pBSK-Nur77, pBSK-Nor1 and pBS34-Nurr1. Primers : Nur77His5/Nur77His32, Nor-1His5/Nor-1His32, Nurr1His5/Nurr1His32) or A20 C-terminal 200 amino acid portion were amplified by PCR (template pCAGGS-A20, primers : A20C5/A20C3). PCR products

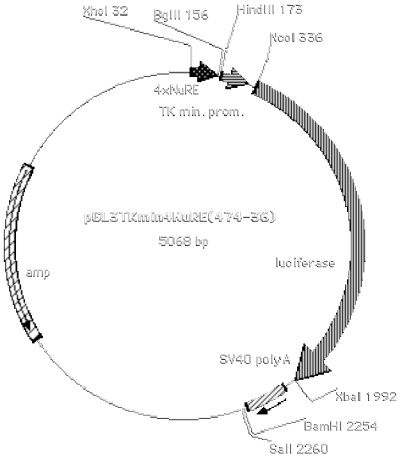
were digested with NheI/XhoI, gel purified and subcloned in NheI/XhoI site of pET28a(+) vector.
Plasmid numbers: pET28a-Nur77His 626-1; pET28a-Nor1His 643-1; pET28a-Nurr1His 644-3; pET28a-A20C 750-2

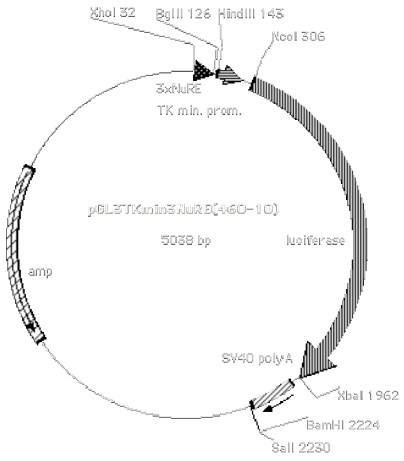
Bcl2 and A20 retroviral expression vectors
Bcl2 cDNA was released form pBabepuro-hBcl2 plasmid with EcoRI vector and subcloned in EcoRI site of pCFG5 IEGZ retroviral expression vector. A20 FLAG-tagged cDNA was released from pCAGGS-A20 plasmid with Xho/HindIII enzymes, blunted, A-tailed and subcloned in pT-Easy vector (Promega) via T-tail mediated cloning. Subsequently A20 cDNA was released from pT-Easy with EcoRI enzyme and subcloned in EcoRI site of pCFG5 IEGZ retroviral expression vector. bcl2/A20 expression vector pCFGIEGZ-hBcl2/ pCFGIEGZ-A20 plasmids numbers : 748-2/740-22

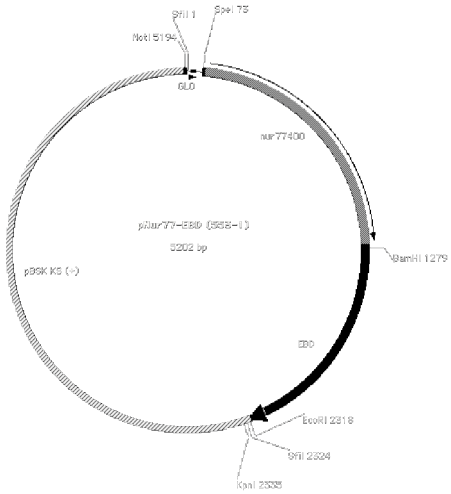
pTLC-Nur77400EBDV/pTLC-Nur77400GBD expression constructs for transgenics
Nur77^{400}EBDV cDNA was released from pNur77400EBDV (340-1) plasmid with SfiI restriction enzyme, blunted and subcloned in BamHI blunted site of pTLC plasmid. Plasmid number: 648-7
Nur77^{400}GBD cDNA was released from pNur77400GBD (510-1) plasmid with SfiI restriction enzyme, blunted and subcloned in BamHI blunted site of pTLC plasmid. Plasmid number: 657-40

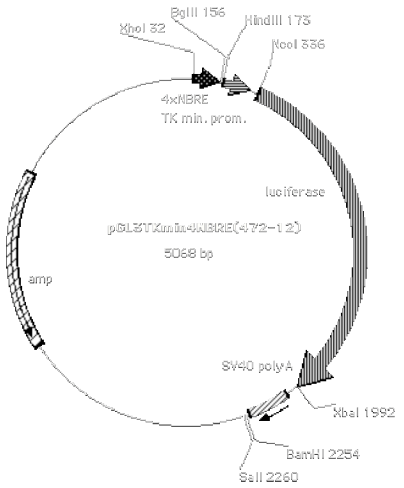
2.1.5 Plasmid Maps

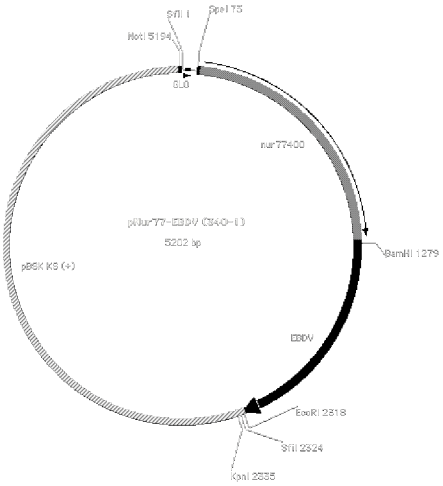











SfiI 1
SpeI 73
NotI 5194
SLO
nur77400
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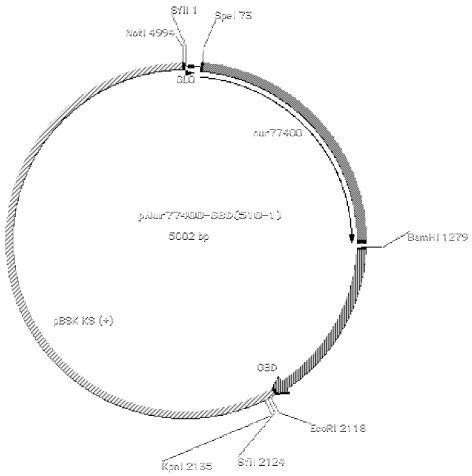
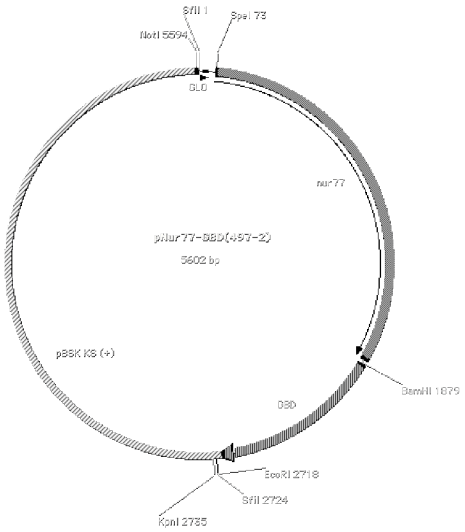
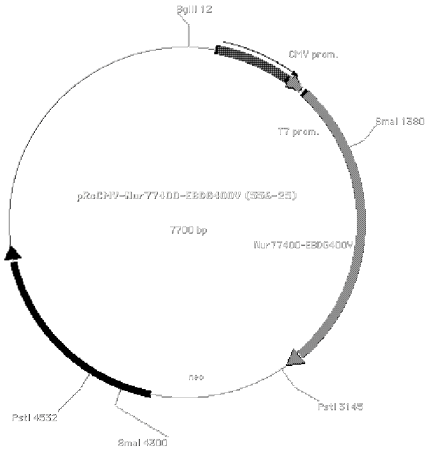
5202 bp
pBSK KS (+)
BamHI 1279
EBDV
EcoRI 2818
SfiI 2824
KpnI 2835


SfiI 1
SpeI 73
NotI 4994
SLO
nur77400
pNur77400-GBD(510-1)
5602 bp
BamHI 1279
pBSK KS (+)
GBD
EcoRI 2118
KpnI 2135
SfiI 2124


SfiI 1
SpeI 73
NotI 5594
SLO
nur77
pNur77-GBD(497-2)
5602 bp
pBSK KS (+)
BamHI 1679
GBD
EcoRI 2718
SfiI 2724
KpnI 2735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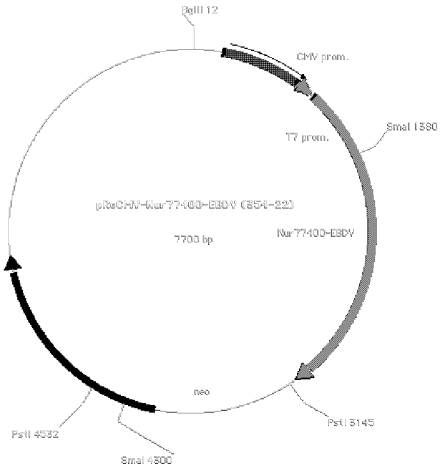
CMV prom.
T7 prom.
SmaI 1880
7700 bp
neo
PstI 5145
PstI 4532
SmaI 4300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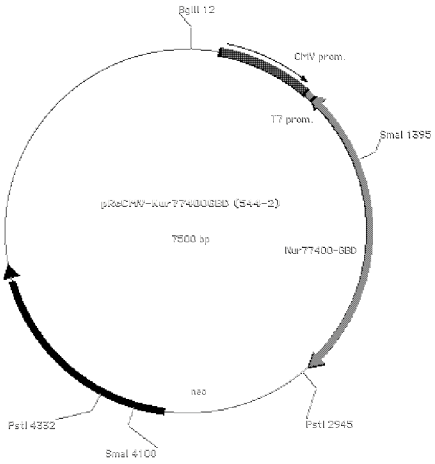
CMV prom.
T7 prom.
SmaI 1880
7700 bp
Nur77400-EBDV
neo
PstI 5145
PstI 4532
SmaI 4300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CMV prom.
T7 prom.
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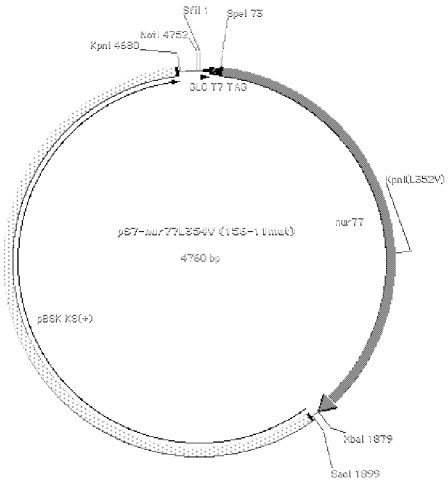
7500 bp
Nur77400-SS
neo
PstI 4332
SmaI 4100
PstI 2945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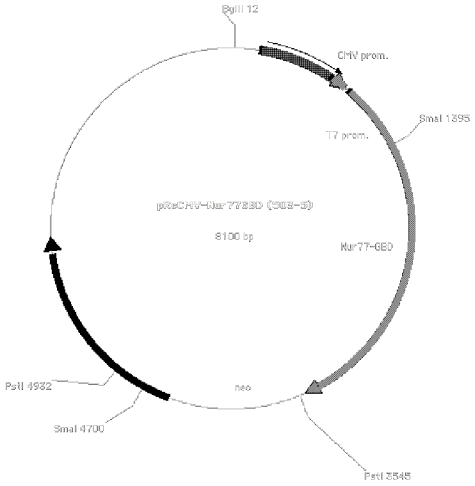
SpeI 73
KpnI 4680
NotI 4752
GLD T7 TAG
KpnI(L352V)
pS7-nur77L354V (154-1 linear)
4760 bp
nur77
pBSK KS(+)
XbaI 1879
SacI 1899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CMV prom.
T7 prom.
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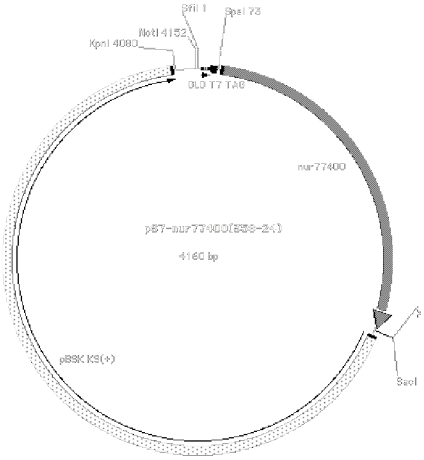
pReCMV-Nur77SS (SS-3)
8100 bp
Nur77-SS
neo
PstI 4932
SmaI 4700
PstI 3545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SpeI 73
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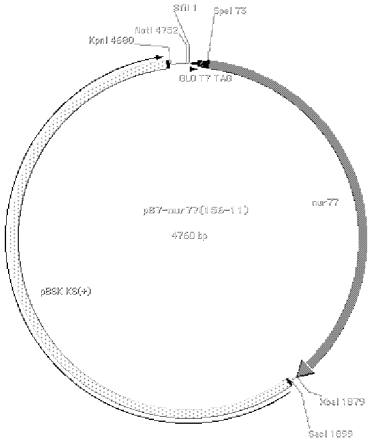
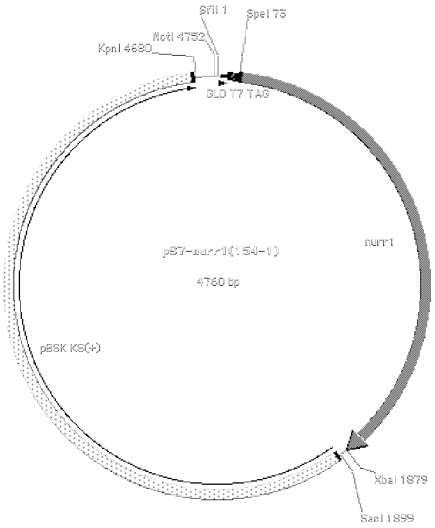
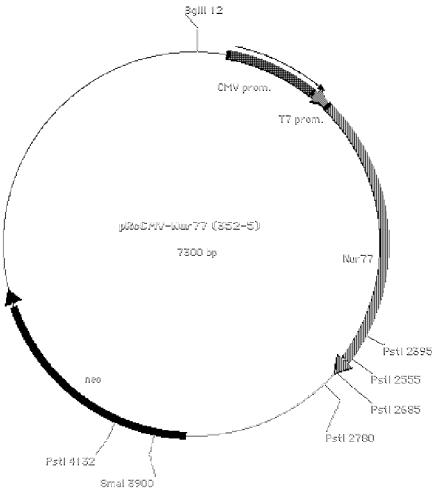
GLD T7 TAG
nur77400
pS7-nur77400(SS3-24)
4160 bp
pBSK KS(+)
SacI


SfiI 1
SpeI 73
NotI 4752
KpnI 4680
GLD T7 TAG
pS7-nur77(154-1)
4760 bp
nur77
pBSK KS(+)
XbaI 1879
SacI 1899


SfiI 1
SpeI 73
NotI 4752
KpnI 4680
GLD T7 TAG
pS7-nur1(154-1)
4760 bp
nur1
pBSK KS(+)
XbaI 1879
SacI 1899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CMV prom.
T7 prom.
pReCMV-Nur77 (352-5)
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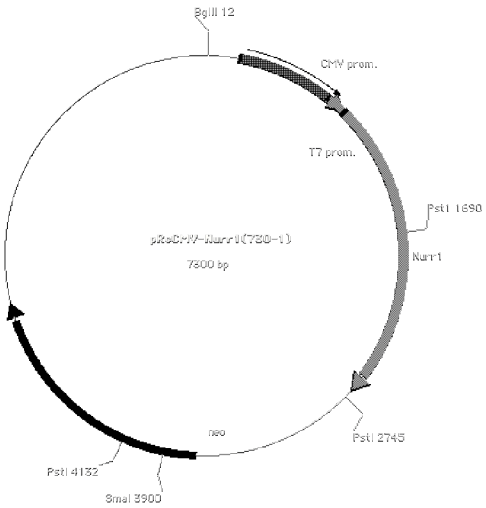
Nur77
PstI 2895
PstI 2555
PstI 2685
PstI 2780
neo
PstI 4132
SmaI 3900


BglII 12
CMV prom.
T7 prom.
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pReCMV-Nurr1(780-1)
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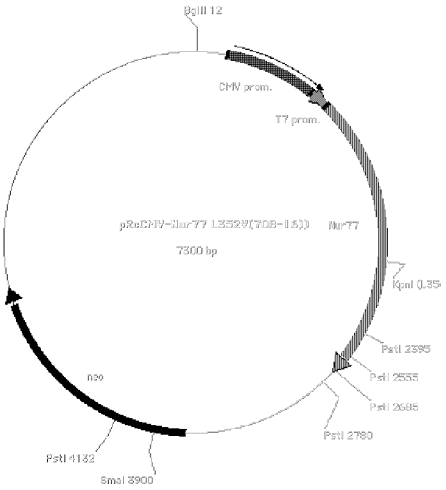
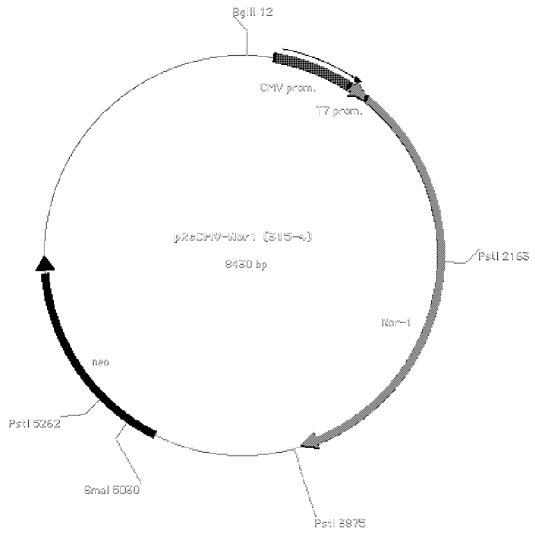
Nurr1
neo
PstI 2745
PstI 4132
SmaI 3900


BglII 12
CMV prom.
T7 prom.
pReCMV-Nur77 (352-5)(708-15))
7300 bp
Nur77
KpnI 0.35
PstI 2395
PstI 2555
PstI 2685
PstI 2780
neo
PstI 4132
SmaI 3900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CMV prom.
T7 prom.
pReCMV-Nur1 (315-4)
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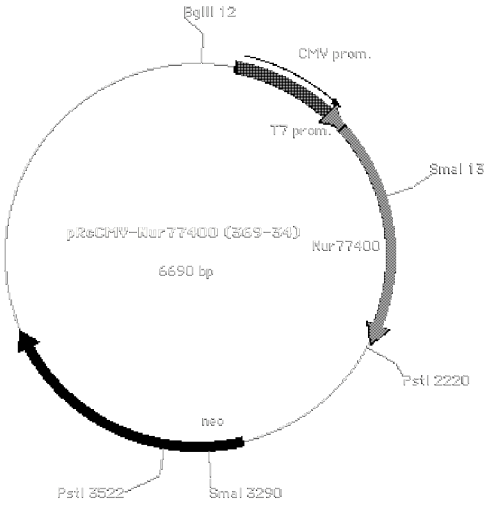
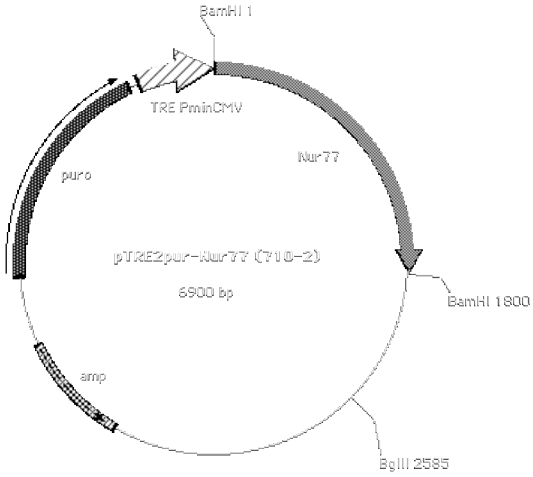
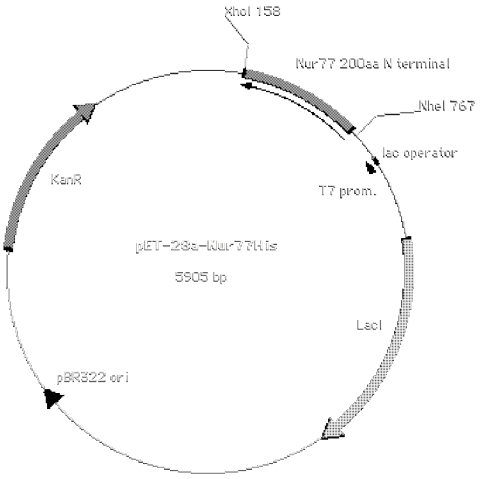
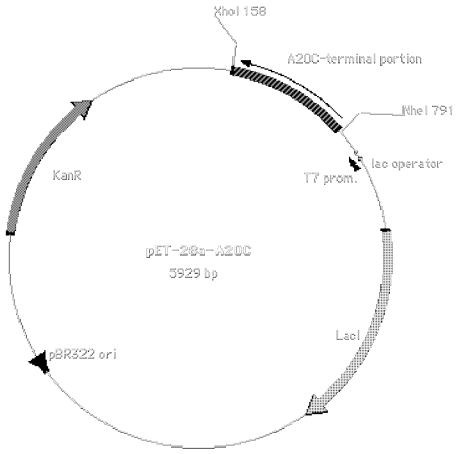
PstI 2163
Nur-1
neo
PstI 5262
SmaI 5030
PstI 3875


BglII 12
CMV prom.
T7 prom.
SmaI 13
pReCMV-Nur77400 (369-34)
6690 bp
Nur77400
PstI 2220
neo
PstI 3522
SmaI 3290


BamHI 1
TRE PminCMV
Nur77
puro
pTRE2pur-Nur77 (710-2)
6900 bp
BamHI 1800
amp
BglII 2585


XhoI 158
Nur77 200aa N terminal
NheI 767
lac operator
T7 prom.
KanR
pET-28a-Nur77His
5905 bp
LacI
pBR322 ori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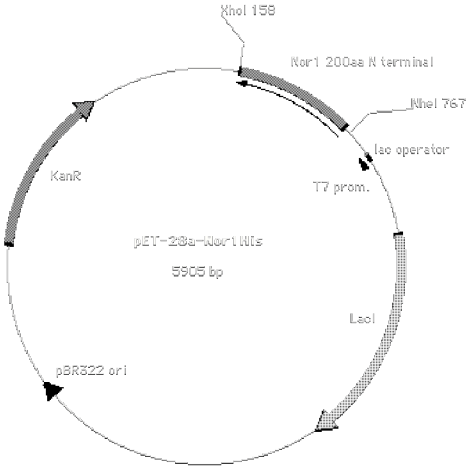
A20C-terminal portion
NheI 791
lac operator
T7 prom.
KanR
pET-28a-A20C
5929 bp
LacI
pBR322 ori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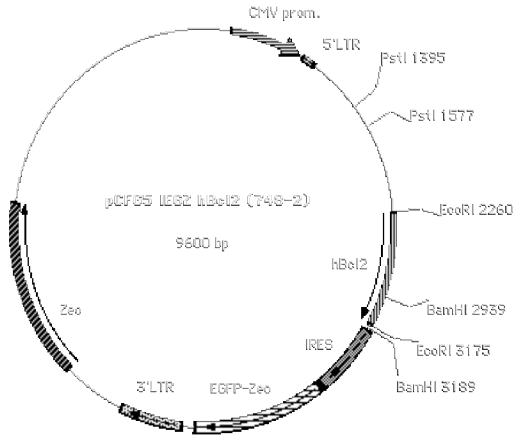
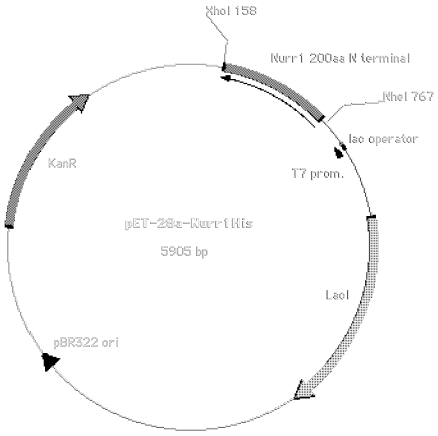
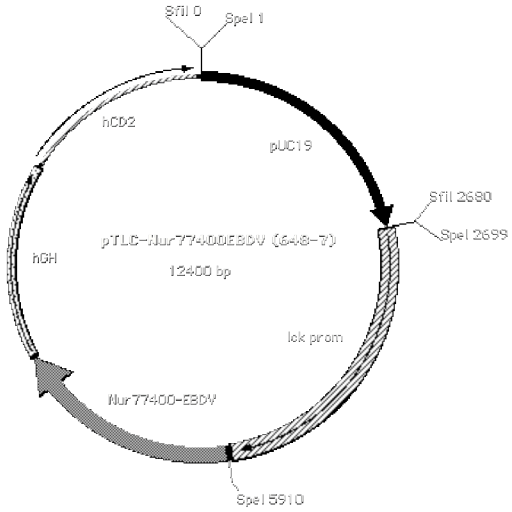
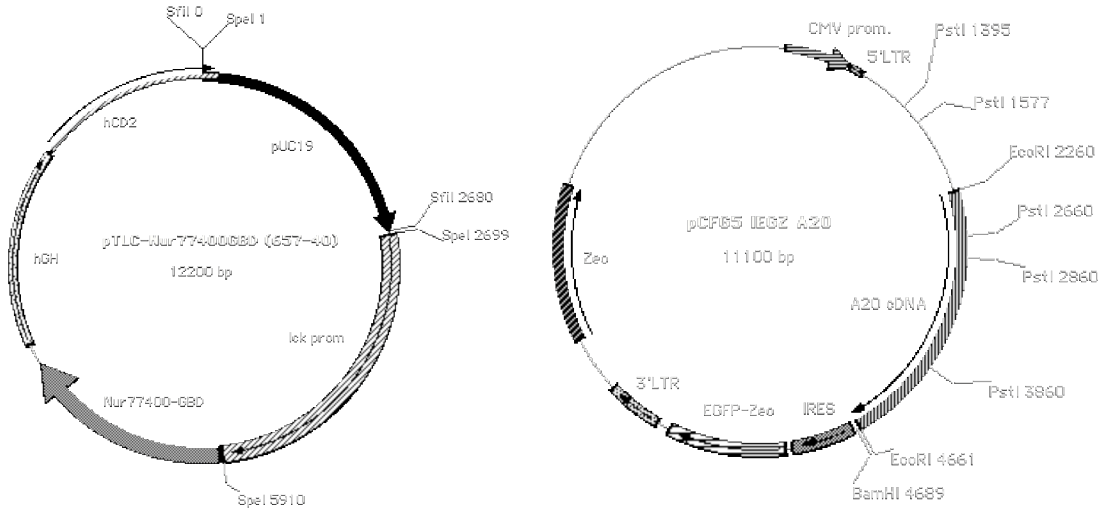
Nor1 200aa N terminal
NheI 767
lac operator
T7 prom.
KanR
pET-28a-Nor1His
5905 bp
LacI
pBR322 ori


CMV prom.
5'LTR
PstI 1395
PstI 1577
pCF05 IRES hBcl2 (748-2)
9600 bp
EcoRI 2260
hBcl2
BamHI 2939
Zeo
IRES
EcoRI 3175
3'LTR
EGFP-Zeo
BamHI 3189


XhoI 158
Nurr1 200aa N terminal
NheI 767
lac operator
KanR
T7 prom.
pET-28a-Nurr1His
5905 bp
LacI
pBR322 ori


SfiI 0
SpeI 1
hCD2
pUC19
SfiI 2680
SpeI 2699
pTLC-Nur77400EBDV (648-7)
hGH
12400 bp
lck prom
Nur77400-EBDV
SpeI 5910



2.1.6 Cell culture - Cell lines and media.

All cells were maintained at 37°C in an incubator (Forma Scientific, Labotect GmbH, Göttingen) in 5% CO_2 and 95% air humidity. All cells were grown in Petri dishes (Greiner Labortechnik, Flikenhausen) of varying sizes depending on the application. Freezing of cells were done by harvesting, washing cells and adding 1 ml of freezing medium (10% DMSO (Fluka Chemie AG, Switzerland) in 20%FCS containing DMEM medium). The cells were transferred to cryovial, left on ice for 1hr and transferred to –80°C for 1 day before finally to liquid nitrogen. To thaw cells, the cryovial was removed from liquid nitrogen and placed at 37°C until the cells had thawed. The cells were transferred to 10 ml of pre-warmed fresh medium followed by light centrifugation before being replated on Petri dishes in fresh medium.

Media for cell culture were purchased at GIBCO BRL. All media contained penicilin and streptomycin (100U/ml; Gibco).

Cell line:	Medium:
Do.11.10 T-cell hybridoma (mouse x mouse) (chicken ovalbumin reactive) (ECACC 85082301)	DMEM, 5% heat inactivated FCS
JURKAT Tet-Off (Clontech)	RPMI, 10% heat inactivated FCS Glutamax, 10 ng/ml Doxycycline (Sigma)
293 HEK	DMEM, 10% heat inactivated FCS
Phoenix retroviral packaging cell line	DMEM, 10% heat inactivated FCS

2.1.7 Mice

Generation of lck-Nur77 (FL-Nur77), mI□B□ mice were previously reported (Weih *et al.*, 1996),(Vallabhapurapu *et al.*, 2001). DN-Nur77 transgenic mice were created similarly as lck-Nur77 line but DN-Nur77 mutant (Woronicz *et al.*, 1994) was cloned into pTLC vector. All animals were housed and bred under the standard conditions with water and food ad libidum in the pathogen free mouse facility of the Forschungszentrum Karlsruhe, Institute of Toxicology and Genetics. The *tnf*$^{-/-}$*lta*$^{-/-}$ double-knockout mice were kindly provided by Dr H. Körner.

For timed pregnancies, mice were mated and the noon of the day vaginal plugs was accepted as 0.5 days post coitus (d.p.c).

Injection of tamoxifen *in vivo*.

Mice were injected i.p. three times every 48 hr with each 100 □l of 10 mg/ml tamoxifen (Sigma) solubilised in 10% ethanol in peanut oil (Sigma). Mice were sacrificed 24 hr after last injection.

2.1.8 Antibodies

Primary antibodies:

Antigen	species	application	source	Cat. No.
Anti-Nur77	rabbit	IF, WB, EMSA		this work
Anti-Nor1	rabbit	WB, EMSA		this work
Anti-Nurr1	rabbit	WB, EMSA		this work
Anti-A20	rabbit	WB, IP		this work
anti-β actin	mouse monoclonal	WB	Sigma	
anti-Nur77	mouse monoclonal	WB	Pharmingen	13471A
anti-c-jun	rabbit	WB	SantaCruz	sc-45
anti-Bcl2	rabbit	WB	SantaCruz	sc-783
anti-CD4-FITC	rat monoclonal	FACS	Pharmingen	553047
anti-CD8-PE	rat monoclonal	FACS	Pharmingen	553032
anti-FasL-biotin	hamster monoclonal	FACS	Pharmingen	555292
anti-FasL	hamster monoclonal	blocking	Pharmingen	28100D
anti-TNFR1	hamster monoclonal	blocking	Pharmingen	557535
anti-TNFR2	hamster monoclonal	blocking	Pharmingen	557534
anti-Fas-PE	hamster monoclonal	FACS	Pharmingen	15405B
anti-Fas	hamster monoclonal	agonist	Pharmingen	15400D
anti-T7 tag-biotin	mouse monoclonal	WB	Novagen	69968-1
anti-IκBα	rabbit	WB	Dr R.Bravo	KD21-5

Secondary antibodies

Antigen	application	source	
anti-mouse Ig-FITC	immunofluorescence	Sigma	F7512
anti-mouse Ig-HRP	WB	DAKO	PO447
anti-rabbit Ig-HRP	WB	DAKO	PO448
streptavidin-HRP	WB	DAKO	PO397
streptavidin-PerCP	FACS	Pharmingen	1303PA

2.1.9 Recombinant proteins

Recombinant mouse and human TNFα and mouse recombinant IL-2 were purchased from PromoCell and used at 25 ng/ml final concentration. Recombinant human TNFR1-Fc protein was obtained from Alexis and used at 5 μg/ml final concentration.

2.1.10 Bacterial strains

DH5α (Gibco), BL21-DE3 (Novagen)

2.2 METHODS

The following routine molecular biology and immunology methods were performed according to standard protocols (Sambrook and Russell, 2001):
Mini plasmid preparation, restriction digests of DNA, spectrophotometric quantification of nucleic acids, phenol: chloroform extraction, preparation of electrocompetent bacteria, ligation reactions, blunting reaction with T4 DNA polymerase, dephosphorylation of plasmids with CIP, basic PCR reactions, electroporation of bacteria; DNA, RNA, protein electrophoresis (SDS-PAGE), precipitation of nucleic acids, Coomassie staining of protein gels, single cell suspensions from mouse lymphoid organs, staining cells for FACS.

The following routine molecular biology manipulations were done by using kits or commercially available reagents according to the manual:
Labelling of DNA by random priming (Rediprime kit, Amersham)
Quantification of proteins by Bradford method (kit, Bio-Rad)
Total RNA extraction (RnaPure reagent, Peqlab)
Maxi plasmid preparation (kit, Qiagen)
Site-directed mutagenesis (Quickchange™ Site-Directed Mutagenesis kit, Stratagene).
Automatic sequencing (kit, Amersham, Vistra sequencer)
Coupled transcription and translation in vitro (T7 TNT kit, Promega)
Labelling of mRNA with ^{33}P for microarray hybridisation (StripEZ kit, Ambion)
Lipofection of 293/Phoenix HEK cells (Lipofectamine-Plus or –2000 reagent, Invitrogen)
The methods described in detail below are only those developed or modified in this thesis.

2.2.1 EXTRACTION AND ANALYSIS OF RNA.

2.2.1.1 RNA isolation from frozen embryonic thymuses.

Thymuses were snap frozen in liquid nitrogen as soon as the animal was sacrificed. At this step, samples can be stored in Eppendorf tubes at -80ºC for further use. Tissues were either homogenized in 2 ml Eppendorfs in 750 µl RNAPure reagent (Peqlab) or in 15 ml Corex tubes in 3 ml RNAPure reagent. The tip of the homogenizer (IKA Labortechnik T25 basic) was cleaned with water and 70% ethanol between samples. The tissues were placed into the tubes containing the RNAPure reagent and homogenization was performed at highest speed.

Following homogenisation, samples were left at room temperature (RT) for 5 min to ensure the complete dissociation of nucleoprotein complexes. Chloroform was added (0.2 ml/ml RNAPure reagent used), samples were vortexed vigorously for 10 sec and were centrifuged at 12000 g for 15 min at 4ºC. The colorless upper aqueous phase was transferred to a new tube and isopropanol was added (0.5 ml/ml RNA Pure reagent used). Samples were left at RT for 5-10 min and then were centrifuged at 12000 g for 15 min at 4ºC. RNA forms a pellet on the side and the bottom of the tube. Supernatant was removed and pellets were washed twice with 1 ml 75% ethanol. Samples were centrifuged at 7500 g for 5 min at 4ºC, supernatants were discarded and pellets were air dried for 5-10 min. Finally, the pellets were resuspended in appropriate volume of ddH_2O and were placed on a thermoshaker (Eppendorf Thermomixer 5436) for 10 min at 60ºC to dissolve the RNA. Samples were further used for concentration estimation and gel electrophoresis.

2.2.1.2 Extraction of Total RNA From Cells

Cells were pelleted in Eppendorf tubes and homogenized in 1 ml RNA Pure reagent by vortexing till the pellet is dissolved. At this step homogenized samples can be stored at -20ºC. The extraction was performed as with the frozen tissue samples (see above section). After the transfer of the aqueous phase to a new tube, 3 µl glycogen (Peqlab) was added.

2.2.1.3 Purification of poly-A tailed mRNA from total RNA.

0.1-1 mg of total RNA (purified by standard RNAPure protocol) was diluted in water to final volume of 500 µl and incubated at 65^{O}C for 10 min. Then 3 µl of biotinylated oligo-$(dT)_{25}$ (MWG-Biotech, 50 pmol/ml) and 13 µl of 20x SSC were added. Tube

content was mixed and incubated 10 min. at RT. Paramagnetic particles (Promega) were washed 3 x 0.3 ml of 0.5x SSC (by collecting particles on magnet stand) then added in 0.1 ml of 0.5x SSC to RNA/oligo mix and incubated 10 min at RT with gentle mixing. Paramagnetic particles were captured on magnetic stand. Supernatant was removed and particles were washed with 4 x 0.3 ml of 0.1x SSC. mRNA was eluted from the particles by resuspending in 1 x 100 µl water and then 1 x 150 µl water.

2.2.1.4 Agarose Gel Electrophoresis of RNA Samples

The quality of RNA samples and genomic DNA contamination were checked by agarose gel electrophoresis. Aliquots of RNA samples (minimum 0.5 µg) were diluted in water and 10x standard gel loading buffer (final 1x concentration) and were loaded on 0.8% (w/v) agarose gels prepared in 1x TAE buffer and containing ethidium bromide to a final concentration of 100 µg/ml for visualization of nucleic acids. Following run, gels were observed by illumination with UV light and photographs were taken using an Eagle Eye (Stratagene) photocamera system.

2.2.1.5 Analysis of RNA by Northern blotting

Electrophoresis of RNA Through Gels Containing Formaldehyde

1.2% agarose gel was prepared by dissolving the corresponding amount of agarose in water and the solution was cooled to 60ºC in a water bath. Appropriate volumes of 5x MOPS buffer (final 1x) and formaldehyde (final 0.5 M) were added under a fume hood. Samples were prepared as follows:

RNA (10 µg-30 µg)	up to 15 µl
5x MOPS buffer	6 µl
Formaldehyde	3 µl
Formamide	30 µl
10x gel loading buffer	6 µl
Final volume	***60 µl***

Samples were incubated at 55ºC for 15 min, chilled on ice and centrifuged briefly. Before loading, 1x MOPS gel running buffer was equilibrated at 4ºC and the gel was pre-run at 130 V for 20 min. Samples were loaded and electrophoresis was performed in the cold room at 130 V (5 V/cm) for 1 h and then the voltage was increased to 160 V for additional 4 h until bromophenol blue migrated 7-8 cm. As a reference, one

sample with ethidium bromide was run and visualized by UV after the run was complete.

5x MOPS buffer: 0.1 M MOPS pH 7.0, 40 mM sodium acetate and 5 mM EDTA pH: 8.0.

10x gel loading buffer: 1 mM EDTA pH 8.0, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 50 % (v/v) glycerol; stable at room temperature for 3 months.

2.2.1.6 Transfer of RNA from Gel to Membrane

The gel was rinsed with DEPC-treated water on a shaker twice for 20 min each to remove formaldehyde, followed by a final wash with 20x SSPE for 20 min. The transfer set up was prepared by placing the gel upside down on a layer of 3MM Whatmann paper (Bender und Hobein) pre wet with transfer buffer and the membrane (Hybond-N+, Amersham) on the gel. Three sheets of pre wet Whatmann paper cut to the size of membrane, paper towels, glass support plate and weight were placed on top and the transfer was performed overnight (O/N). Membrane wrapped in saran wrap was placed in a UV-crosslinker (Stratagene) RNA side-up and RNA was immobilized using Auto-Crosslink programme. The efficiency of transfer was checked by staining the gel with ethidium bromide (0.5 □g/ml) in 20x SSPE for 10 min at RT followed by destaining with ddH_2O for 10 min and photography. In addition, the membrane was stained in 0.03% (w/v) methylene blue in 0.3 M sodium acetate, pH 5.2, for 2 min and was destained in water 3 x 5 min per wash.

20x SSPE: Transfer buffer, 175.3 g NaCl, 27.6 g NaH_2PO_4, 7.4 g EDTA dissolved in 800 ml water and pH adjusted to 7.4 with NaOH.

2.2.1.7 **Preparation of radiolabelled probes**

Nur77 cDNA (BamHI fragment from clone #548 Dr R. Bravo plasmid database and GAPDH cDNA (PstI fragment from clone #202 Dr R. Bravo plasmid database) were run on 1% agarose gel and excised of the gel upon visualization of products by UV. The weight of the slice was determined and gel extraction was performed using QIAquick gel extraction kit (QIAGEN) following the protocol supplied by the manufacturer. DNA was eluted in 30 □l elution buffer. Purified products were further subjected to agarose gel electrophoresis and concentrations were estimated by loading 100 bp DNA ladder marker with known concentration.

Purified DNA fragments were labelled by Rediprime random prime labelling system (Amersham Pharmacia Biotech) according to manufacturer's recommendations. Probes were purified by Sephadex G-50 columns (see EMSA section for detailed description). One µl of this eluate was measured using scintillation counter 1211 Minibeta (Wallac). A probe solution of 400-500 µl with 40000 cpm/µl count was used for hybridisation.

2.2.1.8 Hybridization and Analysis

The membrane was placed RNA-side up in a hybridization tube and was prehybridized in 20 ml of hybridization buffer at 68ºC with rotation for 3-4 h in a hybridization oven / shaker (Amersham). The labelled molecular probe was denatured at 95ºC for 5 min, snap cooled on ice and appropriate amount was pipetted into the hybridization tube for 20-24 h at 65-68ºC. The membrane was washed 3 times, each for 10 min, under low stringency conditions at RT in hybridization oven. This was followed by 2 times 30 min high stringency washes at 65ºC. Finally, the membrane was air dried for 1 min on a Whatmann paper and autoradiography was performed with MP films (Amersham). Quantifications were performed with a Fuji film FLA-3000 fluorescent image analyzer.

Hybridization buffer: 0.5M Na_2HPO_4 pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA

Low stringency wash buffer: 2x SSPE / 0.1% SDS

High stringency wash buffer: 0.2x SSPE / 0.1% SDS

2.2.1.9 First strand cDNA synthesis of total RNA samples

The total RNA (1-5 µg) was added to an Eppendorf tube together with 1 µl oligo $(dT)_{12-18}$ (500 µg/ml) (New England Biolabs) and 1 µl 10mM dNTP mix (Peq Lab) (10 mM each dATP, dGTP, dCTP, dTTP at neutral pH). The volume was completed to 12 µl with sterile ddH_2O. The reaction mixture was heated to 65ºC for 5 min and quickly chilled on ice. Following brief centrifugation, 4 µl 5x first-strand buffer and 2 µl 0.1 M DTT (Invitrogen) were added and the samples were incubated at 42ºC for 2 min. Then, 1 µl (200 U) of Superscript II was added, mixed by pipetting up and down and samples were incubated at 42ºC for 50 min. Following heat inactivation at 70ºC for 15 min, cDNA samples were ready for amplification by PCR. The samples were diluted

5 fold (final volume 100 µl) with sterile ddH_2O before they were used as template for amplification.

2.2.1.10 Semi-quantitative RT-PCR

The reaction mix was prepared by mixing the following per reaction:

5.7 µl ddH_2O
3 µl glycerol (Calbiochem, molecular biology grade)
2 µl 10x PCR buffer (Mg^{2+} free) (Invitrogen)
0.6 µl $MgCl_2$ (50 mM, Invitrogen)
1.5 µl dNTPs (2.5 mM each) (Peq Lab)
0.1 µl [α-^{32}P] dCTP (3000 Ci/mmol; 10 µCi/µl, Amersham)
1 µl sense primer (250 ng/µl for 25mer)
1 µl antisense primer (250 ng/µl for 25mer)
0.1 µl Taq polymerase (0.5 U, Invitrogen)

The above mix was distributed as 15 µl/reaction into PCR tubes and 5 µl cDNA (from 100 µl final diluted volume) was added and mixed. PCR was performed with MJ Research PTC 225 thermal cycler using the following programme:

Denaturation 94ºC 30 sec; Annealing 55^{O}C 30 sec; Extension 72ºC 1 min; for a total of optimised cycle number; 8ºC soak

For determination of the appropriate cycle number, cDNA samples (for most of the primer pairs as a positive sample, cDNA synthesized from total mouse spleen RNA was used) were either left undiluted or diluted 3 and 9 fold respectively and reactions were amplified with different cycle numbers with increments of 3 cycles. The radiolabelled PCR products were separated by 6% polyacrylamide gels. Following electrophoresis (200-250 Volts 2-3, h), gels were dried at 80ºC for 30 minutes using gel drier (Bio-Rad) and exposed using phosphoimager imaging plate (Fuji film FLA-3000). Quantifications were done using Aida software and the linear range was determined. For subsequent analyses with a particular primer pair, the determined cycle number was used and quantifications were always done as described above.

6% polyacrylamide gels: 35 ml ddH_2O, 10 ml acrylamide: bisacrylamide (30 %) (Rotiphorese), 5 ml 5x TBE, 300 µl 10% (w/v) APS, 40 µl TEMED

5x TBE: 54 g Tris-base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH: 8.0) per 1 liter ddH_20

2.2.2 EXTRACTION AND ANALYSIS OF PROTEINS

2.2.2.1 Whole cell extract (WCE) preparation in Laemmli buffer.

Cells were trypsinized and pelleted by centrifugation (2000 rpm 5 min 4ºC) and the pellet was washed once with phosphate buffered saline (PBS). The volume of the pellet was estimated, supernatant was quickly removed and the pellet was resuspended on ice with 5-fold volume of ice-cold suspension buffer. Then an equal volume (5 fold of pellet volume) of 2x SDS gel loading buffer was added as soon as possible without pipetting up and down. The concentration of loading buffer becomes 1x in the final volume. The sample was incubated at 95ºC for 10 min and then kept on ice. The chromosomal DNA was either sheared by sonication (full power between 30 sec and 2 min, Branson sonifier) or by repeated passages through 23-gauge needle and 1 ml syringe. Following centrifugation at 10000 rpm for 10 min at RT, the supernatant was transferred to a new tube and kept at -80ºC for further use. An equal volume of samples (20-40 µl) was then loaded for SDS-PAGE analysis.

Proteinase inhibitors: One tablet in 2 ml ddH_2O gives 25x stock (Complete, Roche).

Suspension buffer: 0.01 M Tris-Cl pH 7.6, 0.1 M NaCl, 0.001 M EDTA pH 8.0, stored at RT without proteinase inhibitors and just before use inhibitor solution was added to a final concentration of 1x.

2x SDS gel loading buffer: 100 mM Tris-Cl pH 6.8, 200 mM DTT (5x dilution from 1M stock), 4% SDS (sodium dodecyl sulphate, electrophoresis grade), 0.2% bromophenol blue and 20% glycerol; this buffer is stored at RT without DTT.

2.2.2.2 Preparation of Nuclear and Cytoplasmic Fractions

Cells were trypsinized and pelleted in an Eppendorf tube (standard scale was for 2-5*10^6 cells). Supernatant was removed and cells were gently resuspended in 400 µl buffer A. The sample was incubated on ice for 15 min and then 25 µl 10% NP-40 was added, vortexed vigorously for 10 seconds and centrifuged at 13000 rpm for 1 min at 4ºC. At this step, the supernatant contains cytoplasmic RNA and proteins and pellet contains nuclei and cell debris. The supernatant (200-300 µl) was transferred to a new tube as ***cytoplasmic*** fraction. The nuclear pellet was washed once with 200 µl Buffer A gently, buffer was removed and 60 µl of buffer C was added <u>without</u> resuspending. The sample was then placed on an Eppendorf shaker for 15 min at 4ºC to ensure vigorous mixing such that pellet remains intact and floats around. Following centrifugation at 13000 rpm for 5 min at 4ºC, 50 µl of ***nuclear*** fraction was recovered

and protein concentration was determined. Cytoplasmic and nuclear fractions were stored at -80ºC. In order to use these fractions for SDS-PAGE, appropriate amounts of protein extracts (minimum 10 µg) were taken and the volume of the samples to be analysed was brought to the same final volume by adding complete buffer C. The same final volume of 2x SDS loading buffer was then added (final 1x), the samples were incubated at 95ºC for 5 min and left to cool to RT before loading.

Buffer A: 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1x proteinase inhibitors and 2.5 mM DTT.

Buffer C: 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 25% glycerol, 1x proteinase inhibitors and 2.5 mM DTT.

Both buffers are stored at 4ºC without proteinase inhibitors and DTT.

2.2.2.3 SDS-PAGE

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Sambrook & Russel, 2001. Resolving (8%) and stacking (5%) gels were prepared as below:

8% resolving gel		*5% stacking gel*
9.7 ml	H_2O	6.8 ml
8 ml	acryl./bisacryl. (30%)	1.7 ml
11.7 ml	1M Tris (pH 8.8)/ 1M Tris (pH 6.8)	1.3 ml
300 µl	10% SDS	100 µl
300 µl	10% APS	100 µl
24 µl	TEMED	10 µl
30 ml	*Final volume*	*10 ml*

Resolving gel solution was prepared and poured between 2 glass plates using the Joey casting system (Peq Lab). The glass plates were marked to provide approximately 9 cm resolving and 2 cm stacking gel. 70% ethanol was poured for a straight boundary between resolving and stacking gels. After polymerization was complete, ethanol was poured off, the surface was washed with ddH_2O and dried with Whatmann paper. Then 5% stacking gel solution was poured on top and an appropriate comb was inserted. Following polymerization, comb was removed and the system was casted to P9DS apparatus (Peq Lab). Tris-glycine buffer (1x) was used as the running buffer and the gel was run until the bromophenol blue in the SDS gel loading buffer migrated out.

1x Tris-Glycine buffer: 25 mM Tris, 250 mM glycine, 0.1% SDS

2.2.2.4 Western blotting - Transfer of Proteins from Gel to Membrane

The transfer was performed by Trans/Blot Cell transfer system (Bio-Rad). For each gel, 4 sheets of Whatmann papers and one membrane were pre-cut as the dimensions of the gel. PVDF membrane (Immobilon^{TM}-P, Millipore) was activated for 15 seconds in methanol, washed in H_2O for 2 min and then placed in transfer buffer for 5 min. Meanwhile the gel was removed and different bands of the loaded prestained Benchtop protein marker (Invitrogen) was recorded by placing a transparent file on the gel and marking the bands. This record was later to be used to mark the corresponding molecular weights on the autoradiogram. The gel was placed on 2 sheets of pre wet Whatmann papers. The membrane was placed on top of the gel, the start was cut on the edge for orientation and rolling over a glass pipette gently prevented air bubbles. The sandwich was completed by placing 2 extra sheets of Whatmann papers and was assembled into the transfer chamber filled with transfer buffer, which was freshly prepared for each transfer. All transfers were carried out at 20 V at RT for 7 h on a magnetic stirrer to ensure continuous mixing of buffer.

Transfer buffer: 39 mM glycine, 48 mM Tris-base, 0.037% SDS (electrophoresis grade), 20% (v/v) methanol pH 8.3.

2.2.2.5 Incubation of PVDF membranes with antibodies.

Following transfer, membrane was placed as protein side up into a container filled with blocking solution and was blocked either for at least 1 h at RT or O/N at 4ºC. Once complete, the membrane was sealed in a nylon bag and primary antibody diluted in blocking solution was added. Following 1 h RT incubation, the membrane was rinsed rapidly twice and then washed 3 times, 10 min each, with TBS-T. The membrane then was incubated similarly with appropriate horseradish peroxidase (HRP) conjugated secondary antibody for 1 h at RT. Following washes as described above, chemiluminescence detection was performed by ECL western blotting detection system (Amersham) according to the manufacturer's instructions. The exposures were performed with Hyperfilm ECL (Amersham).

10x TBS: 250 mM Tris, 1.5 M NaCl, pH adjusted to 8.0 with HCl

TBS-T: 1x TBS in ddH_2O, 0.1% (v/v) Tween-20

Blocking solution: 5% (w/v) non-fat dried milk (Saliter) in TBS-T

2.2.3 Flow cytometry

Cells were harvested and washed with FACS staining buffer (2% FCS in PBS without Ca/Mg) twice at 4°C. Cells were transferred to 96-well plate at 0.5 x 10^6 cells/well/sample, and centrifuged at 1400 rpm for 2 min at 4°C. Supernatant was poured off and FITC- and/or PE- labelled mAb were added, incubated at 4°C for 20 min. Cells were washed twice and analysed on FACS-Star plus flow cytometer (Becton Dickinson).

2.2.4 Purification of recombinant His-tagged proteins

BL21(DE3) bacterial strain was transformed (standard $CaCl_2$-based method) with pET28(a) expression plasmid. Starter cultures were prepared by inoculating 50 ml of LB+kanamycin (40 μg/ml) and incubating overnight in the bacterial shaker in 500 ml Erlenmayer flask. The one litre Erlenmayer flasks with LB+kanamycin medium (4 x 200 ml) were inoculated with 10 ml of starter culture each and incubated until OD600 reached 0.6 (1-2h). Expression of recombinant protein was induced by addition of IPTG to final conc. 1 mM. After 5 hr of induction bacteria were pelleted 7000 rpm, 15 min and frozen in -20^{O}C. Next day all bacterial pellets were thawed on ice and lysed at room temperature with total of 10 ml of lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea, pH 8.0). Lysates were cleared by centrifugation at 4^{O}C, 10000 g, 30 min in 15ml Corex tubes. Then supernatants were transferred to 15 ml Falcon tube and 2 ml of Ni-NTA resin equilibrated with lysis buffer were added. Tubes were rotated then for 1 hr at RT. After that resin/lysate mix was applied to poly-prep column (Bio-Rad). Columns were washed 2 x 8 ml of wash buffer (comp. as lysis buffer but pH 6.3). Proteins were eluted 5 x 0.5 elution buffer I (comp. as lysis buffer but pH 5.9) and 8 x 0.5 ml elution buffer I (comp. as lysis buffer but pH 4.5). Out of each eluted fraction 5 μl was taken for Bradford protein assay. Fractions containing significant amount of protein were pooled, diluted to 0.5-1 mg protein/ml with elution buffer II and finally dialysed in 3-12 ml Slide-a-Lyser cartridges (PerBio) against 2 liters of dialysis buffer (20 mM Tris-Cl pH 8.0, 150 mM NaCl, 2 M urea) overnight at RT. After that protein concentration was determined and 10 μg of protein was subject for SDS-PAGE and Coomassie staining to determine the quality. Antigens in this form were used to immunised rabbits (done at Moravian Biotechnology), 4-5 mg of protein per rabbit, 2 rabbits per antigen.

Lysis buffer: 100 mM NaH_2PO_4, 10 mM Tris-Cl, 8M urea, pH 8.0 (with HCl)

Wash buffer: as above but pH 6.3

Elution buffer I: as above but pH 5.9

Elution buffer II: as above but pH 4.5

always adjust pH before use

Dialysis buffer : 20 mM Tris-Cl pH 8.0, 150 mM NaCl, 2 M urea

2.2.5 *In vitro* translation of Nur77/Nor-1/Nurr1 proteins.

In vitro translation was done according to TNT T7 kit manual (Promega). pRcCMV vector, pRcCMV-Nur77, pRcCMV-Nor1, pRcCMV-Nurr1 plasmids were used.

2.2.6 Electro-mobility shift assay (EMSA).

2.2.6.1 Preparation of Probes

Following single stranded oligonucleotides were used:

Oct-1-up	5'-GATCCTGTCGAATGCAAATCACTAGAA A-3'
Oct-1-lo	5'-GATCTTTCTAGTGATTTGCATTCGACTC-3'
Ig□-up	5'-GATCCAGAGGGGACTTTCCCACAGGA-3'
Ig□-lo	5'-GATCTCCTCTGGGAAAGTCCCCTCTG-3'
NBRE-up	5'-GATCC TCGTGCGAAAAGGTCAAGCGCTA-3'
NBRE-lo	3'-GAGCACGCTTTTCCAGTTCGCGATCTAG -5'

2.2.6.1.1 Annealing of oligonucleotides

Following reaction mix was prepared:

2 □l of each single-stranded oligo (100 pmol/□l)

200 □l TE (pH: 7.5)

2 □l NaCl (5M)

(1 pmol/□l oligo concentration in the final volume of 206 □l)

The reaction was incubated at 80ºC for 15 min and left at RT overnight.

Radioactive labelling of the annealed oligonucleotides

Following reaction mix was prepared:

10 □l ddH_2O
2 □l of annealed oligo (2 pmol total)
2 □l 10x REACT 2 buffer (Invitrogen)
2 □l dNTPs (0.5 mM each without dCTP)
3 □l □^{32}P-dCTP (10□Ci/□l)

1µl Klenow fragment (6.5 U, Invitrogen) as a total volume of 20 µl and was incubated at RT for 20 min.

2.2.6.1.2 Probe purification.

10 µl of blue dextran solution (0.7% (w/v) in water) was added to the labelling mix. Sephadex G-50 (Sigma) in TE buffer was used to prepare 1 ml column in 1 ml syringe the bottom of which was plugged with glass wool. Labelling mix with blue dextran was added on the column and unincorporated radioactivity was left in the column by eluting 200-400 µl blue solution (DNA with blue dextran) in TE. One µl of this eluate was measured using scintillation counter 1211 Minibeta (Wallac). A total of 20000-50000 cpm per reaction was used for EMSA.

2.2.6.2 EMSA Assay

Following solutions are required:

5x Binding Buffer (BB): 50 mM Tris-HCl, 250 mM NaCl, 5mM EDTA, 25% glycerol; kept at RT and before use DTT is added to a final concentration of 25 mM.

Buffer C with 1x proteinase inhibitors and 2.5 mM DTT

Unspecific competitors: Sonicated calf thymus DNA (1 µg/µl) for Igκ bandshift or poly-dI-dC homopoymer (1 µg/µl; Sigma) for NBRE/Oct-1 bandshift assays.

Rabbit anti-Nur77/Nor-1/Nurr1 polyclonal antisera for EMSA

anti-Nur77	626-1 (rabbit 18)
anti-Nor-1	643-1 (rabbit 24)
anti-Nurr1	644-3 (rabbit 19)

All working solutions and binding reactions should be prepared on ice, 5x BB and Buffer C were prepared on ice before setting up the binding reactions. Below are the components of a binding reaction:

5 µl ***nuclear extract solution*** (2-4 µg extract, volume was completed to 5 µl with Buffer C) or 3 µl TNT reaction (in vitro translated proteins)

4 µl ***5x BB*** (final 1x)

Competitor (0.4 µg (0.4 µl/reaction) calf thymus DNA (1/10) or 2 µg (2 µl/reaction) dIdC (1/2) for 4 µg nuclear extract or 10 ng calf thymus DNA for 3 µl of TNT reaction)

1 µl ***antiserum*** (for antibody challenge reactions only)

Oligo (2 µl depending on the count, 20000-50000 cpm total)

ddH_2O to complete the volume to ***20 µl***

Preferentially, premixes were prepared for each oligo shift by adding 5x BB, competitor and ddH_2O the total amounts of which were determined according to the total number of reactions. Then, the mix was distributed to precooled tubes, followed by addition of appropriate volumes of buffer C and protein extract for each sample. For antibody challenge reactions, 1 µl of appropriate antiserum (see above for antisera used) was added and the reactions were incubated on ice for 10 min. The radioactively labelled probe was then added and the reactions were kept at RT for 20 min. Once complete, 2 µl 10x gel loading buffer per reaction was added and the reaction mix was loaded onto 5% gel. While the binding reactions were incubated, the wells of the gel were washed and the gel was pre-run at 150 V for 10 min using 0.25x TBE as running buffer. A typical run is at 150 V for 2-3 h at RT. Then the gel was dried at 80ºC for 30 min and exposure was performed using Hyperfilm MP (Amersham).

5% polyacrylamide gels: 8.3 ml acrylamide: bisacrylamide (30%), 2.5 ml 5x TBE (final 0.25x), 39.2 ml ddH_2O, total volume 50 ml.

2.2.7 Induction of apoptosis by CD3 crosslinking.

Do.11.10 cells were plated at 0.03 x 10^6 cells/ml. 48 hr later cells were replated in fresh medium at density of 0.2 x 10^6 cells/ml (400 µl/24 well format) or 1 x 10^6 cells/ml for other formats. αCD3 antibodies were immobilised on plastic: antibodies were diluted to 0.5 µg/ml in 50 mM Tris-Cl pH 9.0 buffer and overlaid on plastic

plates/dishes (CellStar, Greiner). After overnight incubation at 4^{O}C plates/dishes were washed once with PBS and then used directly. In some experiments death receptor blocking reagents were added: rhTNFR1-Fc (Alexis) – 5 μg/ml, agonistic/neutralising antibodies – 10 μg/ml.

2.2.8 Staining cells with annexin-V-biotin for FACS analysis.

Cells (0.1-1 x 10^6 cells/staining reaction) were harvested into 96 well plates (V shape, Greiner). Spun down 1300 rpm for 3 min. Cells were washed once with 200 μl of annexin-V incubation buffer (I) and resuspended in 100 μl of annexin-V staining solution (II). After incubation on ice for 15 min cells were washed twice with 200 μl of incubation buffer. Then cells were resuspended in SA staining solution (III) and incubated on ice for 10 min. Cells were washed twice with incubation buffer resuspended in 100 μl incubation buffer and analysed by FACS.

incubation buffer (I): 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$

annexin-V staining solution (II): dilute 10 μl of annexin V-biotin (Roche) stock in 1000 μl of incubation buffer (I) (sufficient for ten samples)

Straptavidin (SA) staining solution (III): dilute 5 μl of SA-PE or SA-Cy or SA-PerCP stock (Pharmingen) in 1000 μl of incubation buffer (I) (sufficient for ten samples)

2.2.9 Cytospins and immunofluorescence analysis.

Cells were resuspended in PBS containing 20% FCS at density 1 x 10^6 cells/ml. Cells (100 μl) were immobilised on glass slides (SuperFrostPlus, Menzel-Gläser) by the use of standard cytospin rotors. Centrifugation was done at 4^{O}C, 5 min, 500 rpm with brake off. After spin cells were directly fixed in acetone/methanol mix 50:50 for 3 min at RT. Slides were briefly washed in PBS. Cell containing slide area was marked with PAP pen (DAKO). Primary anti-Nur77 antibodies were applied at 1:100 dilution of crude rabbit sera in 3% BSA/PBS solution. Incubation was done in humidified chamber at 37^{O}C for 2-3 hr. Slides were then washed 3 x 3 min in PBS. Secondary anti-rabbit-FITC antibodies were applied at 1:100 dilution in 3% BSA/PBS solution. DAPI was included at 0.1 mg/ml in secondary antibody solution. Incubation was done at room temperature in the dark for 45 min-1 hr. Slides were then washed 3 x 3 min in PBS. Slides were then mounted in PVA medium and after overnight incubation at 4^{O}C analysed with Zeiss Axiovert fluorescent microscope.

2.2.10 Luciferase assay.

Cells were harvested into 15 ml Falcon tubes (Greiner), washed 2 x 2 ml with PBS Mg^-/Ca^-, lysed in 1 ml of 1x passive lysis buffer (Promega) per 1 x 10^6 cells/ml. Then lysates were precleared by centrifugation for 5 min, 1500 rpm, at RT. Assays were done by mixing 50 µl of lysate and using single injector to inject 100 µl of assay solution (for 6 ml of assay solution (suitable for 50 assays): 5.6 ml GlyGly buffer, 400 µl luciferin stock (1 mM in GlyGly buffer), 80 µl ATP (100 mM), 4 µl DTT (1 M). Measurement was done for 20 sec. in Berthold luminometer.

GlyGly buffer: 25 mM GlyGly, 15 mM $MgSO_4$, 4 mM EGTA, pH 7.8

2.2.11 Beta-galactosidase assay

Cells were lysed 24-48 hr after transfection in 1x Passive Lysis Buffer (Promega, use 1 ml per 2 x 10^6cells). In a plastic cuvette 5-100 µl of extract, 1 ml of B/Z buffer and 0.2 ml of ONPG substrate (2 mg/ml ONPG in buffer B/Z) were combined. Reactions were incubated at 37^{O}C until yellow colour developed. Reactions were stopped by addition 0.5 ml of 1M Na_2CO_3 and then OD420nm was determined as a measure of beta-galactosidase activity.

B/Z buffer: 60 mM Na_2HPO_4, 40 mM $NaHPO_4$, 10 mM KCl, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol (added just before use)

2.2.12 Hybriziations with high density LifeGid filters.

Prehybridization and hybridization of LifeGrid high-density filters were done according to LifeGrid manual (Incyte) with modifications.

Prehybridization was done with 40 ml of hybridization buffer (Incyte) for 3-4 hr at 42^{O}C. Pure mRNA in 250 µl was used for probe synthesis for LifeGrid hybridizations according to Strip-EZ kit manual. For hybridisation 15 ml of hybridisation buffer, 50 µl of herring sperm DNA (Incyte) replaced perhybridization buffer. After 15 min. denatured probe (in 10 mM EDTA, Ambion) was added and hybridization continued for another 20-24 hr at 42^{O}C with 8 rpm rotatory speed. After that time hybridization buffer was discarded, filters were rinsed with 200 ml 2x SSC at RT. Then following washes were performed: 1x 200 ml wash solution (I) at 68^{O}C, 1-2x wash solution (II) at 68^{O}C.

Three hybridization experiments were performed in the way that after each round probe was degraded and washed off the filter and for subsequent hybridizations probes and filters were swapped so that each filter at the end was hybridized twice with the same probe and once with the other probe. After each round of hybridization filters were exposed to Phosphoimager screens for 16-24 hr and then scanned at 50 μm resolution. The resulting images were imported to AIDA software (Raytest) to identify spots of differing intensity (scheme of experiment and an example of LifeGrid patterns after hybridization are shown on Figure 13). The signal intensity for each individual spot on the filter was normalised to actin signal. Filter patterns from –Dox and +Dox probe hybridizations were compared and signals showing differential intensity in all 3 independent experiments were identified (Table I). Only upregulated genes have been characterised (stronger signal in –Dox probes).

Rinsing solution: 2 x SSC

Wash solution I: 2 x SSC, 1% SDS, prewarmed 68^{O}C

Wash solution II: 0.6 x SSC, 1% SDS, prewarmed 68^{O}C

2.2.13 Rapid PCR based test for presence of the transgene in stably transfected cells.

For rapid detection of transgene inserted in genomic DNA 0.5-2 x 10^6 cells were lysed in 300 μl PBND buffer with 0.1 mg/ml proteinase K at 55^{O}C for 3 hr. Then proteinase K was inactivated at 95^{O}C for 10 min. 2-4 μl of resulting DNA preparation was used for standard PCR with appropriate primers.

PBND buffer: 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 2.5mM $MgCl_2$, 0.1mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20

2.2.14 Generation of recombinant retrovirus.

Phoenix amphotropic retroviral packaging cell line was plated at 1.5 x 10^6 cells/10cm dish in 8 ml of medium. After 48 hr of incubation cells were transfected by the use of Lipofectamine 2000 (Invitrogen), 5 μg of plasmid (pCFG5 IEGZ) was diluted in 1 ml DMEM medium, 40 μl Lipofectamine 2000 reagent was diluted in 1 ml DMEM medium and mixed with diluted plasmid DNA. Complexes were allowed to form for 20 min at RT. Then conditioned medium was aspirated from cells and 6 ml of fresh medium was added to cells gently to avoid extensive cell detachment and lipid/DNA complexes were added drop wise to the dish. After 48 hr of incubation supernatants

were collected and after filtration through 45 μm polyacetate cellulose (Renner) filter were used to infect target cells.

2.2.15 Infection of Do.11.10 with recombinant retrovirus.

2 x 10^6 Do.11.10 cells were resuspended in 2 ml of medium containing retroviruses (see previous section) and 2 μl of polybrene were added (5 mg/ml in water, Sigma). Cell suspension was transferred to 12 well plates and spun at room temperature at 1000 g for 3 hr. After that cells were transfected to 10 ml of fresh medium. After additional 24 hr of incubation cells were selected with 300 μg/ml of zeocin for 10 days. Cells were then expanded and analysed as pools.

2.2.16 Transient transfection of 293/Jurkat/Do.11.10 cells by electroporation.

Cells were plated in the following ways:

293: 5 x 10^6 cells in 20 ml of medium (15 cm dish).

JURKAT: 1 x 10^5 cells/ml in 20 ml (15 cm dish).

Do.11.10: 1 x 10^5 cells/ml in 20 ml (15 cm dish).

After 48 hr (293, Do.11.10) or 72 hr (Jurkat) cells were harvested (in case of 293 trypsinised) and desired amount of cells was resuspended in DMEM (no FCS). For 293: density 3 x 10^6 cells per 350 μl for standard transfection or density 6 x 10^6 cells per 350 μl if transfection is to be split in halves (e.g stimulation with hormones). For Jurkat: density 4 x10^6 cells per 350 μl for standard transfection or density 8 x 10^6 cells per 350 μl if transfection is to be split in halves (e.g stimulation with hormones). For Do.11.10: density 5 x 10^6 cells per 350 μl for standard transfection or density 8 x 10^6 cells per 350 μl if transfection is to be split in halves (e.g stimulation with hormones). For each transfection DNA was mixed with 350 μl of cell suspension in an Eppendorf. Incubated at room temperature for 5 min and transferred to 0.4 cm electroporation cuvette (BioRad/Peqlab). Then cuvettes were electroporated with at 960 μF/250 V (GenePulser BioRad). After 3 min of incubation at room temperature cell suspension was recovered with glass Pasteur pipette and plated in 5 ml of prewarmed complete medium (6 cm) or split into two 6 cm dishes to perform EBD/GBD ligands stimulations. In each transfection 10 μg of Nur77 reporter plasmid and 0.1-10 μg of expression plasmid were used. In each transfection containing Nur77

reporter gene fixed amount of LacZ expression vector (pRcCMV-LacZ) was added to normalise for transfection efficiency.

2.2.17 Development of Tet-Off Nur77 Jurkat cell line.

Jurkat Tet-Off T cells (Clontech) were plated 0.1 x 10^6 cells/ml. After 48 hr 8 x 10^6 cells were suspended in 350 µl RPMI medium, 20 µg of pTRE2pur-Nur77 plasmid (710-2) was added and after mixing tranfered to 0.4 cm electroporation cuvette and electroporated at 960 µF/250 V (BioRad GenePulser). Cells were removed from cuvette with Pasteur pippette and transferred to 20 ml of standard culture medium containing 1 µg/ml of doxycycline. Then cells were counted diluted to 15000 cells/ml in medium (with doxycycline 1 µg/ml) containing puromycin (1 µg/ml). Cells (200 µl = 3000 cells) were then aliquoted into U shaped 96 well plates. After two weeks single clones were picked from wells, expanded and analysed.

Single clones were expanded in 10 ng/ml Dox containing medium. Then cells (50 x 10^6) were washed 5 x 10ml RPMI medium and replated in +/- 10 ng/ml Dox containing medium for 48 hr. Then WCE extracts were prepared and Western blotting identified clones showing inducible Nur77 expression.

2.2.18 Stable transfection of 293 cells.

Cells were electroporated as described above with the exception that cells were plated in 20 ml of fresh medium (15cm dish) at the end. After 48 hr of incubation cells were trypsinised, harvested and then plated at 1 x 10^4 cells /dish (10 ml in 10 cm dish) in a medium containing G418 (0.8 mg/ml of 70% active compound). After two weeks cells were expanded as pools and analysed.

EBD/GBD ligands were obtained from Sigma. All were reconstituted with ethanol and used at 10^{-6} M final concentration: tamoxifen (4-OHT) (EBD synthetic antagonist); hexestrol (EBD synthetic agonist); estradiol (EBD natural agonist); dexamethasone (Dex) (GBD synthetic agonist)

3 RESULTS

3.1 PART ONE: Analysis of expression and function of Nur77 nuclear orphan receptors in TCR-induced apoptosis.

3.1.1 The regulation of Nur77 expression by TCR in Do.11.10 cells.

Nur77 regulation during AICD of T cells has been previously studied (Woronicz *et al.*, 1994; Woronicz *et al.*, 1995). The experiments described in that report, however, did not include thorough time course analysis of Nur77 induction by TCR. In order to extend those studies and to validate the suitability of the Do.11.10 cell system to study Nur77 function, *nur77* mRNA induction in response to TCR stimulation has been determined. To get insight into signalling pathways involved in Nur77 regulation downstream of TCR, pharmacological activators of protein kinase C (phorbol ester - PMA) and calcium signalling (calcium ionophore - ionomycin) were used in addition to anti-CD3 monoclonal antibodies (αCD3 Ab). Do.11.10 cells were stimulated with PMA, ionomycin, combination of PMA/ionomycin or αCD3 Ab, total RNA isolated and subject to Northern blotting with *nur77*- and *GAPDH*-specific molecular probes (Figure 1A). Both PMA and ionomycin treatment induced *nur77* mRNA transiently but with different kinetics. PMA-induced *nur77* mRNA peaked at 1 hr and returned to basal levels at 4 hr. On the other hand ionomycin induced *nur77* mRNA reached plateau at 1 hr, stayed unchanged till 4 hr and returned to basal levels at 8 hr. Combined PMA plus ionomycin usage or αCD3 Ab treatment led to prolong *nur77* mRNA upregulation (Figure 1A). Interestingly, sustained *nur77* mRNA induction (beyond 8 hr) correlated with the ability of the stimulant to induce apoptosis (Figure 1A column graphs above Northern blot pictures).

In parallel, DNA binding to NBRE was determined. Apoptosis-inducing stimulation (combined PMA and ionomycin) led to prolonged DNA binding (Figure 1B, right panel). Surprisingly, PMA alone was not capable of inducing NBRE binding despite its ability to induce NF-κB translocation (Figure 1B, left panel) and *nur77* mRNA upregulation (Figure 1A, leftmost panel). It is known that NBRE DNA-binding activity is composed of both Nur77 and Nor-1 ((Cheng *et al.*, 1997) and Figure 4D). To check the response of Nur77 homologues to PMA plus ionomycin, mRNA levels of *nur77*, *nor-1* and *nurr1* from stimulated Do.11.10 cells were

determined by RT-PCR. PMA induced *nur77* mRNA but failed to increase the levels of both *nor-1* and *nurr1* mRNA (Figure 1C).

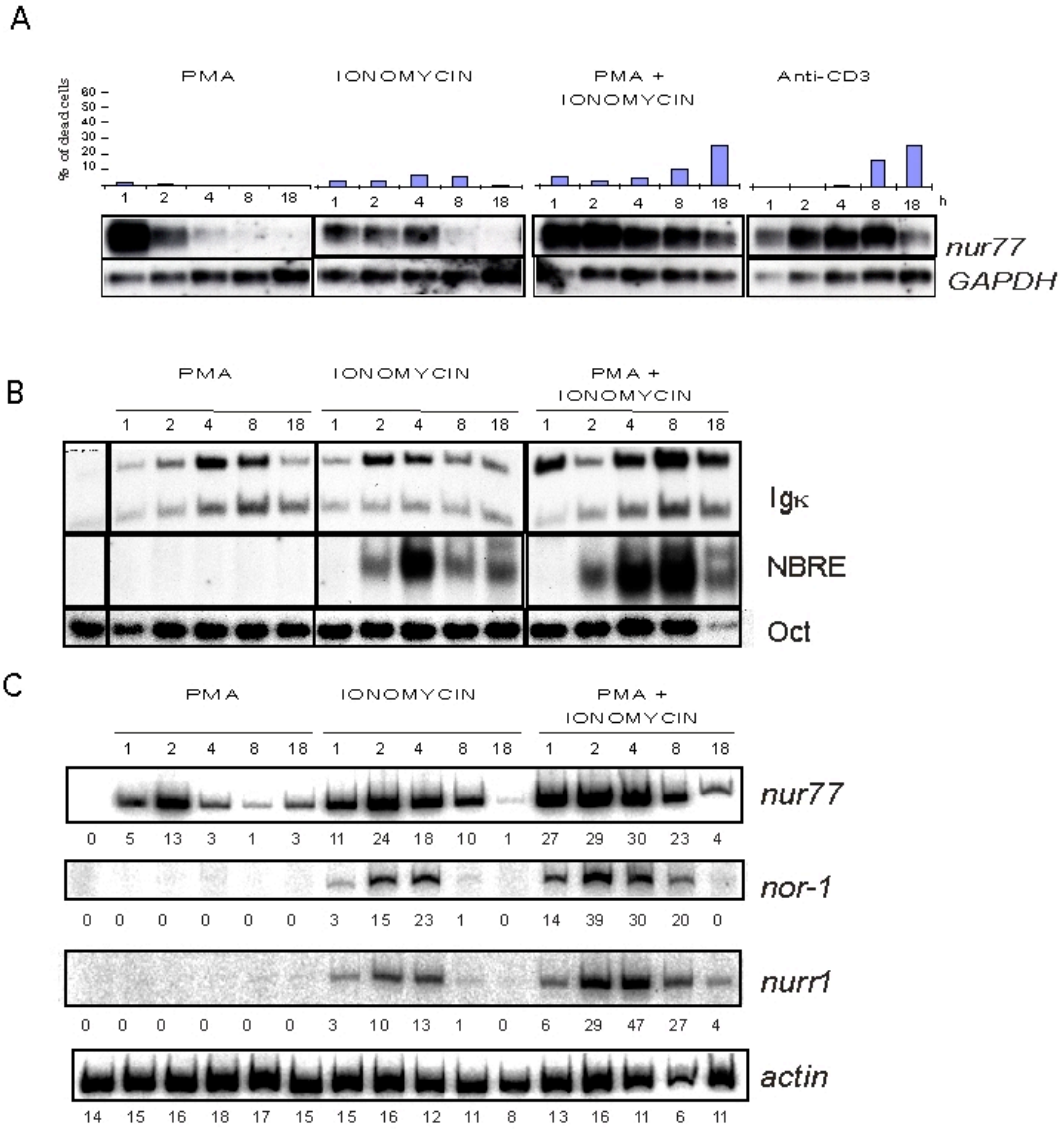


FIGURE 1. Differential regulation of *nur77* and its homologues by non-apoptotic and apoptotic stimuli in Do.11.10 hybridoma - predominant involvement of calcium signalling. (A) *Nur77* mRNA levels in cells stimulated with indicated reagents. Total RNA was subject to Northern blotting with *nur77* and *GAPDH* (loading control) specific probes. *Nur77* mRNA was undetectable in unstimulated Do.11.10 cells (B) Nur77 DNA binding under different stimulation conditions. Nuclear extracts were subject to EMSA assays with NBRE, Ig (NF-B specific) and Oct probes. (C) RT-PCR analysis of mRNA levels of *nur77* and homologues under different stimulation conditions. Numbers under bands represent Phosphoimager units.

Treatment with ionomycin, on the other hand, resulted in similar transient induction of all Nur77 family members. Combination of PMA and ionomycin increased mRNA levels of all Nur77 family members and induction was prolonged under these conditions. These results suggest that expression of Nur77 family members is regulated predominantly by calcium pathway. PKC on the other hand seems to have

two effects on Nur77 induction. It is capable of transiently inducing *nur77* mRNA expression and also enhances the effect of ionomycin at both mRNA and DNA-binding level.

3.1.2 Overexpression of Nur77 does not induce apoptosis in Do.11.10 cells.

Since Nur77 is capable of inducing apoptosis in immature thymocytes *in vivo* (Calnan *et al.*, 1995; Weih *et al.*, 1996) and high levels of sustained expression of Nur77 correlated with apoptosis in hybridomas it was hypothesised that Nur77 overexpression alone in transformed T cells could also result in apoptosis. Before addressing this question, an assay to measure Nur77 transcriptional potential had to be established. This was achieved by the generation of specific reporter gene constructs. Two Nur77 DNA response elements have been described in the literature – NBRE (Wilson *et al.*, 1991) and NuRE (Philips *et al.*, 1997a). NBRE binds Nur77 monomers whereas NuRE binds Nur77 homodimers. Three or four copies of either NBRE or NuRE encoding oligonucleotide multimers were subcloned in front of the minimal TK promoter placed into pGL3-Basic vector. Figure 2A shows schematically the structure of Nur77 reporter constructs. In order to check the ability of these reporter constructs to monitor Nur77 transcriptional activity 293 HEK cells were transfected with either empty vector or Nur77 encoding expression vector. All reporter constructs showed increased luciferase activity in response to Nur77 (Figure 2B and 2C). The construct containing four NuRE response elements showed highest inducibility (57-fold) and was chosen as standard Nur77 reporter gene in all subsequent experiments.

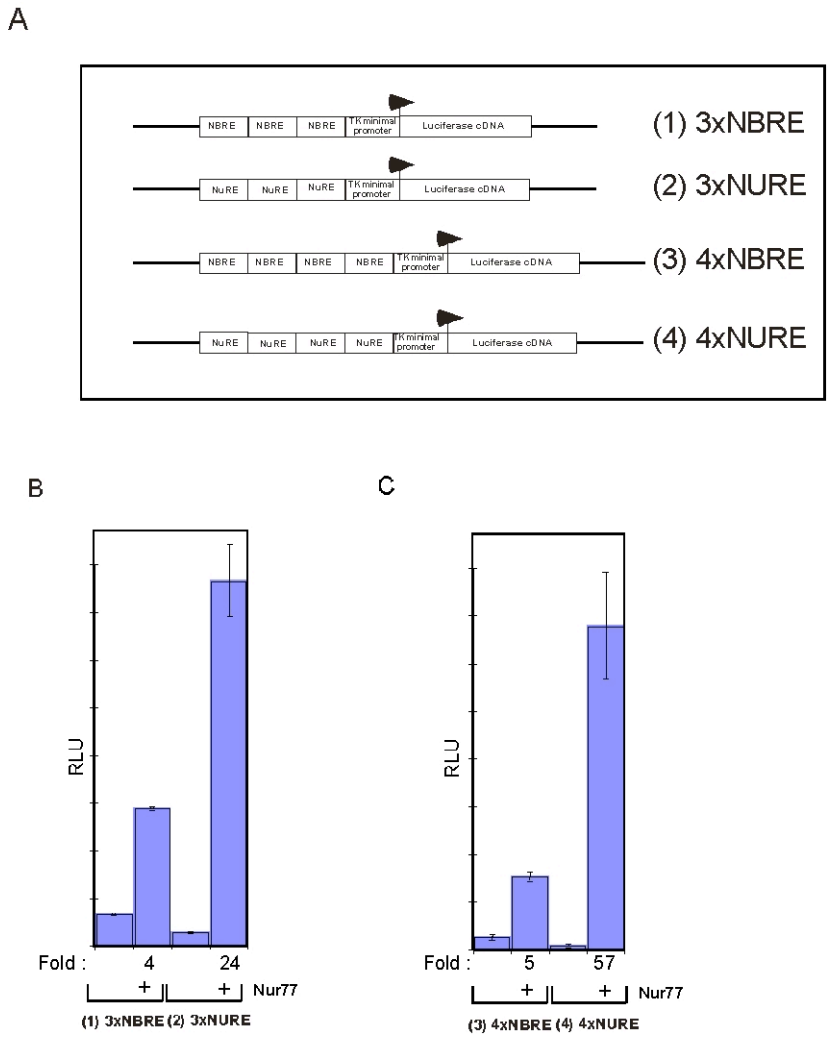


FIGURE 2. Construction of Nur77-dependent reporter gene system. (A) Schematic representation of reporter gene constructs. (B) and (C) reporter constructs were transiently transfected into 293 cells alone or in combination with Nur77 expression vector. Columns show normalised luciferase activity. Error bars indicate standard deviation from duplicate samples.

To test whether Nur77 overexpression alone is sufficient to induce apoptosis, cDNA encoding full-length Nur77 was transfected into Do.11.10 hybridoma cells. A GFP expressing plasmid was included to mark transfected cells. Between 10-20% of all cells were GFP positive in each transfection (Figure 3A). Annexin-V staining was used to distinguish apoptotic cells by flow cytometry. Anti-CD3 treatment resulted in a shift of cells from annexin-V negative to annexin-V positive window indicating efficient induction of apoptosis. Transfection of Nur77 alone, however, did not change the fraction of annexin-V positive cells (Figure 3A). Nur77 was transcriptionally active as shown in Nur77 reporter gene assays (Figure 3B). Nur77 was also not sufficient to trigger upregulation of FasL mRNA, a necessary step in

AICD of T cell hybridomas (Figure 3C). In summary, Nur77 overexpression alone seems not sufficient for induction of apoptosis in transformed Do.11.10 T cells.

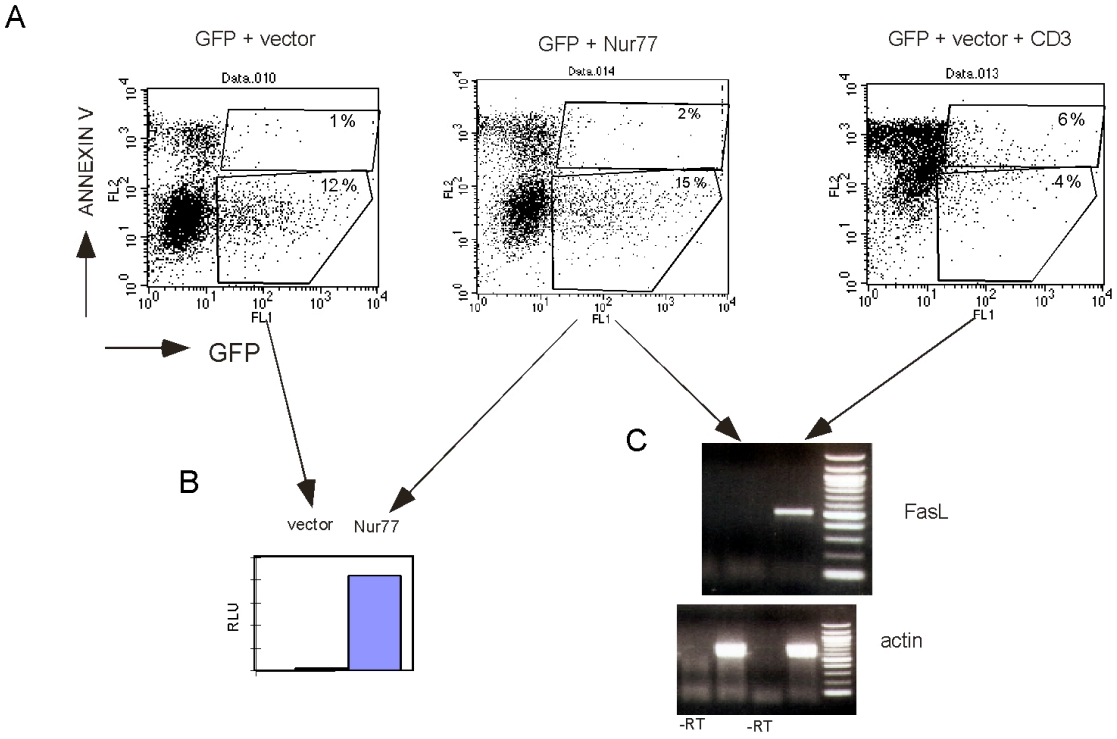


FIGURE 3. Overexpression of Nur77 is not sufficient to induce apoptosis in Do.11.10 cells. (A) Do.11.10 cells were transiently transfected with pEGFP plasmid and either empty vector or Nur77-expressing plasmid. Cells were left untreated or stimulated with □-CD3 Abs overnight. Cultures were stained with annexin V-PE and analysed by FACS. (B) Cells were transfected with Nur77 reporter gene and either vector or Nur77-expressing plasmid. After overnight incubation cells were lysed and luciferase activity was measured. (C) Total RNA from cultures shown in middle and right panel of (A) was extracted, cDNA was synthesised and RT-PCR reactions were performed to determine *FasL* and *actin* mRNA levels.

3.1.3 Nur77 nuclear function in Do.11.10 cells.

For many years since the original description of Nur77 involvement in TCR-mediated apoptosis (Liu *et al.*, 1994; Woronicz *et al.*, 1994) it was hypothesised that Nur77-mediated transcription is necessary for apoptosis induction. In the year 2000, however, a LNCaP prostate cell system was described in which Nur77 translocated to mitochondria and participated in apoptosis by non-genomic function (Li *et al.*, 2000). Thus, the critical question arose whether Nur77 is also a mitochondrial protein in T cells. In Do.11.10 hybridomas DNA binding of Nur77 was observed before and during induction of apoptosis (Figure 1B). This result suggests that Nur77 promotes apoptosis in this cell system by transcribing target genes or at least by some other nuclear function. To validate this hypothesis it was necessary to rule out the Nur77

translocation to mitochondria in TCR-stimulated Do.11.10 cells. Since there are no antibodies commercially available that are specific for individual Nur77 family members (except Nur77 specific mAb reactive in Western blots from Pharmingen) rabbit polyclonal sera directed against Nur77, Nor-1 and Nurr1 have been raised. The first 200 N-terminal amino acids from Nur77 and homologues have been chosen as source of antigen for immunisation of rabbits since they are not evolutionary conserved (Figure 4A). Polyclonal rabbit sera were first tested in EMSA assay with *in vitro* translated Nur77 homologues and NBRE oligo as a probe. Sera from rabbit 18 (anti-Nur77), 24 (anti-Nor-1), and 19 (anti-Nurr1) were capable of selectively abolishing the binding of cognate transcription factor to DNA (Figure 4B). Sera from rabbits 78 (anti-Nur77), 76 (anti-Nor1), and 75 (anti-Nurr1) did not affect the NBRE binding, indicating low titres (data not shown). Polyclonal sera were also tested in Western blots with *in vitro* translated Nur77 homologues. Sera from rabbit 18 (anti-Nur77), 24 (anti-Nor-1), and 19 (anti-Nurr1) were capable of selectively recognising cognate transcription factor with minimal cross-reactivity (Figure 4C). In addition antisera were tested in EMSA with extracts from thymocytes stimulated with PMA/ionomycin. After four hours of PMA/ionomycin treatment abundant NBRE binding was visible (Figure 4D). Pre-treatment with anti-Nur77 antiserum reduced the NBRE binding slightly, anti-Nor-1 antiserum markedly reduced NBRE binding whereas anti-Nurr1 antisera did not have any effect. This result suggests that NBRE binding activity in PMA plus ionomycin stimulated thymocytes is composed of Nor-1 and minor amounts of Nur77, which is agreement with, published report (Cheng *et al.*, 1997). Taken together these data demonstrate successful generation of rabbit polyclonal antisera directed against individual Nur77 family members which show high degree of specificity and can be used for EMSA, Western blotting and immunofluorescence studies (see below).

In order to reveal subcellular localisation of endogenous Nur77, cytoplasmic and nuclear extracts from Do.11.10 cells stimulated with □□CD3 Abs were subject to Western blotting with anti-Nur77 antibodies. The cJun protein was chosen as marker of nuclear fraction and an unspecific band cross-reacting with anti-cJun antibody, which was present exclusively in cytoplasmic fraction demonstrated the purity of preparations.

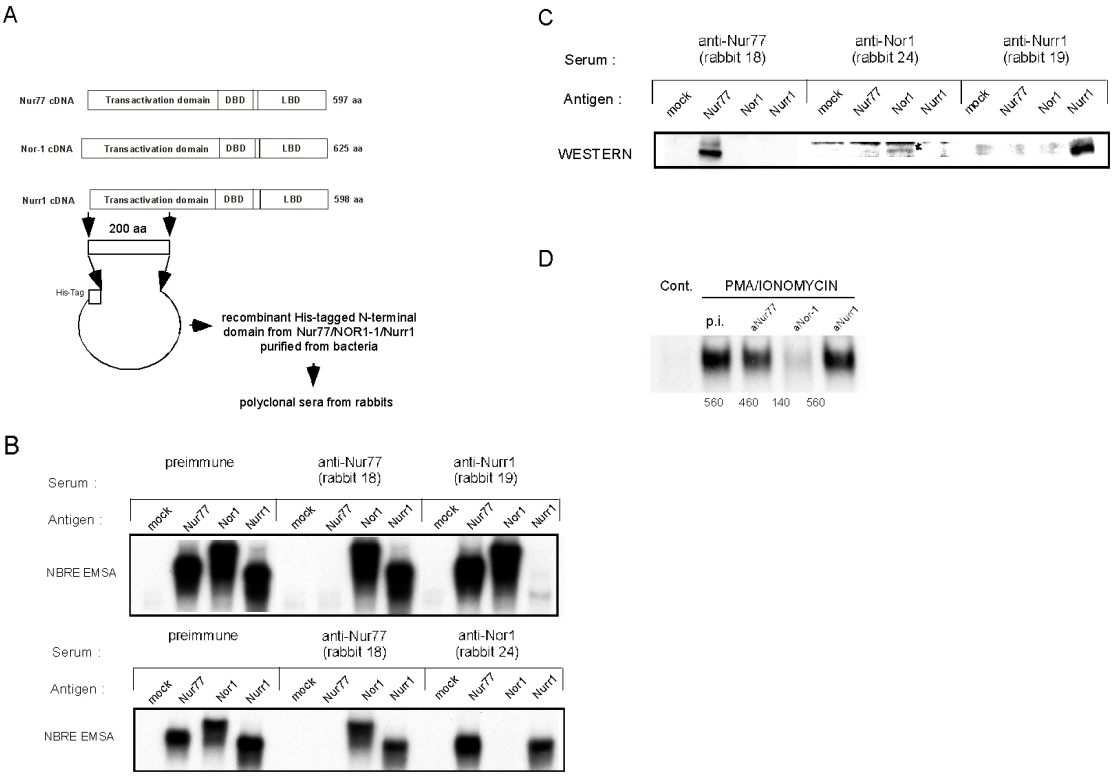


FIGURE 4. Polyclonal antibodies directed against N-terminal domains of Nur77 family transcription factors show high degree of specificity in EMSA and Western assays. (A) Strategy to generate anti-Nur77, anti-Nor-1 and anti-Nurr1 antibodies. (B) EMSA test of antibody specificity. *In vitro* translated Nur77 homologues were used for EMSA with NBRE oligo in the presence of preimmune serum or anti-Nur77, anti-Nor-1 and anti-Nurr1 antibodies. (C) Western blot test of antibody specificity. *In vitro* translated Nur77 homologues were resolved on SDS-PAGE, blotted and probed with indicated antisera. (D) Nuclear extracts from thymocytes unstimulated (Cont.) or treated with PMA/ionomycin for 4 hr were used for EMSA with NBRE oligo in the presence of preimmune serum (p.i.) or anti-Nur77, anti-Nor-1 and anti-Nurr1 antibodies. Numbers indicate Phosphoimager quantification of NBRE complexes.

Nur77 signal was detected only in nuclear fractions at all time points tested (Figure 5A). Under these conditions, the mitochondrial cytochrome c protein localised exclusively in cytoplasmic fractions, arguing for lack of translocation of Nur77 to mitochondria in T cell hybridomas (Figure 5B). In order to confirm the biochemical data on Nur77 subcellular localisation, Nur77 distribution pattern was studied by immunofluorescence. Nur77 was detected only in TCR-stimulated cells and perfectly overlapped with DAPI nuclear stain (Figure 5C). Taken together, these data demonstrate that endogenous Nur77 protein localises to the nucleus of TCR-stimulated T cell hybridomas. Although it cannot be ruled out that a minor Nur77 fraction localised to mitochondria these results clearly argue against a quantitative translocation of Nur77 to mitochondria downstream of TCR.

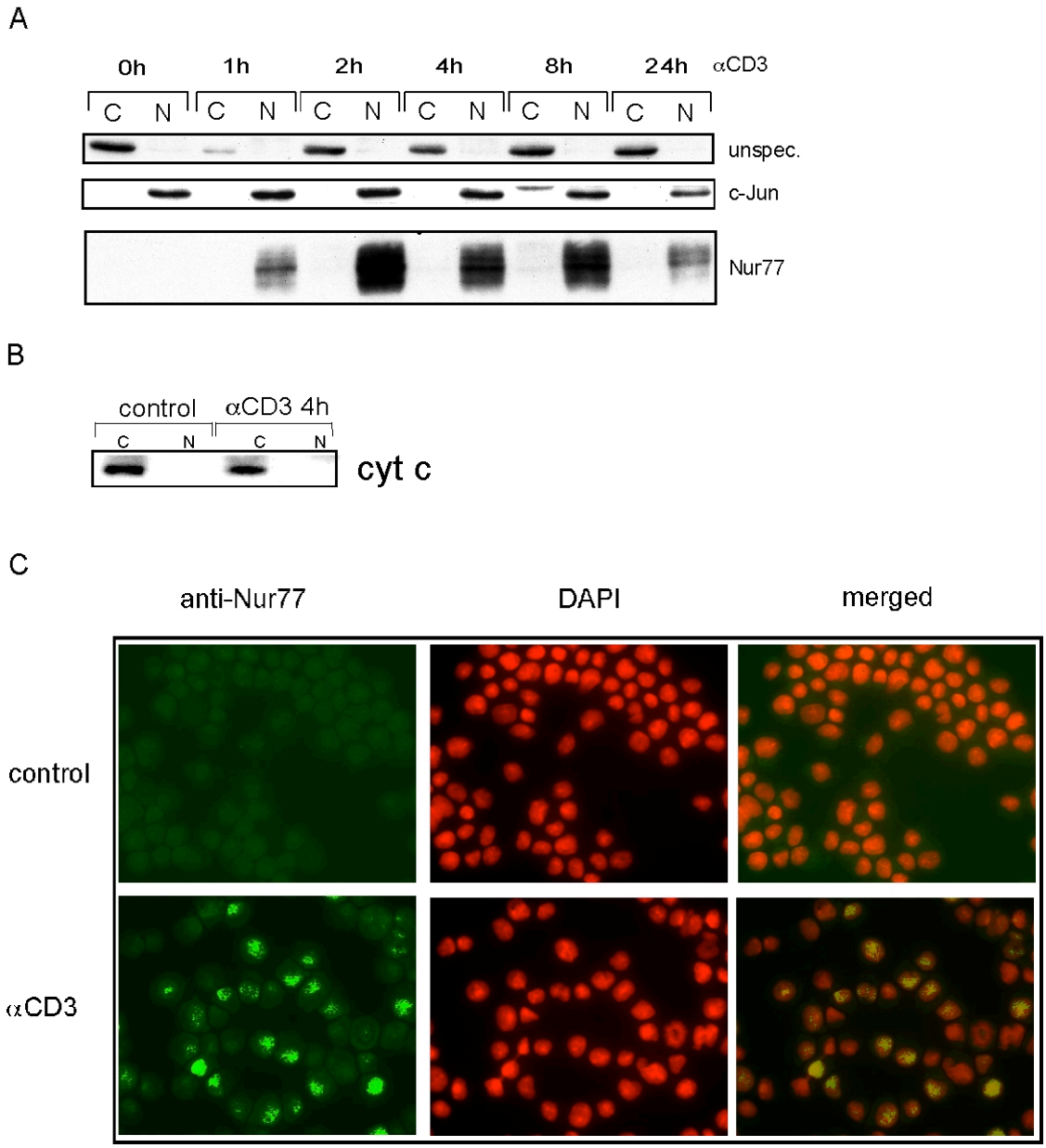


FIGURE 5. Nur77 induced by TCR signals in Do.11.10 cells localises to the nucleus.
(A) Levels of Nur77 in cytoplasmic (C) and nuclear (N) extracts of Do.10.11 cells stimulated with anti-CD3 Abs were determined by Western blot. Membrane was re-probed with anti-cJun antibody to demonstrate purity of nuclear fractions. Unspecific band cross-reacting with anti-cJun antibodies was used as cytoplasmic marker (unspec.) (B) Samples as in (A) were probed with anti-cytochrome c antibodies. Only 0 and 4hr time points are shown. (C) Analysis of Nur77 subcellular localisation by immunofluorescence. Cells cultured in the absence or presence of anti-CD3 Abs for 4 hr, spun on glass slides, fixed and probed with anti-Nur77 followed by anti-rabbit-FITC secondary antibodies. DAPI was used to visualise nucleus and is shown in red.

It was previously reported that Nur77 mitochondrial action could be blocked by the anti-apoptotic Bcl2 protein. Since in T cell hybridomas Nur77 localises to nucleus it was important to check the influence of Bcl2 on AICD. Retroviruses transducing empty vector or human Bcl2 cDNA were generated and used to infect Do.11.10 cells.

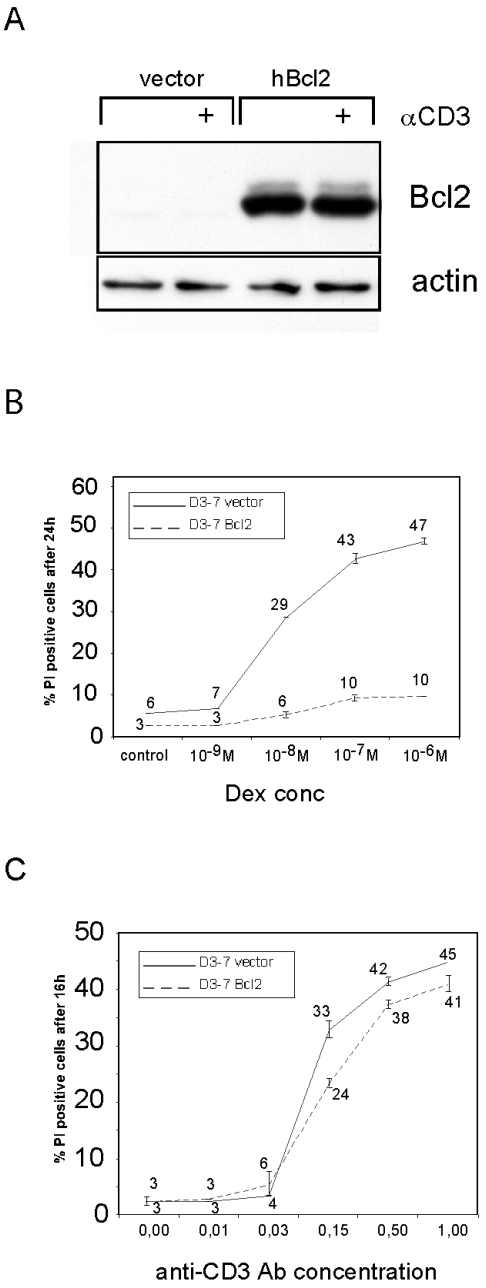


FIGURE 6. Overexpression of Bcl2 protein in Do.11.10 blocks Dex but not anti-CD3-induced apoptosis. (A) Bcl2 levels were determined by Western blotting of whole cell extracts from control and Bcl2-transfected pools. Actin signal demonstrates equal loading. (B) Do.10.11 control or Bcl2-expressing pools were incubated with indicated dexamethasone concentration for 24 hr. After incubation propidium iodide was added to the cultures and percentage of dead cells was determined by flow cytometry. (C) Do.10.11 control or Bcl2-expressing pools were incubated with indicated anti-CD3 Abs concentrations for 16 hr. Cell death was measures as in (B).

After primary infection cells were selected as pools, expanded and whole cell extracts were used for Western blotting with anti-Bcl2 antibodies. Vector-transduced Do.11.10 cells showed very low Bcl2 expression (faint band visible on longer exposure). Bcl2-transduced cells showed strong signal demonstrating high Bcl2 overexpression (Figure 6A). The levels of endogenous and transgenic Bcl2 protein did not change

upon TCR stimulation. Since it was reported that dexamethasone induces apoptosis in T cell hybridomas and this form of PCD is Bcl2 sensitive (Ivanov *et al.*, 1997) control and Bcl2-expressing hybridoma pools were tested for the sensitivity to Dex. As expected, overexpression of Bcl2 rendered Do.11.10 completely Dex resistant (Figure 6B). The sensitivity to AICD, however, remained almost unchanged (Figure 6C). These results demonstrate that TCR-mediated apoptosis of T cell hybridomas is not Bcl2 sensitive and together with the Nur77 localisation experiment argue against Nur77-mediated cytochrome c release at the level of mitochondria as a major mechanism of cell death induction.

3.1.4 Characterisation of novel Nur77 target genes.

The finding that Nur77 transcription factor localises to the nucleus in TCR-stimulated Do.11.10 cells opened the way to address one of the most interesting issues regarding the function of Nur77 - the nature of target genes. In order to characterise Nur77 target genes several independent strategies were employed.

3.1.4.1 Using transgenic mice overexpressing dominant-negative Nur77 in T cells to clone Nur77 target genes – a loss-of-function approach.

The first strategy relied on the usage of previously created transgenic mice strain overexpressing DN-Nur77 (dominant negative Nur77) protein in T cells (Weih F. - unpublished). Thymocytes from similar transgenic mouse strains were recently reported to be partially resistant to apoptosis induced by *in vivo* administered anti-CD3 Abs (Zhou *et al.*, 1996). The reason for this could be the failure of anti-CD3 Abs to fully induce Nur77 target genes in transgenic thymocytes and therefore mRNA population from transgenic thymocytes induced through CD3 could differ from mRNA population from wild-type thymocytes after stimulation. This could be employed to identify target genes of Nur77 (i.e. genes not inducible by CD3 in transgenic thymocytes) via profiling of differential mRNA expression (e.g. DNA microarrays).

Overexpression of DN-Nur77, however, does not result in a strong phenotype. Defects in negative selection were reported in such transgenic mice but this did not translate into autoimmunity (Calnan *et al.*, 1995). In contrast, overexpression of full length Nur77 in immature thymocytes of transgenic mice (FL-Nur77 line) results in massive apoptosis (Weih *et al.*, 1996). These mice have very small thymus and lack

mature T cells in the periphery. DN-Nur77 abolishes Nur77 transcriptional activity in transfected cells and rescues from TCR-mediated apoptosis (Woronicz *et al.*, 1994). To establish the functionality of the DN-Nur77 transgene, DN-Nur77 tg mice were crossed with FL-Nur77 transgenic mice and the phenotype of double-transgenic animals was analysed. Thymocytes and splenocytes from DN-Nur77, FL-Nur77 and DN-Nur77/FL-Nur77 mice were checked for CD4/CD8 expression by flow cytometry. In addition, thymus cellularity was measured. DN-Nur77 transgenic mice had normal thymus size and CD4/CD8 profile in both the thymus and the periphery (Figure 7B, left panels).

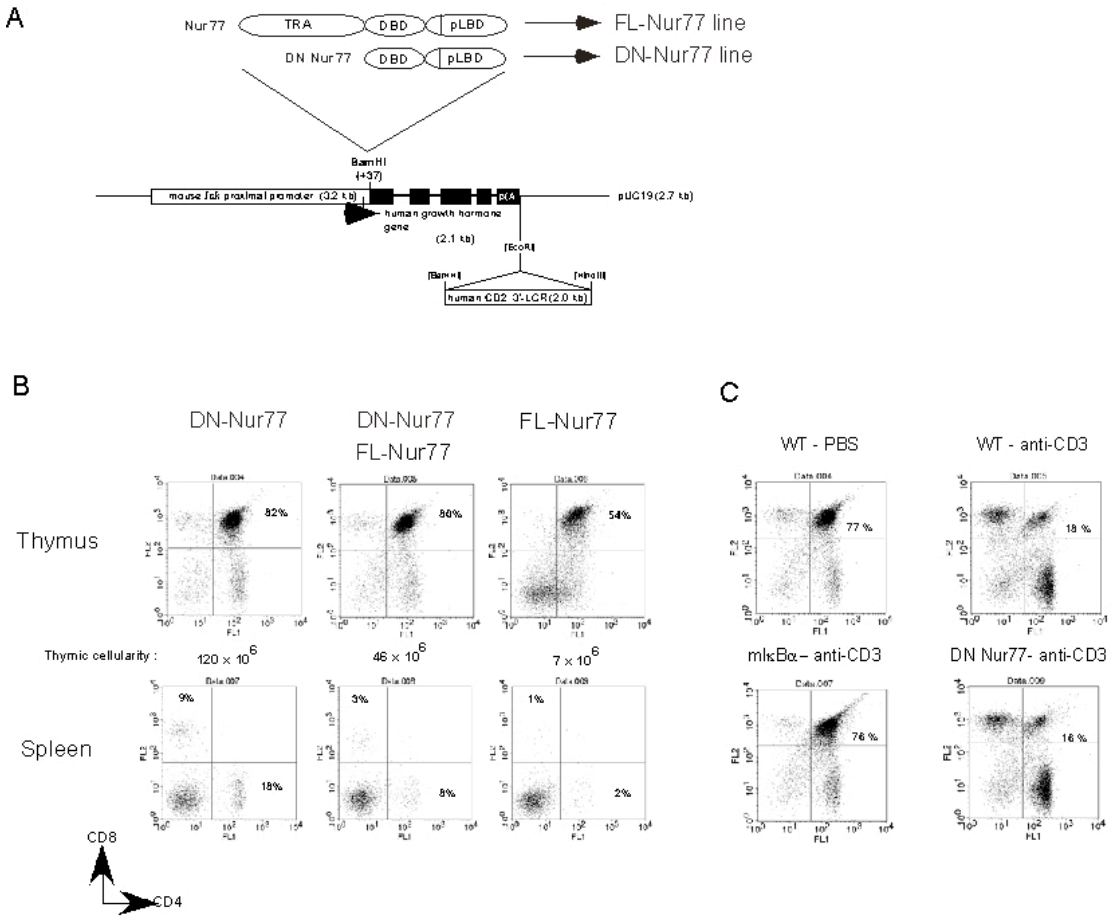


FIGURE 7. DN-Nur77 transgene partially rescues phenotype of Nur77 transgenic (FL-Nur77) mice. (A) Schematic representation of DNA constructs used to generate transgenic lines. (B) FACS CD4/CD8 T cell profiles in thymus and spleen of indicated transgenic mice (percentage of DP or SP T cells is indicated respectively). Cellularity of the thymus is indicated. (C) DN-Nur77 transgene does not protect DP thymocytes from apoptosis induced by anti-CD3 Abs. Mice of indicated genotype were injected i.v. with anti-CD3 Abs. CD4/CD8 thymocyte profiles were analysed 36 hr post injection by FACS as in (B). mI□B□ is a transgenic mice line overexpressing mI□B□ transgene in T-cells.

In contrast, FL-Nur77 animals had greatly reduced cellularity in thymus, few DP cells and virtually no mature T cells (Figure 7B, right panels). Double transgenic animals

showed almost normal CD4/CD8 thymocyte profile with only slightly reduced cellularity and reduced but present mature T cells in the periphery (Figure 7B, middle panels). Thus, DN-Nur77 transgene is able to antagonise the activity of full length Nur77. In order to establish whether DN-Nur77 transgene protects against apoptosis induced by TCR, mice were injected with αCD3 Abs. Wild-type and homozygous DN-Nur77 mice were injected with either PBS or αCD3 Ab. Thirty-six hours after injection thymi were removed and thymocytes were analysed for CD4/CD8 staining by flow cytometry. Surprisingly, DN-Nur77 transgenic thymocytes were as sensitive as wilt-type to αCD3 administration (Figure 7C). Recently, overexpression of superinhibitory IκBα (mIκBα) molecule in T cells was shown to rescue thymocytes from TCR-mediated deletion (Hettmann *et al.*, 1999). To demonstrate that the αCD3 antibody was not overdosed, mIkBa-transgenic mice were injected with αCD3. Indeed, the mIκBα transgene protected against anti-CD3 Abs induced thymocyte deletion (Figure 7C, lower left dotplot). Since the DN-Nur77 transgene did not protect from apoptosis induced by αCD3 administration *in vivo* the usage of DN-Nur77 mice to clone TCR-induced Nur77 targets seems not feasible.

3.1.4.2 Creating conditional Nur77 expression systems based on EBD/GBD fusion proteins to clone Nur77 target genes – a gain-of-function approach.

The second strategy employed to identify Nur77 target genes relied on the application of inducible expression systems, in which activity of a transcription factor of interest can be selectively induced or inhibited. Such systems can serve as a means of characterisation of target genes by direct comparison of mRNA profiles before and after activation/inactivation of transcription factor under study.

One of the established strategies to generate an inducible activation system is to fuse the gene of interest to the ligand-binding domain (LBD) from estrogen (EBD) or glucocorticoid receptors (GBD) (Brocard *et al.*, 1998; Eilers *et al.*, 1989). The resulting fusion protein is inactive in the absence of dedicated ligand (estrogens for EBD; glucocorticoids for GBD). Addition of the hydrophobic ligand results in the release of chimeric protein from a complex with heat shock proteins unmasking the activity of the protein fused to LBD. The major drawback of this approach is that fusion of heterologous proteins to EBD/GBD frequently results in a chimeric protein,

which retains only partial activity. High expression levels may compensate this loss but to reach high expression it is important to reduce the size of chimeric protein to minimum. The rationale therefore was to use minimal Nur77, retaining full transactivation potential on both types of DNA response elements. Domains necessary for Nur77 transcriptional activity have been previously mapped and Nur77 C-terminal putative LBD domain was found unnecessary for transactivation of NBRE-based reporter gene (Paulsen *et al.*, 1992)(Davis *et al.*, 1993). These data were, however, obtained before the discovery that Nur77 can also bind to DNA as a homodimer (Philips *et al.*, 1997a). It was therefore important to determine whether the putative LBD was necessary for Nur77 transactivation from homodimer response element (NuRE). Nur77 and its C-terminal truncation mutant ($Nur77^{400}$) were tested for their ability to induce the transcription of NuRE based Nur77 reporter gene in transiently transfected 293 cells. $Nur77^{400}$ mutant showed moderately increased transcriptional potential compared to full-length Nur77 (Figure 8B). These results demonstrate that deletion of 200 amino acids from C-terminus does not hamper Nur77 homodimerization and transcriptional activation.

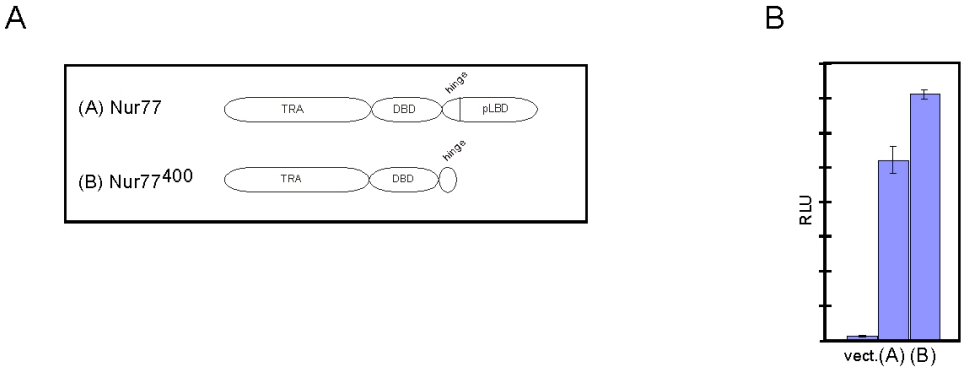
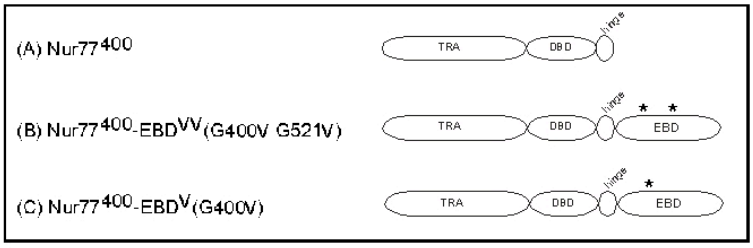


FIGURE 8. The putative LBD of Nur77 is not required for transactivation and homodimer formation. (A) Schematic representation of Nur77 constructs. (B) 293 cells were transiently transfected with Nur77 reporter gene and either vector or constructs encoding Nur77 as shown schematically in (A). Luciferase activity in cell lysates is plotted (RLU). Error bars in the graph indicate standard deviation from duplicate samples.

In order to generate conditional Nur77 chimeric proteins, Nur77 or $Nur77^{400}$ mutant were fused to the N-terminus of two different mutated EBDs (estrogen binding domains) from human estrogen receptor – EBD^{V} and EBD^{VV}. EBD^{V} contained one point mutation (G400V) resulting in reduced affinity for a natural estrogen - estradiol. EBD^{VV} contained two point mutations (G400V and G521V) resulting in selective loss

of the ability to bind estradiol but not synthetic estrogen antagonists or agonists. These EBD mutants were chosen since EBD^{V} harboured only one mutation affecting the EBD function to a minimal extent. EBD^{VV}, on the other hand, can be induced by a large variety of synthetic estrogen antagonists and agonists remaining unresponsive to natural ligands, which is critical for application *in vivo*. The resulting fusion proteins (Figure 9A shows schematically their structures) were transiently transfected into Jurkat T cells, 293 HEK cells and Do.11.10 cells together with a Nur77 reporter gene. After overnight incubation different EBD ligands were added for additional 24 h. After that time cells were harvested and luciferase assays were performed to determine Nur77 transcriptional activity. Nur77 full length fused to EBDs did not show significant transcriptional activity in response to any of the EBD ligands (not shown). $Nur77^{400}$ fusion proteins, however, demonstrated very effective ligand-dependent transcriptional activation in Jurkat T cells (Figure 9B, upper panel). In 293 cells the extent of Nur77 activation by EBD ligands was clearly diminished (Figure 9B, middle panel). In Do.11.10 cells fusion proteins did not show significant activation by EBD ligands (Figure 9B, lower panel). These results demonstrate generation of $Nur77^{400}$-EBD^{VV} conditional allele, which responds to synthetic estrogens and antiestrogens but in a cell type-specific manner. The $Nur77^{400}$-EBD^{VV} fusion proteins responded particularly well to hexestrol in Jurkat T-cells.

A



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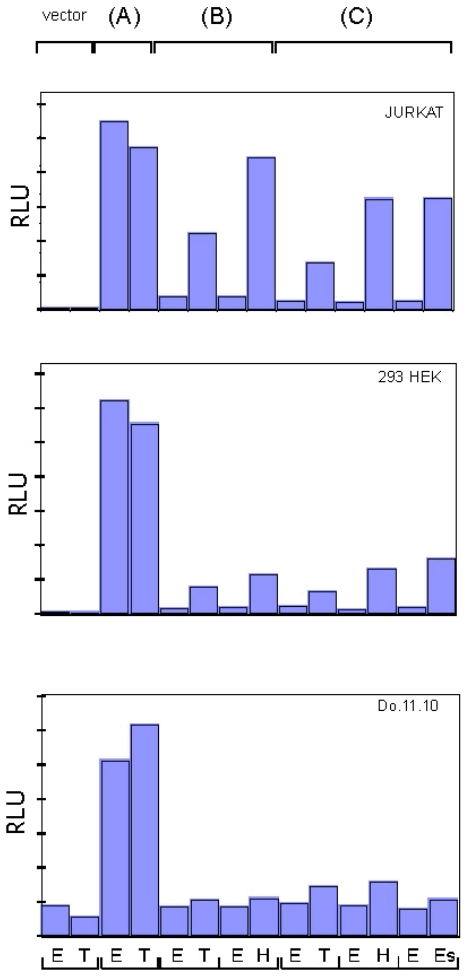


FIGURE 9. Nur77^{400}-EBD fusion proteins direct ligand-dependent Nur77 transcriptional activity in a cell type-specific manner. (A) Schematic representation of Nur77/EBD fusion protein constructs. (B) Indicated cell types were transiently transfected with Nur77 reporter gene and either vector or Nur77/EBD encoding constructs as shown in (A). Each transfection was split into halves and after overnight incubation EBD ligands were added: E - ethanol vehicle control, T - 4-hydroxytamoxifen, H - hexestrol, Es - □-estradiol. After additional 24 hr of incubation cells were lysed and luciferase activity was determined (RLU).

Since Nur77^{400}-EBD/EBD^{V} fusion proteins worked only in one cell type it was important to search for Nur77 activation strategy, which would show a broader applicability. Nur77 or Nur77^{400} mutant were therefore fused to GBD (glucocorticoid ligand binding domain from human glucocorticoid receptor). This GBD contained the I747T point mutation leading to the loss of ability to bind natural glucocorticoids (e.g. cortisol) but retaining responsiveness to synthetic glucocorticoid - dexamethasone. The resulting fusion proteins (Figure 10A shows schematically their structures) were transiently transfected into Jurkat T cells and 293 HEK cells together with Nur77 reporter gene. After an overnight incubation the synthetic agonist dexamethasone (Dex) was added for additional 24 hr. After that time cells were harvested and luciferase assays were performed to determine Nur77 transcriptional activity. Both Nur77-GBD and Nur77^{400}-GBD showed Dex-dependent Nur77 transcriptional activity in both cell types (Figure 10B). In addition, basal activity of GBD fusion proteins was much lower than in the case of EBD fusion proteins (compare Figure 9B with Figure 10B, cultures treated with vehicle). In order to create a Dex-dependent *in vitro* Nur77 activation system Nur77^{400}-GBD chimeric protein was stably transfected into 293 cells. Nur77^{400}-GBD chimeric protein was chosen for stable transfection since it had smaller molecular weight (compared to Nur77-GBD), which should facilitate higher expression levels. Resulting cell pools were transiently transfected with Nur77 reporter gene and subsequently treated with Dex. The Nur77^{400}-GBD expressing cell pool showed Dex-dependent Nur77 transcriptional activity with basal level of transcription matching the control pool (Figure 10C). This result documents the creation of a conditional system for Nur77 expression, which is strictly Dex dependent. The ultimate goal was, however, to achieve the conditional Nur77 expression in T cells. This was unsuccessful since both Nur77^{400}-EBD^{VV} and Nur77^{400}-GBD fusions could not be stably expressed in Jurkat T cells and Nur77^{400}-EBD^{VV} was inactive in Do.11.10. In addition, Nur77-GBD fusions could not be tested in Do.11.10 cells since Dex induced apoptosis in these cells (see Figure 6B).

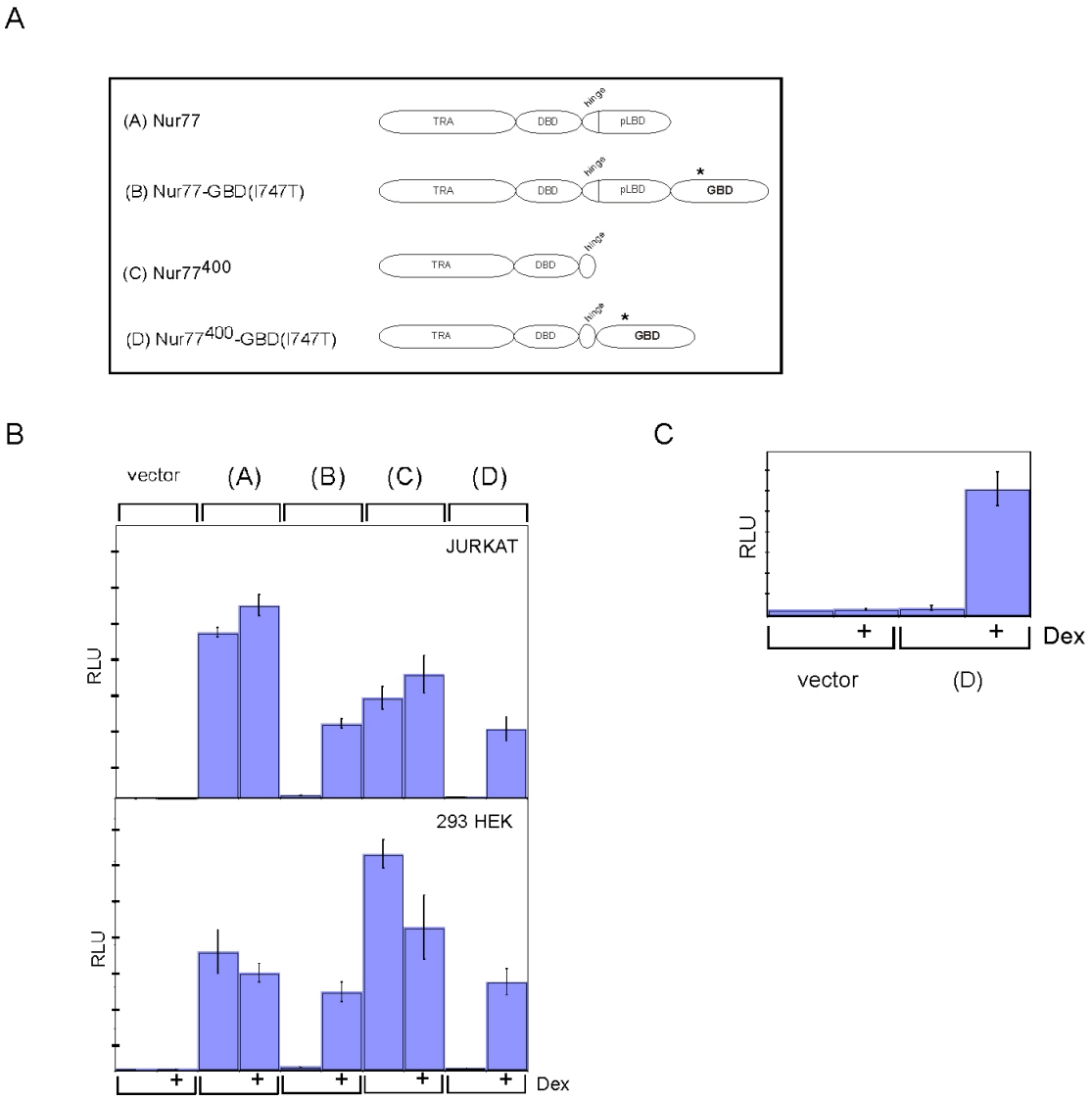


FIGURE 10. Establishment of Dex-dependent Nur77 conditional activation system. (A) Schematic representation of DNA constructs encoding Nur77/GBD fusion proteins. (B) Indicated cell types were transiently transfected with Nur77 reporter gene and either vector or Nur77/GBD fusions depicted in (A). Each transfection was split in halves and after overnight incubation vehicle (ethanol) or dexamethasone (+) was added. After additional 24 hr of incubation cells were lysed and luciferase activity was measured (RLU). (C) 293 cells were stably transfected with either empty vector or Nur77400-GBD fusion protein. Cell pools were transiently transfected with Nur77 reporter gene and treated with Dex as described for (B). Error bars represent standard deviation from duplicate samples.

The phenotype of Nur77 transgenic mice is very dramatic. These animals show severe thymus atrophy and lack mature T cells in the periphery. It is, however, difficult to determine at which step of T cell differentiation Nur77 acts. In order to generate a more suitable model to study Nur77 function in developing T cells conditional expression of Nur77 in the thymus was attempted. A cDNA encoding Nur77^{400}-EBD^{VV} or Nur77^{400}-GBD chimeric proteins were subcloned in the pTLC expression

vector (Figure 11A). This vector has been previously shown to direct copy number dependent and integration site independent expression of the transgene in T cells (Vallabhapurapu *et al.*, 2001). The resulting constructs were used to generate transgenic mice.

Three founder lines (screened by PCR of tail DNA) were obtained (not shown) with pTLC-Nur77^{400}-EBD^{V} construct. Line 7 had low copy, whereas lines 8 and 15 had high transgene copy numbers integrated as judged by transgene-specific PCR in relation to an endogenous reference gene (not shown). Only line 8, however, showed normal germ line transmission (17 transgenic pups out of 25 born pups). Line 7 showed infrequent germ line transmission (1/20) and line 15 no germ line transmission at all (0/36). It was therefore so far only possible to analyse mice from line 8. In order to demonstrate transgene expression, thymocytes from non-transgenic and transgenic littermates from line 8 were isolated and whole cell extracts were subject to SDS-PAGE followed by Western blotting with anti-Nur77 specific monoclonal antibodies. Expression of Nur77^{400}-EBD^{VV} was detected only in transgenic thymocytes (Figure 11B). Levels of transgenic fusion protein were significantly lower than endogenous Nur77 protein. This could only result in a mild phenotype, if any, since it was noticed before that only high Nur77 expressors produced a clear phenotype (Dr Falk Weih – personal communication). Analysis of thymus cellularity and CD4/CD8 thymocyte profiles from non-transgenic and transgenic mice did not reveal any differences, indicating that the transgene did not influence T cell development in the absence of EBD^{VV} ligands (data not shown). To establish whether Nur77^{400}-EBD^{VV} transgenic mice have a ligand inducible phenotype, groups of control and age matched transgenic mice mice, were injected with estrogen receptor antagonist - tamoxifen. It was expected that injection of tamoxifen resulted in activation of latent Nur77 activity in transgenic thymocytes, leading to the induction of apoptosis in immature thymocytes and establishing a characteristic phenotype with a marked reduction in the of DP thymocytes population.

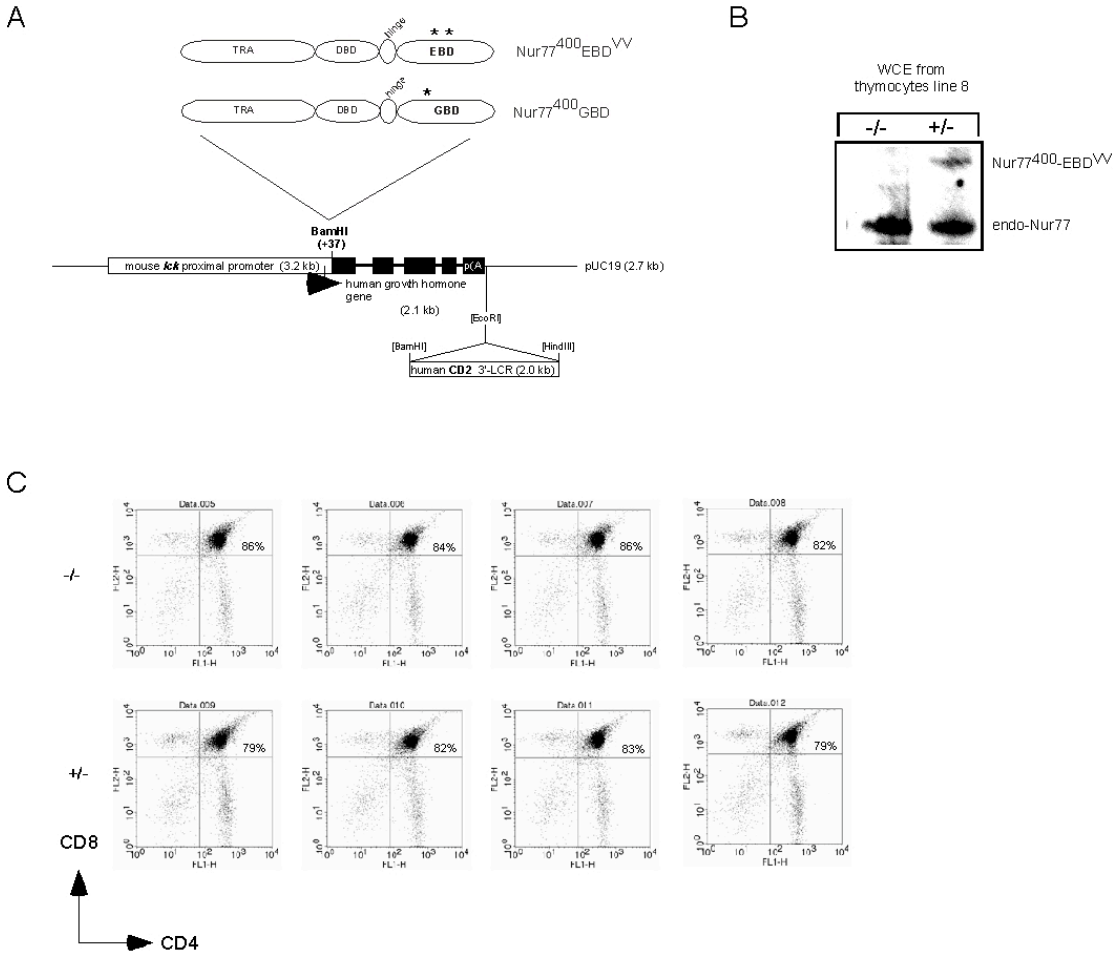


FIGURE 11. Generation and analysis of transgenic mice expressing Nur77^{400}-EBD^{VV} fusion protein in thymocytes. (A) Schematic representation of transgene constructs used to generate transgenic mouse lines. (B) Expression of Nur77^{400}-EBD^{VV} fusion protein in WCE from transgenic thymocytes was determined with with anti-Nur77 monoclonal antibodies. (C) Four control (-/-) and four transgenic (-/+) mice from line 8 were injected with 4-hydroxytamoxifen. CD4/CD8 thymocyte profiles were analysed by FACS. Percentage of DP immature thymocytes is indicated.

Thymocyte suspensions from injected mice were stained with αCD4/αCD8 monoclonal Abs and analysed by flow cytometry. The average percentage of DP cells in control (non-transgenic) animals was 85% (+/- 2%) (Figure 11C, top). In transgenic thymocytes the average percentage of DP cells was 81% (+/- 2%) (Figure 11C, bottom). This result suggests that *in vivo* activation of Nur77^{400}-EBD^{VV} fusion protein results in deletion of some DP cells but the extent of deletion was very low, indicating that the strategy to establish a transgenic model to study Nur77 function by using the Nur77^{400}-EBD^{VV} conditional system could work but transgenic lines expressing significantly higher levels of the chimeric protein have to be generated.

With pTLC-Nur77^{400}-GBD construct five founder lines (screened by PCR of tail DNA) were obtained (not shown). However, Dex induces apoptosis in immature

thymocytes (Reichardt *et al.*, 1998). To be able to use Dex as ligand to activate $Nur77^{400}$-GBD fusions in thymocytes an advantage will be taken from the glucocorticoid receptor (GR) mutant mice. This mouse strain bears a point mutation in DNA-binding domain of GR (GR^{dim} mutation), which results in lack of the DNA-binding of GR. These mice are viable but thymocytes show resistance to Dex-mediated apoptosis (Reichardt *et al.*, 1998). Therefore $Nur77^{400}$-GBD tg mice have to first be bred on the $GR^{dim/dim}$ genetic background before Dex injections *in vivo* could be performed to look for inducible phenotype.

3.1.4.3 Conditional expression of Nur77 *in vitro* in the Tet-Off system.

Since the attempts to generate conditional Nur77 alleles with EBD/GBD fusion proteins resulted only in partial success, the Tet-Off system (Clontech) of conditional expression has been used to obtain regulated Nur77 expression in T cells. Tet-Off system relies on the usage of artificial bacterial regulatory proteins (e.g. TetR-VP16), which respond to teteracycline derivatives. The gene of interest is placed under the control of CMV minimal promoter and multimerised tet-operator sequences. This artificial promoter is activated by TetR-VP16 fusion protein. Addition of tetracycline (or its derivative doxycycline; Dox) to the culture medium and binding to the TetR-VP16 protein induces allosteric change so that it loses the ability bind operator sequences and transcription stops. Removal of tetracycline/Dox results in restart of transcription (Gossen and Bujard, 1992).

A full length Nur77 cDNA was subcloned in pTRE2pur vector under the control of tet-operator sequences and resulting plasmid was electroporated into Tet-Off Jurkat T cells (contain stably integrated plasmid expressing TetR-VP16 fusion protein, Clontech). Several independent puromycin resistant clones were selected, expanded and checked for Dox-dependent expression of Nur77 protein. Two clones (J17-9, J17-8) showed dramatic Nur77 overexpression upon Dox removal and two other clones (J17-3, J17-1, J17-6) low levels of Nur77 expression (Figure 12A). Majority of tested clones did not show any Nur77 expression. Clone J17-8 showed the lowest basal level and highest inducible levels (best regulatory window) and was selected for further work. In order to determine Nur77 expression kinetics and cellular localisation in this system cells were cultured in the absence of Dox and nuclear and cytoplasmic fractions were subject to Western blotting with anti-Nur77 Abs. Anti-cJun Abs were

used to demonstrate purity of nuclear fractions. Nur77 expression reached plateau 10 hours after Dox removal and at all time points Nur77 signal was visible only in the nuclear fraction (Figure 12B). Nur77 nuclear localisation was also demonstrated by immunofluorescence (Figure 12C). To show that overexpressed Nur77 is transcriptionally active J17-8 clone was transfected with Nur77 reporter gene and plated in the presence and absence of Dox. Cells were lysed after 48 hours and luciferase assays were performed to measure Nur77 activity. In the absence of Dox 12-fold increase in luciferase activity was measured (not shown). These data demonstrate creation of a cell culture system of conditional Nur77 expression in Jurkat T cells. In this cell system, Nur77 is highly overexpressed and localises to nucleus where it is transcriptionally competent. It is important to notice, however, that Nur77 activation did not lead to any phenotypic consequences as far as cell viability and growth are concerned (not shown).

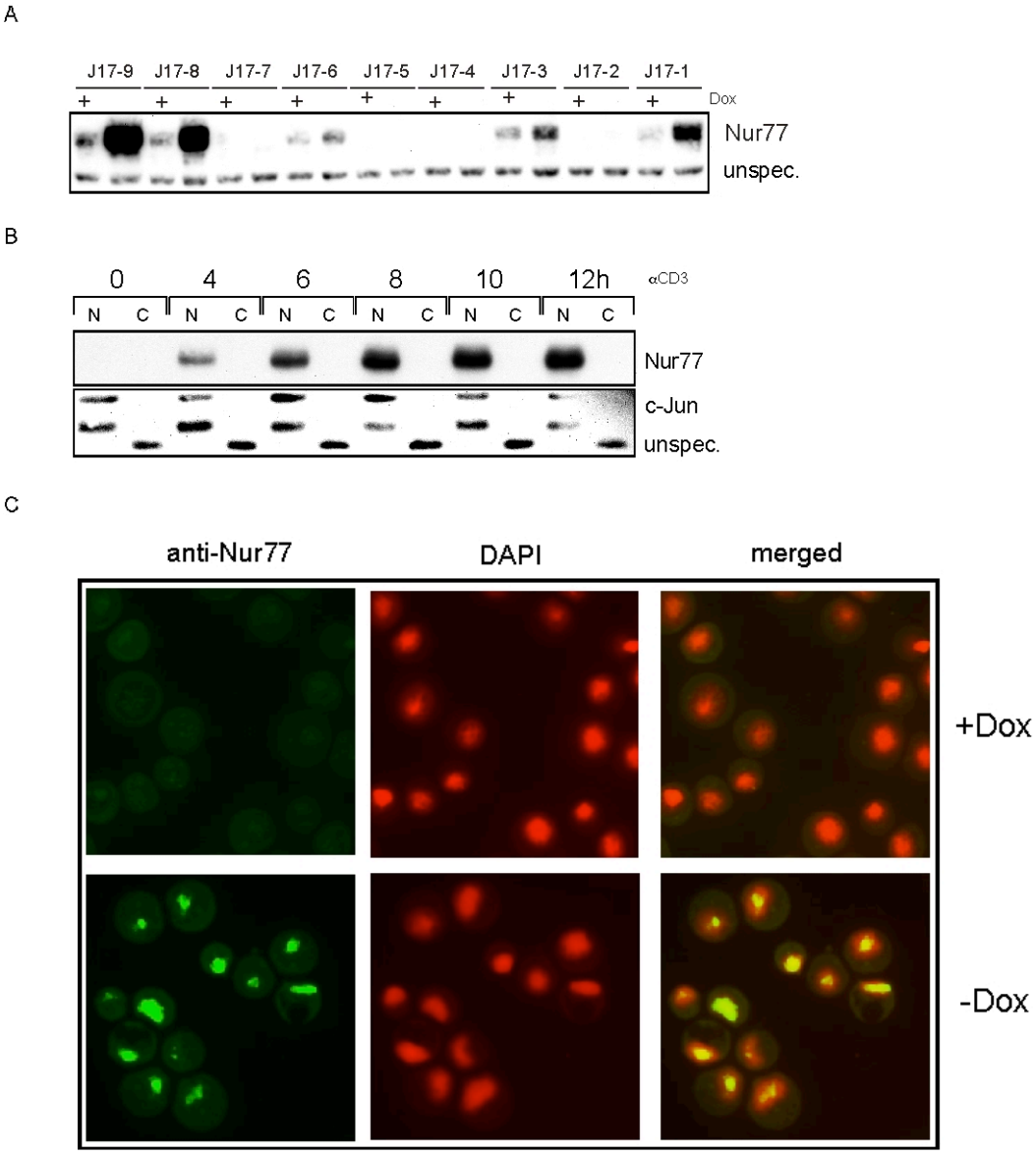


FIGURE 12. Conditional expression of Nur77 in Tet-OFF system in Jurkat T cells. (A) Jurkat T cell clones stably transfected with Nur77 expression vector under control of tet-operator sequences were screened for inducible Nur77 expression by Western blotting. (B) Kinetics of expression and subcellular localisation of overexpressed Nur77 in clone J17-8. Cells were cultured in the absence of doxycycline for indicated time, lysed and nuclear (N) and cytoplasmic (C) extracts were prepared. Nur77 localisation was determined by Western blotting. Membrane was re-probed with anti-cJun Abs to demonstrate purity of nuclear fractions. Unspecific band cross-reacting with anti-cJun Abs demonstrates purity of cytoplasmic preparations (unspec.). (C) Analysis of Nur77 subcellular localisation by immunofluorescence. Cells were cultured in the absence or presence of Dox for 12h. Cells were then spun on glass slides, fixed and incubated with anti-Nur77 Abs followed by anti-rabbit-FITC secondary antibodies. DAPI was added to visualise the nucleus and is shown in red.

3.1.4.4 Characterisation of genes induced by Nur77 activation in Jurkat T cells - a second gain-of-function approach.

Conditional expression of Nur77 in Tet-Off Jurkat T cells was established (see above). Since Nur77 was transcriptionally active as measured by dedicated reporter gene it was reasoned that some yet unknown genes could be induced upon Nur77 activation. In order to characterise such genes, clone J17-8 was cultured in the absence and presence of Dox for 9 hours. Labelled cDNA from –Dox and +Dox cultures was hybridised to LifeGrid high-density filters containing 8000 double spotted human cDNAs (Incyte). Figure 13 shows the scheme of the experiment. Several genes were induced in cultures lacking Dox (Table I). To confirm differential expression PCR primers specific for the enolase-□, hIPF1, hVIP and KIAA0261 protein mRNAs were designed. RT-PCR reactions were performed with cDNAs from –Dox and +Dox cultures and specific gene primers. Differential expression was confirmed for KIAA0261 gene. The mRNAs for enolase-□ did not show clear differential expression (Table 1 and data not shown). Primers for hIPF1 and hVIP failed to generate PCR products with cDNAs from J17-8 (-Dox) clone used as template.

Table I. Genes responding to Nur77 activation in Tet-Off system.

Gene name	Accession	Fold induction Experiment - spot1/spot2[a]	Differential expression confirmed by RT-PCR[b]	comments
enolase-□	BE621812	1 - 5/2 2 - 5/2 3 - 5/2	-	
IPF1 – insulin promoter factor	NM_000209	1 – 8/- 2 – 4/2 3 – 6/5	N/D (amplification failed)	
VIP – vasoactive intestinal peptide	XM_004381	1 – 4/7 2 – 4/16 3 – 3/4	N/D (amplification failed)	
killer cell lectin-like receptor subfamily A, member 1	NM_006611	1 – 2/6 2 – 4/6 3 – 4/6	N/D	This locus does not exists in mouse genome
KIAA0261	AW402845	1 – 2/6 2 – 3/7 3 – 3/5	+	Similar to wapl: „wings-apart like“ protein

a – values indicate of how much stronger was the signal from spots representing indicated genes on the filter in the –Dox cultures compared to +Dox cultures.
b – (-) indicates lack of difference in the mRNA levels between –Dox and +Dox samples measured by RT-PCR. (+) indicates more than 2-fold increase in the mRNA levels in –Dox samples compared to +Dox samples measured by RT-PCR. (N/D) indicates failure to amplify PCR product with selected primers

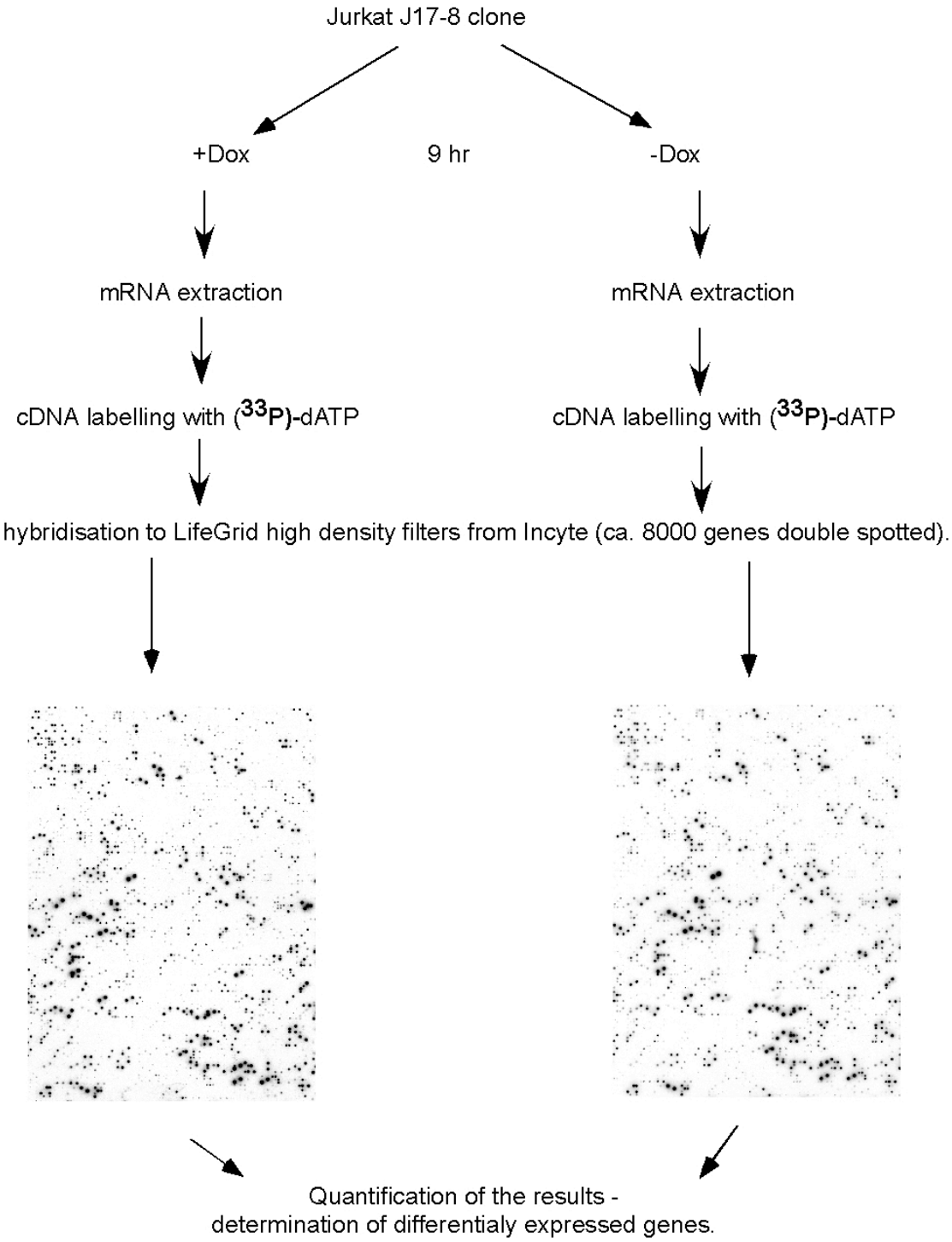


FIGURE 13. Schematic representation of the experiment designed to identify Nur77 target genes in Jurkat T cells by Tet-Off conditional expression system.

To check whether these putative Nur77 target genes are upregulated in FL-Nur77 transgenic thymuses levels of *nur77*, *enolase-*, *IPF* and *VIP* mRNA were determined by RT-PCR in embryonic thymi (14.5, 16.5, 18.5 d.p.c) from both wild-type and FL-Nur77 mice. Endogenous *nur77* expression in wild-type thymi was strongest on day 14.5 and later reduced by ca. 10-fold (Figure 14, upper left panel). In contrast, Nur77

expression in transgenic thymi was identical to wild-type at day 14.5 but dramatically increased at later time points (Figure 14, upper right panel). The Nur77-induced phenotype was apparent for the first time at day 17.5 by TUNEL stain (detects apoptotic cells) and at day 18.5 as judged by thymus size (not shown). *Enolase* and *VIP* mRNA levels were similar in wild-type and transgenic thymi. *IPF* mRNA was not detected in both thymi. These results suggest that neither enolase-α nor VIP is the mediator of Nur77 function in transgenic thymocytes.

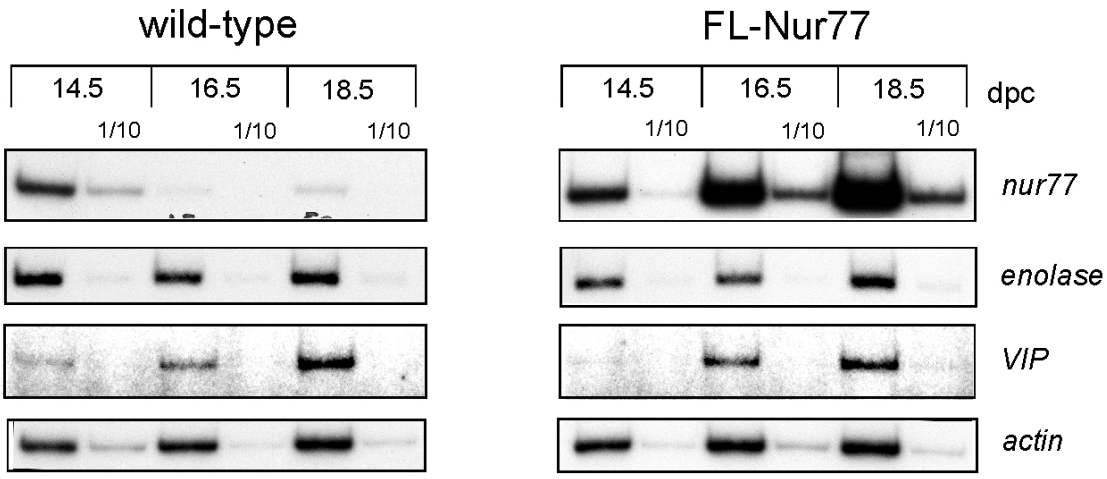


FIGURE 14. RT-PCR analysis of Nur77, Enolase and VIP expression in wild-type and FL-Nur77 transgenic embryonic thymuses. Embryonic thymi were isolated from embryos of indicated times of gestation. cDNA generated from total thymus RNA was used for RT-PCR with Nur77, Enolase and VIP specific primers. 10-fold dilution of cDNA demonstrates quantitative amplification (1/10 marked lanes).

In order to address whether up regulation of *enolase*, *Vip* and KIAA0261 mRNAs are induced by Nur77 activation in a non-T cells 293 HEK cells stably transfected with Nur77^{400}-GBD fusion protein (Figure 10C) were used. 293 cells were treated with Dex for 4 hr and 24 hr or left untreated and mRNA levels of *enolase*, *VIP* and KIAA0261 were analysed by RT-PCR. Dex treatment failed to affect the basal levels of *enolase* and KIAA0261 gene mRNA and *VIP* mRNA was undetectable (data not shown). These results suggest that in 293 cells Nur77 is not sufficient to induce upregulation of those mRNAs.

3.2 PART TWO: Analysis of the function of Rel/NF-κB transcription factors in TCR-mediated apoptosis.

3.2.1 Inhibition of NF-κB by overexpression of non-degradable mIκBα leads to increased sensitivity to TCR-induced apoptosis.

To investigate the role of NF-κB in the T cell hybridoma model of AICD nuclear translocation of NF-κB was inhibited. This was achieved by overexpression of a non-degradable T7-tagged mutant IκBα molecule (Vallabhapurapu *et al.*, 2001). Several Do.11.10 neomycin resistant clones were isolated and two, termed Do/κB^{pos}(1) and (2), did not show any expression of the mIκBα transgene (Figure 15A, upper panel) and were used as controls in all subsequent experiments. Two other clones, termed Do/κB^{neg}(1) and (2), showed strong mIκBα expression with concominant decrease of endogenous IκBα levels (Figure 15A, upper panel). To assess efficiency of NFκB inhibition nuclear extracts were prepared from cells activated with αCD3 Abs were subject to EMSA experiments with NF-κB specific oligo probe. Whereas NF-κB was strongly induced in control cells, mIκBα expressing clones showed no inducible NF-κB binding (Figure 16B). In control cells, majority of NF-κB binding was composed of RelA and p50 (Zeller, 2001). Minor amounts of RelB were also detected. At 8 hr of stimulation an additional band was apparent in control cells but not in NF-κB negative cells, which probably is the c-rel protein (Zeller, 2001).

To demonstrate lack of NF-κB transcriptional activity down stream of TCR, Do.11.10 cells were stimulated with αCD3 Ab and induction of the classical NF-κB target gene *nfkb2* (encoding NF-κB1 p100/p52) was determined by RT-PCR. Expression of the *nfkb2* gene was induced in control cells with kinetics closely matching NF-κB binding detected by EMSA (Zeller, 2001). Induction of *nfkb2* was almost completely abolished in NF-κB negative clones (Zeller, 2001). Thus, overexpression of the mIκBα transgene in Do.11.10 hybridoma cells results in a complete block of NF-κB activation downstream of the TCR.

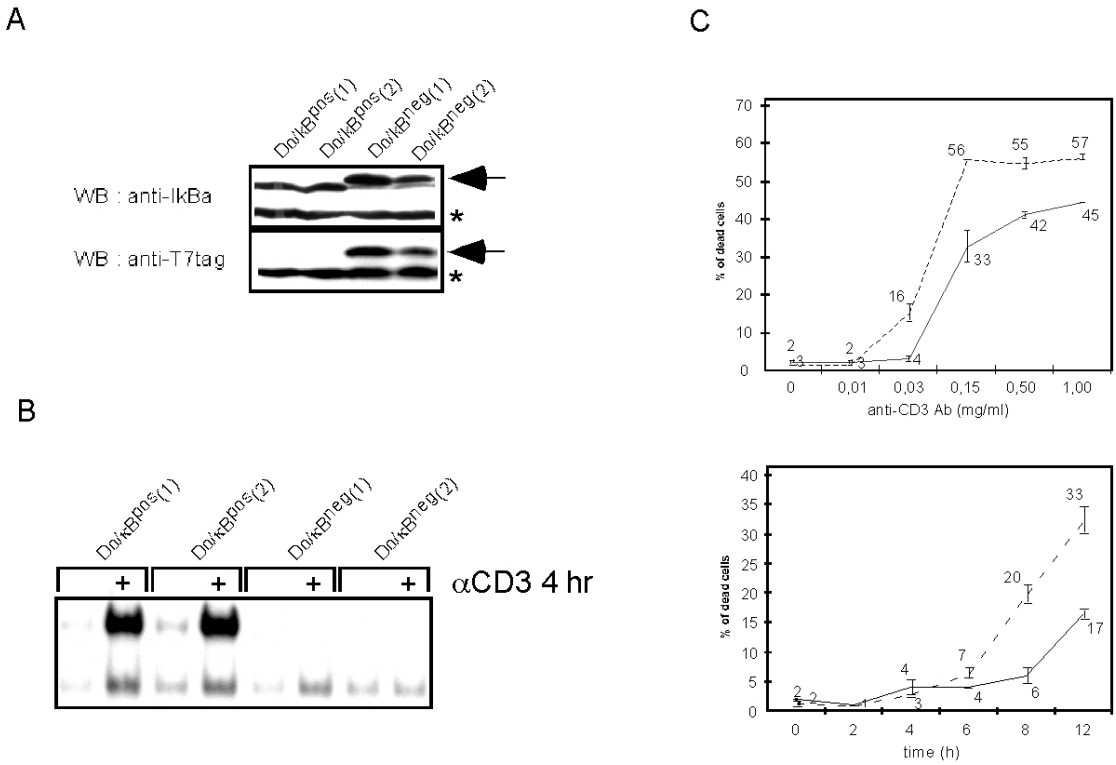


FIGURE15. Inhibition of NF-κB by overexpression of non-degradable mIκBα in T-cell hybridomas leads to increased sensitivity to TCR induced apoptosis. (A) Expression levels of total IκBα in Do/κB^{pos}(1)/(2) (lanes 1 and 2) and Do/κB^{neg}(1)/(2) (lanes 3 and 4) clones were determined by Western blotting with anti-IκBα antibodies (upper panel). Expression levels of exogenous non-degradable clones were determined by Western blotting with anti-T7-tag antibodies. An asterisk(*) indicates unspecific band used here as loading control. (lower panel) (B) Activation status of NF-κB was determined by EMSA using nuclear extracts from Do/κB^{pos}(1) (left part) and Do/κB^{neg}(1) (right part) clones and Igκ probe (C) Anti-CD3 induced apoptosis in Do/κB^{pos}(1)/(2) (solid line) and Do/κB^{neg}(1)/(2) (dashed line) clones was measured as a function of anti-CD3 Ab concentration (15 hr of incubation, upper panel) or as a function of time (1 μg/ml, lower panel). Apoptotic cells were stained with propidium iodide and analysed by flow cytometry. One representative of five independent experiments is shown. Error bars indicate standard deviation form duplicate samples. (Figure 15C lower panel was reproduced from Zeller, 2001).

Since NF-κB is strongly induced by apoptotic TCR signalling in Do.11.10 cells the question whether NF-κB contributes to regulation of apoptosis has been addressed. The cell death sensitivity and kinetics in the absence and presence of NF-κB have been compared and a moderate but significant increase in sensitivity to AICD in NF-κB-deficient cells was noticed (Figure 15C, upper panel). The cell death kinetics was relatively slow so that only after approx 8 hr of induction first apoptotic cells were detectable (Figure 15C, lower panel). Surprisingly, cell death in the NF-κB-deficient cells occurred approx. 2 hr earlier than in wild-type cells. These data show that lack of NF-κB results in increased sensitivity to TCR-induced apoptosis in Do.11.10 hybridoma cells.

3.2.2 TCR induces NF-κB dependent expression of anti-apoptotic A20 protein.

Since there was an increase in the sensitivity to TCR mediated apoptosis in NF-κB deficient cells it was hypothesised that defect in the expression of anti-apoptotic gene(s) could be responsible for observed phenotype. NF-κB is a very well documented principal regulator of genes protecting cells from TNF mediated apoptosis (Karin and Lin, 2002) so the regulation of known NF-κB anti-apoptotic targets genes in response to TCR was tested (Zeller, 2001). To filter out clone-to-clone variations in gene expression Do/κB^{pos}(1) and (2) or Do/κB^{neg}(1) and (2) clones were mixed and activated with αCD3 Abs or combination of PMA plus ionomycin. Total RNA from stimulated cells was converted to cDNA and used as template for semiquantitative RT-PCR. Expression of the *egr3* gene, which is induced in an NF-κB independent manner, was measured as a control for efficient TCR signalling.

Table II. Expression of selected anti-apoptotic NF-κB target genes in αCD3-stimulated Do.11.10 T cell hybridomas.

Gene	Induced by anti-CD3	NF-κB dependent	Induced by PMA + Iono	NF-κB dependent
TRAF 1	-[a]		N/D[e]	
TRAF 2	-		N/D	
IAP 1	+[b]	+[d]	N/D	
IAP 2	-		N/D	
XIAP	not expressed[c]		N/D	
Bfl-1/A1	+	-	+	-/+[f]
Bcl-x_L	-		+	-/+
MnSOD	-		N/D	
A20	+	+	N/D	
Gly96	+	-	N/D	
c-FLIP	-		N/D	

[a,b] indicates either unaltered (-) or increased (+) mRNA levels upon anti-CD3 induction

[c] XIAP mRNA was detected (mouse brain cDNA served as a positive control ; not shown); [d] Dependence on NF-κB was determined by comparing Do/κB^{pos} and Do/κB^{neg} clones ; [e] N/D, not determined ; [f] Inducibility by PMA plus ionomycin was only partially dependent on NF-κB ;

Table II was created on the basis of the RT-PCR results described in Zeller, 2001.

Table II shows the genes analysed and their dependence on NF-κB. Interestingly, only *cIAP1* and *A20* were both TCR inducible and completely NF-κB dependent whereas a partial dependence on NF-κB was observed for the PMA plus ionomycin induced expression of the genes encoding Bfl-1/A1 and Bcl-X_L (Table II and Figure 16A). Since *cIAP1* has recently been shown to perform also pro-apoptotic functions (Li *et al.*, 2002) further studies were focused on *A20* as genuine anti-apoptotic gene. The defect in TCR-mediated *A20* mRNA inducibility was reconfirmed in individual Do.11.10 clones (Zeller, 2001). To demonstrate that A20 protein levels follow the amount of mRNA, anti-A20 Abs directed against its C-terminal portion were raised. Western blot analysis demonstrated strong A20 protein induction at 4 hr of αCD3 stimulation in control but not in NF-κB negative cells (Figure 16B). The finding that A20 is induced by TCR signals conflicts with the reported TCR-induced downregulation of *A20* mRNA in thymocytes and mature naïve T cells (Tewari *et al.*, 1995). To address this, *A20* mRNA and protein levels were measured in primary T cell blasts. There was a clear TCR-dependent induction of both *A20* mRNA (Zeller, 2001) and protein in this system (Figure 16D). Similar results were obtained with CD4 and CD8 T cell blasts (not shown). This data indicate that TCR-induced A20 expression is a general feature of mature T cell blasts (see below) and in T cell hybridoma it is under NF-κB control.

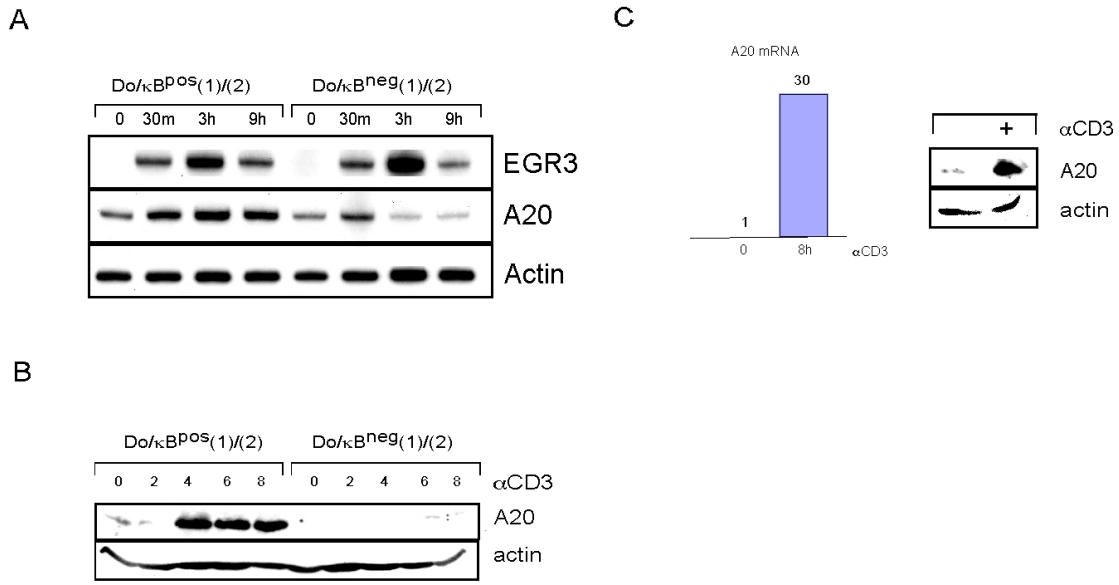


FIGURE 16. NF-κB is necessary for TCR-mediated induction of *A20* gene in Do.11.10 and primary T cells. (A) Steady state *egr3*, *A20* and β-actin mRNA levels for were determined by RT-PCR in pooled Do/κB^{pos}(1)/(2) (left panel) and Do/κB^{neg}(1)/(2) (right panel) clones after indicated time of anti-CD3 treatment. (B) Expression levels of endogenous A20 protein in Do/κB^{pos}(1)/(2) (left panel) and Do/κB^{neg}(1)/(2) (right panel) clones were determined by Western blotting with anti-A20 antibodies after indicated time of anti-CD3 treatment. Lower panel shows β-actin signal used as loading control. (C) Quantification of *A20* mRNA levels in T cell blasts from wild-type B6 mice after indicated times of αCD3 treatment (left panel). Quantification RT-PCR results was done by Phosphoimager and normalised to actin levels. Right panel shows A20 protein levels and actin signal used as loading control. (Figures 16A and 16C left panel were reproduced from Zeller, 2001).

3.2.3 Overexpression of A20 protein in NF-κB-negative cells rescues both sensitisation to TNF and enhanced AICD phenotypes

If A20 was responsible for observed sensitisation to AICD, reexpression of A20 in NF-κB-deficient cells should rescue the phenotype. To test this hypothesis recombinant retroviruses transducing either empty vector or full-length Flag-tagged *A20* cDNA were generated and used to infect both Do/κB^{pos} and Do/κB^{neg} clones. Consistent with published reports (Beg and Baltimore, 1996) Do/κB^{neg} cells were found to be very sensitive to TNF and overexpression of A20 made them completely resistant to TNF (Zeller, 2001). This demonstrates that amount A20 protein in transfected cells was sufficient for effective protection from TNF and that more than 95% of cells in the pools expressed A20. The influence of overexpressed A20 on AICD in both Do/κB^{pos} and Do/κB^{neg} cells was also measured. Overexpression of A20 had no effect on AICD in control cells (Figure 17C). In NF-κB deficient cells, however, reconstitution with A20 lowered the extent of apoptosis to the level seen in control cells. These results show that reexpression of A20 in Do/κB^{neg} cells is

sufficient to rescue both the TNF sensitivity and enhanced AICD in response to TCR crosslinking. A20 overexpression showed no effect on Dex mediated apoptosis in both cell pools (Zeller, 2001). In order to determine the total levels of A20 protein at the time of AICD induction Western blots with total cell extracts from clone mixes used for apoptosis measurement studies were performed. Total amount of A20 protein in control cells were much lower than those detected in cells overexpresing A20 (Figure 17A). Moreover an αCD3 induced increase in steady-state levels of transgenic A20 was noticed even in the absence of NF-κB (Figure 17A). In order to directly determine the A20 rate of synthesis and rate of degradation pulse and pulse-chase experiments were performed. There was a 3-fold increase in the synthesis of A20 upon αCD3 crosslinking but the half-life of A20 (approx. 4 hr) was not affected by αCD3 treatment (not shown).

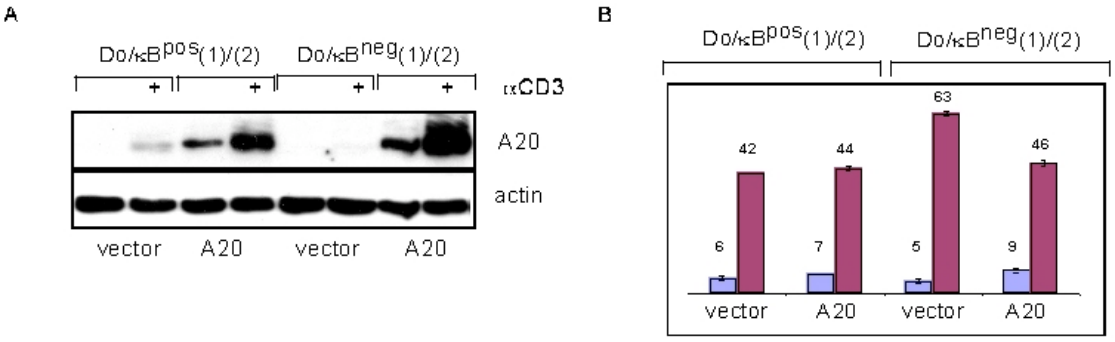


FIGURE 17. Overexpression of A20 protein in Do/κB^{neg} clones rescues increased AICD in the absence of NF-κB. (A) Expression levels of total (endogenous and transgenic) A20 protein in pooled Do/κB^{pos} (left panel) and Do/κB^{neg} clones (right panel). A20 levels in controls (Vector) or A20-transduced clones (A20) were determined by Western blotting. Cells were either left untreated or stimulated for 7 hr with anti-CD3 Ab (+). (B) Quantification of αCD3 induced apoptosis. Pools of clones were treated for 15 hr with anti-CD3 Ab and percentage of apoptotic cells was determined by propidium iodide staining and flow cytometry. Error bars indicate standard deviation from duplicate samples.

3.2.4 Inhibition of NF-κB in Do.11.10 cells results in a switch from Fas- to TNF-dependent AICD.

In NF-κB-negative cells death in response to αCD3 treatment starts 2 hr earlier than in control cells and then continues with similar kinetics (Figure 15C, lower panel). This results in overall slight increase of apoptotic rate in NF-κB deficient cells at 16 hr of αCD3 treatment. Such an effect suggested that cell death in NF-κB negative cells proceeds by different mechanism than in control cells. To test the involvement of death receptors from TNFR superfamily, specific blocking reagents were included in αCD3-treated cultures. In agreement with published data FasL was a principal cell

death inducer in control Do.11.10 cells ((Wang *et al.*, 2001) and Figure 18A, left upper panel) but unexpectedly blocking TNF reduced the levels of AICD in Do.11.10 cell also by approx. 50%. Reagents blocking TRAIL and TWEAK were ineffective (data not shown). Surprisingly, cell death in NF-κB negative cells was only slightly reduced by anti-FasL Ab but very effectively by TNFR-Fc (Figure 18A, right upper panel). These results suggest that in the absence of NF-κB, Fas pathway operates inefficiently but instead cells die by TNF. This cell death involves therefore so-called bystander apoptosis, an undesired effect for physiological AICD. Control cells overexpressing A20 did not show altered AICD mechanism (Figure 18A, left lower panel) demonstrating specificity of A20 function despite very high levels of expression. NF-κB-negative cells reconstituted with A20 showed normal AICD hardly differing from control cells (Figure 18A, right lower panel). This data demonstrate that with respect of AICD A20 is capable of completely taking over NF-κB function.

The observation that TNF was involved in AICD of Do.11.10 cells was unexpected since A1.1 hybridoma cells were reported to rely exclusively on Fas system and not on TNFR pathway (Wang *et al.*, 2001). Other studies also performed with Do.11.10 cells, however, suggest that TNF sensitises to Fas-mediated apoptosis (Elzey *et al.*, 2001). Do.11.10 hybridomas were therefore stimulated with TNF and agonistic anti-Fas mAb. While neither of these reagents was capable of inducing cell death in Do.11.10 cells on its own, the combined stimulation resulted in extensive apoptosis (Figure 18B). Thus, TNF is capable of stimulating Fas pathway, offering an explanation for the observed influence of TNF on AICD of Do.11.10 hybridoma.

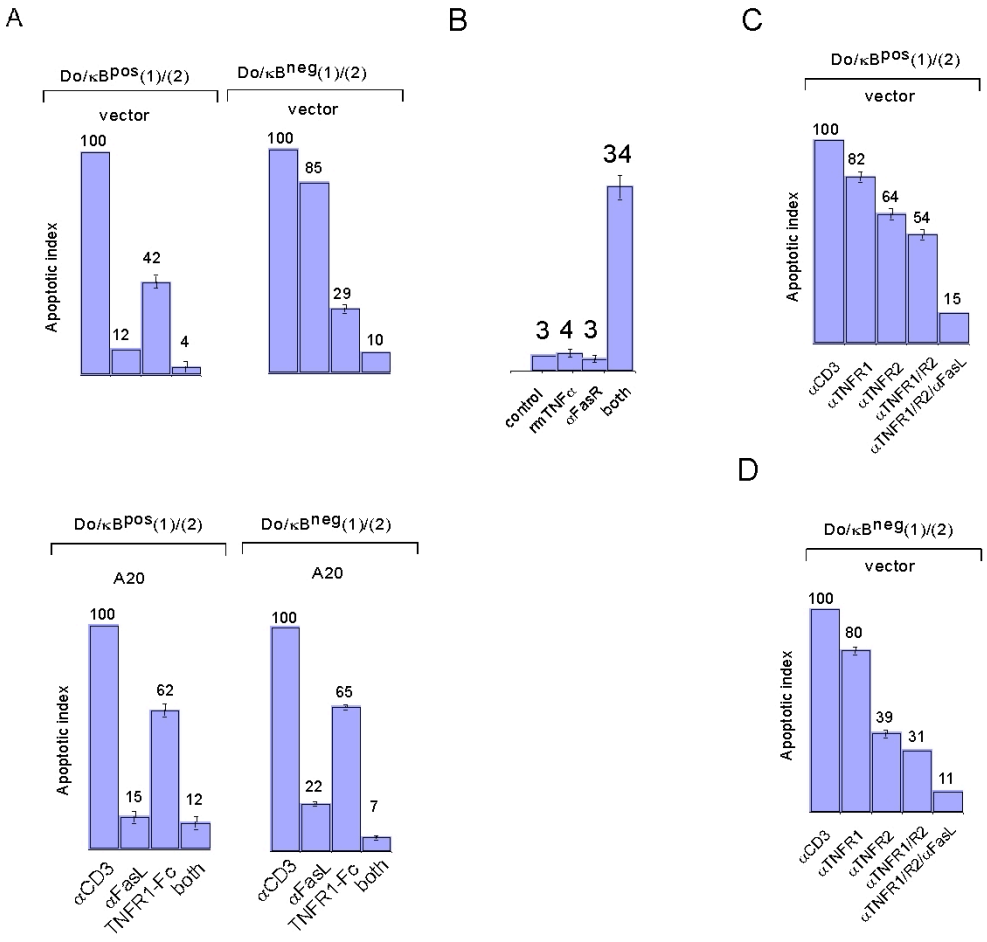


FIGURE 18. Inhibition of A20 induction by blocking NF-κB in Do.11.10 cells results in a switch from Fas- to TNF-dependent AICD. (A) Pools of Do/κB^{pos} and Do/κB^{neg} clones were stimulated with αCD3 Ab for 16 hr in the absence or presence of indicated death receptor blocking reagents. Apoptotic cells were detected by flow cytometry and data are shown as „apoptotic index". Cell death in cultures treated with αCD3 alone (αCD3) was set to 100% and the apoptosis that remained in the presence of indicated death receptor blocking reagents is plotted as percentage of total αCD3 mediated death to demonstrate the relative contribution of the Fas and TNFR signalling pathways. The absolute levels of apoptosis were similar to Figure 15C (not shown). Vector - clones stably transduced with empty vector ; A20 - clones stably transduced with A20 expression plasmid. (B) Pooled Do/κB^{pos} vector transduced clones were either left untreated (control), stimulated overnight with murine TNF (mTNFα), anti-Fas agonistic Abs or both. Apoptotic cells were detected with flow cytometry. Percentage of apoptotic cells in cultures is plotted (C), (D) The individual contribution of TNFR1 and TNFR2 to αCD3 induced cell death was analysed with Ab specifically blocking TNFR1 or TNFR2. Quantification was performed as in (A).

Do.11.10 cells express both TNFR1 and TNFR2 (not shown). To examine which TNFRs are involved in Do.11.10 model of AICD, cells were stimulated with αCD3 in the absence and presence of αTNFR1/R2 blocking mAb. In control cells as well as in NF-κB-negative cells anti-TNFR2 Ab had much more pronounced effect, implying that TNFR2 mediates the majority of the TNF signalling (Figure 18C and D). It is important to mention that Do/κB^{neg} cells also died after treatment with human TNF,

which triggers only TNFR1 on mouse cells (not shown). The predominant involvement of TNFR2 in this AICD model maybe due to the fact that TNFR2 is a high affinity receptor, which is preferentially triggered under conditions of limiting endogenously produced TNF (Lewis *et al.*, 1991).

3.2.5 NF-κB downstream of TNFRs upregulates Fas gene in αCD3-stimulated Do.11.10 cells but this effect is dispensable for efficient Fas-dependent cell death.

To address the question why Do/κB^{neg} cells show greatly reduced Fas-dependent cell death, expression of FasL and Fas was analysed, which are both putative NF-κB target genes (Kasibhatla *et al.*, 1999) (Zheng *et al.*, 2001). No defect in anti-CD3-induced *FasL* mRNA levels or FasL cell surface expression was observed in NF-κB deficient clones (Figure 19A and B, upper panels). While basal level of *Fas* mRNA or Fas surface expression was also unchanged, αCD3-induced upregulation of *Fas* mRNA and Fas surface expression were abolished in Do/κB^{neg} compared to Do/κB^{pos} cells (Figure 19A, lower panel and Fig. 19B, middle panel).

Since TNF contributed to the regulation of AICD in Do.11.10 cell system and Fas is induced by TNF in fibroblasts (Zheng *et al.*, 2001) the involvement of endogenous TNF in Fas regulation in Do.11.10 cells was analysed by employing αTNFR1 or αTNFR2 neutralising mAb. Inclusion of αTNFR1/R2 neutralising Abs during AICD of Do/κB^{pos} cells lead to greatly reduced induction of *Fas* mRNA (Figure 19B, left part of middle panel) and complete block of the αCD3-induced Fas upregulation on the cell surface (Figure 19C). Both TNFRs were involved with slightly larger contribution of TNFR2. These results demonstrate that Fas is TNF inducible gene and this depends on NF-κB in T cells. It was important to determine if the observed lack of Fas induction by αCD3 was responsible for the reduced Fas-dependent AICD in Do/κB^{neg} cells. An advantage was taken of the observation that both vector transduced Do/κB^{pos} cells and Do/κB^{neg} cells reconstituted with A20 show efficient AICD, which is Fas dependent (Figure 18A). The αCD3-mediated Fas upregulation in these cells was tested by FACS and shown that Fas is not induced in NF-κB negative cells reconstituted with A20, despite apparently normal AICD (Figure 19D). These observations suggest that basal level of Fas expression is not limiting for normal

AICD and rule this out as the reason for reduced contribution of FasL to AICD of NF-κB negative cells.

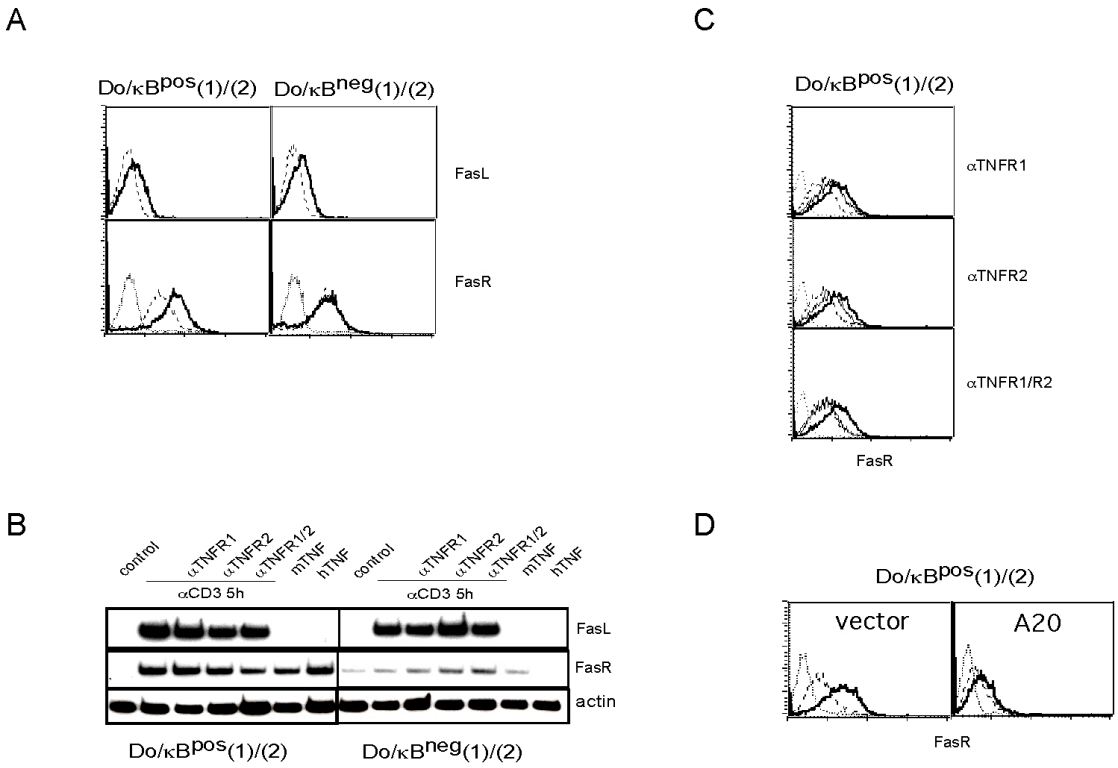


FIGURE 19. Regulation of FasL and Fas expression in Do.11.10 cells by NF-κB, TNF, and A20.(A) NF-κB is required for αCD3 stimulated, but not basal, expression of Fas. Do/κB^{pos} and Do/κB^{neg} cells were treated with αCD3 Abs for 16 hr (FasL) or 6 hr (Fas). Surface expression of FasL and Fas was analysed by flow cytometry. Thick solid lines indicate induced levels of expression, dashed lines indicate basal level of expression, dotted lines indicate fluorescence of unstained cells. (B) NF-κB downstream of TNFRs is required for Fas induction by endogenous TNF secreted in response to anti-CD3 stimulation. Do/κB^{pos} and Do/κB^{neg} cells were treated with αCD3 Ab for 6 hr in absence or presence of indicated anti-TNFR blocking antibodies or treated alone with murine or human TNF. Total RNA was subject for RT-PCR analysis to determine induction of *FasL* (upper panel), *Fas* mRNA (middle panel). Expression of β-actin is shown as amplification control (lower panel). (C) Both TNFR1 and TNFR2 contribute to αCD3 stimulated Fas surface expression in D0.11.10 cells. Do/κB^{pos} cells were stimulated with αCD3 for 6 hr in the presence of αTNFR1 (upper panel), αTNFR2 (middle panel), αTNFR1 plus αTNFR2 mAb (lower panel). Thick solid lines indicate induced levels in the absence of αTNFR mAbs, thin solid lines indicate induced levels in the presence of αTNFR mAbs, dashed lines indicate basal level of Fas expression, dotted lines indicate fluorescence of unstained cells. (D) A20 does not restore αCD3 induced Fas expression in Do/κB^{neg} cells. Control Do/κB^{pos} cells (vector) and Do/κB^{neg} cells stably expressing A20 were stimulated with αCD3 for 6 hr and Fas levels were detected by flow cytometry. Line legend as in (A).

3.2.6 NF-κB downstream of both TCR and TNF receptors is necessary for maximal induction of *A20* mRNA but not A20 protein.

To examine how much of the αCD3-induced NF-κB activity was dependent on endogenous TNF Do.11.10 cells were stimulated with αCD3 mAb in the absence and

presence of αTNFR1/R2 neutralising Abs and nuclear extracts were analysed in EMSA. In the absence of TNFR1 signalling the NF-κB induction profile hardly changed as compared to control cultures (Figure 20A, lower left panel). Blocking of TNFR2 however resulted in significant reduction in NF-κB inducibility particularly at 4 hr and 8 hr of stimulation (Figure 20A, lower right panel). Blocking of both TNFRs revealed that majority of NF-κB induced after 2 hr of activation is mediated by endogenous TNF (Figure 20A, upper right panel), although there was also TNF-independent NF-κB induction, which was even more pronounced after 4 hr and 8 hr.

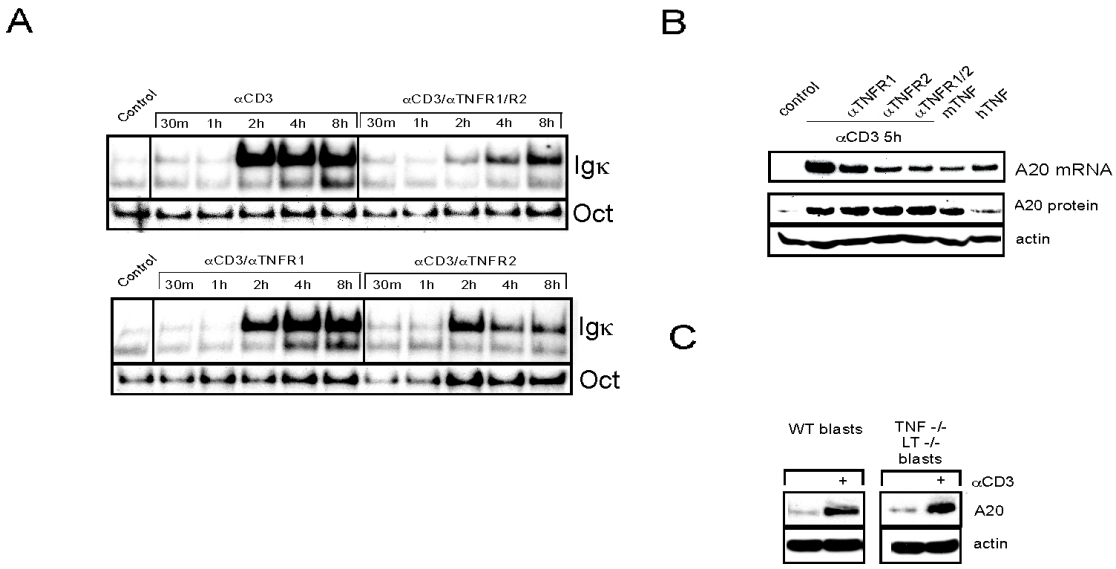


FIGURE 20. Anti-CD3-stimulated secretion of TNF contributes to both maximal NF-κB binding and *A20* mRNA induction, but does not affect A20 protein levels. (A) Nuclear extracts from Do.11.10 cells were prepared and analysed in EMSA as in Figure 15 with the exception that αTNFR neutralising mAbs were included during stimulation period as indicated. Oct probe demonstrates equal loading. (B) *A20* mRNA levels (upper panel) were analysed by RT-PCR as described in Figure 19B. Protein extracts from indicated cultures were subject to Western blotting with anti-A20 Abs (middle panel) Actin protein levels are shown as a loading control (lower panel). (C) T-cell blasts from wild type and *tnf*$^{a/-}$, *lta*$^{-/-}$ double-knockout mice were either left untreated or stimulated for 7 hr with αCD3 Abs (+). Whole cell extracts were prepared and subject to Western blotting with anti-A20 Abs (upper panel). The actin signal demonstrates equal loading (lower panel).

Since *A20* is a classical TNF-responsive gene and hybridoma cells secreted significant amounts of TNF upon TCR crosslinking, the contribution of TNF to induction of *A20* mRNA and protein in Do/κB^{pos} cells was determined. Cells were stimulated with αCD3 Abs in the absence and presence of αTNFR blocking mAbs. Both anti-TNFRs antibodies lowered αCD3 induced *A20* mRNA levels (Figure 20B, upper panel). In the presence of αTNFR2 blocking mAbs the effect of αTNFR1 mAbs was hardly

visible arguing for major involvement of TNFR2 in TNF signalling. Despite clear reduction of *A20* mRNA levels TNFR mAbs did not affect induced A20 protein levels (Figure 20B, middle panel).

To clearly demonstrate that TCR signalling also induces A20 expression independent of TNF, T cell blasts from *tnf*$^{-/-}$*lta*$^{-/-}$ double-knockout mice, which are deficient in any known TNFRs ligand were analysed (Korner *et al.*, 1997). Anti-CD3-induced A20 levels were comparable between wild-type and *tnf*$^{-/-}$*lta*$^{-/-}$ T cell blasts, indicating that activation of the TCR induces A20 independent of TNFR signalling (Fig. 20C).

4 DISCUSSSION

The Activation Induced Cell Death (AICD) is a process in which T cell receptor (TCR) induces apoptosis in activated T cells in the peripheral lymphoid system. AICD serves to remove potentially harmful self-reactive T cells and to limit T cell expansion during an immune response. AICD operates in a strictly clone specific fashion where cells stimulated through TCR but not bystander cells undergo apoptosis. Defective AICD is a cause of autoimmune syndromes and the understanding of the molecular mechanisms of TCR-mediated apoptosis is therefore of great medical importance (Lenardo *et al.*, 1999). The Nur77 nuclear orphan receptor and Rel/NF-κB proteins constitute two families of transcription factors, which are involved in TCR-mediated cell death (Dudley *et al.*, 1999; Liu *et al.*, 1994; Woronicz *et al.*, 1994). Molecular mechanisms, by which these proteins contribute to AICD remain, however, obscure. In the work presented here the function of Nur77 nuclear orphan receptors and Rel/NF-κB proteins in TCR-mediated apoptosis was studied by the application of *in vitro* models of AICD.

4.1 Regulation of Nur77 and its homologues in Do.11.10.

In this work the correlation between Nur77 expression and apoptosis induction was studied in detail. PMA or ionomycin (non-apoptotic stimuli) upregulate *nur77* mRNA transiently whereas αCD3 Abs or PMA plus ionomycin (apoptosis-inducing stimuli) treatment leads to prolonged induction. This is in agreement with the report published previously, where αCD3 Abs induced prolonged *nur77* mRNA upregulation, whereas PMA only transiently induced *nur77* mRNA expression (Woronicz *et al.*, 1994). Consistent with published data (Woronicz *et al.*, 1994; Woronicz *et al.*, 1995), there was little difference between NBRE binding at 2 hr and 4 hr after addition of either ionomycin or PMA/ionomycin. In addition, the observation that ionomycin is sufficient to induce significant NBRE binding fits into the current model of *nur77* promoter regulation (Youn and Liu, 2000). In this model a critical regulatory step is a calcium-mediated dissociation of transcriptional co-repressor Cabin 1 from MEF2D transcription factor bound to *nur77* promoter.

The major novel finding in this work is the dramatic difference in NBRE binding at 8 hr of stimulation between ionomycin and PMA plus ionomycin treated cells. Together

with the observation that PMA plus ionomycin, but not PMA alone, trigger transcription of *nor-1* and *nurr1* genes these results demonstrate that correlation between Nur77 family members expression and apoptosis is of a quantitative nature. Thus, a non-apoptotic stimulation ionomycin induces clear but transient NBRE binding whereas apoptosis-inducing stimulation triggers prolonged NBRE binding. Interestingly, apoptosis is first detectable after the 8 hr of stimulation. Taken together these results suggest that apoptotic signal delivered through TCR allows Nur77 proteins to be maintained in DNA binding competent state in the nucleus at the moment when apoptosis is induced. On the other hand, under conditions were no apoptosis occurs Nur77 binding is down regulated before the onset of apoptosis.

4.2 Overexpression of Nur77 in transformed T cells is not sufficient to induce apoptosis.

Since Nur77 overexpression under the control of *lck*-proximal promoter in transgenic mice results in massive thymocyte apoptosis (Weih *et al.*, 1996) it was tempting to hypothesise that Nur77 overexpression alone would induce apoptosis in T cells. This, however, was not the case since both Do.11.10 and Jurkat T cells did not show any growth suppression or signs of apoptosis when transfected with *nur77* cDNA. The ionomycin also failed to induce apoptosis despite inducing a significant NBRE DNA binding. These results are consistent with the model in which hybridomas need a second signal, in addition to Nur77 expression, to undergo apoptosis. Similar results were obtained recently in LNCaP prostate cancer cells (Li *et al.*, 2000). In that report transient transfection of Nur77 into LNCaP cells results in nuclear accumulation of Nur77 but apoptosis does not occur unless the cells are treated with PMA or ionomycin. This is in contrast to RatA1 fibroblasts, which undergo apoptosis solely upon Nur77 overexpression (Masuyama *et al.*, 2001). Thus, it appears that transformed T cells are resistant to Nur77 induced apoptosis. Since transformed cells are characterised by deregulated cell cycle, lack of contact inhibition and independence on growth factors it is possible that these features are responsible for resistance to Nur77 induced apoptosis. In addition, one of the mechanisms contributing to tumor development is the evasion of apoptosis by transformed cells (Wright *et al.*, 1994). One of the possible parameters for differential susceptibility to Nur77 could be the basal activity of Akt kinase. Akt was shown to directly

phosphorylate Nur77 and abolish its transcriptional activity (Masuyama *et al.*, 2001). Cell lines showing high Akt kinase levels could be protected against Nur77 action.

4.3 Nur77 promotes apoptosis by two distinct mechanisms correlating with either mitochondrial or nuclear Nur77 translocation.

Nur77 orphan nuclear receptor is an immediate early gene induced by variety of stimuli (see Introduction) some of which are capable of inducing apoptosis. Nur77 is necessary for TCR mediated apoptosis of T cell hybridomas (Liu *et al.*, 1994; Woronicz *et al.*, 1994). It was also implicated in cancer drug induced PCD (Li *et al.*, 2000). In the latter case, cancer drug induces quantitative translocation of Nur77 to mitochondria followed by cytochrome c release and PCD. This can be blocked by anti-apoptotic Bcl2 protein. The mechanism described was the first demonstration of the involvement of transcription factor in the regulation of apoptosis at the level of mitochondria. The authors provide convincing evidence of mitochondrial targeting of Nur77. They were also able to uncouple Nur77 transcriptional function from its mitochondrial role. Nur77 mutant lacking DNA binding domain and large portions of flanking sequences is translocating to mitochondria where it induces apoptosis. It was hypothesised that Nur77 mitochondrial targeting is the only mechanism by which Nur77 promotes apoptosis and thus no Nur77 target genes exist, at least in the context of apoptosis.

In this work the above-mentioned suggestions were challenged. It was demonstrated for the first time that Nur77 induced by TCR signals in Do.11.10 cells translocates quantitatively to the nucleus and stays there throughout apoptosis induction and execution. Nur77 was not detected in mitochondria at any time preceding the induction of apoptosis. Consistently with published data (Ivanov *et al.*, 1997) this form of apoptosis is also resistant to Bcl2 overexpression, further arguing that the mechanism of Nur77 action downstream of TCR is distinct from its role in prostate cancer cells treated with cancer drugs. It is, however, still not clear if nuclear retention of Nur77 during AICD of hybridomas is accompanied by transcription of target genes or simply required for yet another non-genomic function of Nur77 in the nucleus. For example, estrogen and androgen receptors, for long considered to be conventional transcription factors, are capable of activating MAP kinases independently of their

transcriptional functions (Peterziel *et al.*, 1999). Nevertheless, these results pave the way for the search for Nur77 target genes in TCR-mediated apoptosis.

4.4 Attempts to characterise Nur77 target genes.

Since Nur77 overexpression per se is not sufficient to induce apoptosis in transformed T cells *in vitro,* a loss-of-function approach to characterise Nur77 targets downstream of TCR was attempted. This was done with the help of transgenic mice overexpressing DN-Nur77 in T cells. DN-Nur77 transgene partially rescued the phenotype of FL-Nur77 tg mice and thus demonstrated its functionality. Nevertheless, injection of αCD3 Abs resulted in normal DP thymocyte deletion in the DN-Nur77 transgenic mice. This results conflict published data showing that DN-Nur77 mutant overexpressed under the control of TCRα chain enhancer results in the resistance of DP thymocytes to αCD3 Abs *in vivo* treatment (Zhou *et al.*, 1996). While the promoters chosen to control the expression of transgenes differed (TCRα chain enhancer versus *lck*-proximal promoter) it is not clear how this could affect the TCR mediated apoptosis. The DN-Nur77 transgenic mice used here for antibody injection experiments were homozygous for the transgene. One possibility would be that a recessive mutation caused by transgene integration affected the rescue from TCR induced apoptosis. This could have occurred if for example the transgene disrupted a gene responsible for setting the cell's sensitivity to αCD3 antibodies (e.g. component of TCR or TCR signalling complex). One can imagine that homozygous transgenic thymocytes are more sensitive to TCR mediated apoptosis and therefore the protection provided by the transgene is not visible any more. To rule out such an effect injection of αCD3 Abs should be repeated with heterozygous animals since these were the mice in which the rescue from Nur77 induced phenotype was originally observed. Since DN-Nur77 homozygous thymocytes did not show resistance to αCD3 Abs other approaches to characterise Nur77 targets had to be tried.

Since the loss-of-function approach to clone Nur77 target genes was abandoned a gain-of-function approach based on Nur77 conditional expression both *in vitro* and *in vivo* was attempted. In order to achieve these Nur77 conditional alleles were created by fusing $Nur77^{400}$ truncation mutant to EBD. $Nur77^{400}$-EBD fusion proteins were tested in three cell types and ligand dependent transcriptional activity could be demonstrated only in Jurkat T cells. Such a result was unexpected since folding

problems from which fusion proteins suffer frequently would rather occur in cell type-independent fashion. One explanation for this finding could be that Nur77^{400}-EBD fusion proteins have lower affinity for some protein necessary for full transcriptional potential as compared to Nur77^{400} mutant. Such a hypothetical protein could then be expressed at different levels in distinct cell types and in Jurkat T cells high expression levels could compensate lowered affinity of Nur77^{400}-EBD. Clearly the applicability of Nur77^{400}-EBD proteins has to be determined individually for each experimental system. Generation of Nur77-GBD and Nur77^{400}-GBD fusion proteins resulted in Dex-dependent Nur77 transcriptional activity. This data was in agreement with previous reports obtained with Nur77^{400} fused to wild-type GBD (Davis *et al.*, 1993). Furthermore Nur77^{400}-GBD chimeric protein could be stably expressed in 293 HEK cells, which lead to establishment of *in vitro* system for Nur77 conditional activation. This system in combination with Nur77 controlled expression in Tet-Off system could be useful to discriminate between genes responding to Nur77 activation in T cells and non-T cell lines (see below). Taken together these results demonstrate successful generation of Nur77 ligand activated alleles, which will facilitate the studies on the Nur77 function.

Overexpression of Nur77 in T cells of transgenic mice leads to massive apoptosis (Weih *et al.*, 1996). Unfortunately, the severity of the phenotype of FL-Nur77 mice makes it difficult to characterise Nur77 target genes. In order to generate a controlled model system, in which Nur77 activity could be induced by exogenous ligand, Nur77^{400}-EBD fusion protein was expressed in transgenic mice under *lck*-proximal promoter. Only one transgenic line was available for analysis and these mice showed very low transgene expression in thymocytes. Consistent with this *in vivo* activation of Nur77^{400}-EBD chimera by injection of tamoxifen did not lead to gross phenotypic abnormalities. The expected outcome of the experiment was that long-term activation of Nur77 in transgenic thymocytes would induce apoptosis similar to transgenic mice overexpressing full length Nur77 constitutively (FL-Nur77 line). The only measurable effect of tamoxifen injection was a small reduction in the percentage of DP thymocytes in transgenic as compared to control mice. This effect was tamoxifen induced since there was no difference between control and transgenic littermates in DP thymocyte counts in freshly prepared thymi (not shown). These results suggest that the chosen strategy to obtain *in vivo* conditional model of Nur77 function could

work provided that transgenic founders expressing higher amounts of Nur77^{400}-EBD will be characterised.

4.5 Novel putative Nur77 target genes.

Comparison of mRNA profiles between control and Nur77 overexpressing Jurkat T cells (+/- doxycycline culture conditions) revealed genes induced in response to Nur77 activation. Out of around 8000 genes represented on LifeGrid high-density filters only four shown reproducible induction in response to Nur77 activation (table I). These are α-enolase (ENO1), insulin promoter factor 1 (IPF1), vasoactive intestinal peptide (VIP) and KIAA0261 (Wapl homologue). This result shows that Nur77 activation did not cause large-scale changes in mRNA composition in Jurkat cells. The reasons for the recovery of so few Nur77-responsive genes could be that for many genes Nur77 may be a necessary but not sufficient regulatory protein. In this case a loss-of-function approach, like the use of transgenic mice or cells expressing DN-Nur77, may be more productive. Alternatively, since LifeGrid high-density filters contain only a fraction of all estimated human transcripts, it is possible that many Nur77-responsive genes are simply not represented on the filters and remained undetected in this experimental setting. The drawback of this approach was the lack of positive control, for example a known Nur77 target gene, which would provide the information on the performance of the screening method.

4.5.1 *ENO1* as Nur77 target.

The human ENO1 gene encodes enolase-α. Human genome contains three separate genes encoding enolases: muscle-specific, neuron-specific and a ubiquitously expressed isoforms of this glycolytic enzyme (Giallongo *et al.*, 1990). *ENO1* gene encodes ubiquitously expressed isoform of enolase and is a rare example of bifunctional gene. Recently it has been discovered that *ENO1* mRNA is alternatively translated to two protein products: 48 kD enolase and 37 kD polypeptides. The 48 kD variant corresponds to enolase whereas 37 kD protein is identical with the previously characterised c-myc binding protein (MBP-1) (Feo *et al.*, 2000), (Subramanian and Miller, 2000). MBP-1 was shown to bind to the *c-myc* oncogene promoter, serving as transcriptional repressor of *c-myc* gene. Enolase-α is preferentially cytoplasmic

whereas MBP-1 localises to the nucleus. MBP-1 overexpression induces apoptosis in 3T3 fibroblasts (Ray, 1995). Interestingly, Nur77 overexpression also induces PCD in RatA1 fibroblasts (Masuyama *et al.*, 2001). These observations suggest that enolase could be a mediator of Nur77 activity in certain experimental settings. There was, however, no increase in steady-state *ENO1* mRNA levels in FL-Nur77 transgenic thymi. *ENO1* mRNA is highly abundant but not T cell lineage specific transcript. Therefore one cannot formally rule out the possibility that strong *ENO1* mRNA expression originating from thymic stromal cells masks FL-Nur77 transgene mediated *enolase* mRNA upregulation in thymocytes. It appears, however, unlikely that Nur77 induces apoptosis in the thymocytes through transcriptional induction of *ENO1* gene.

4.5.2 *IPF1* gene as Nur77 target.

The insulin promoter factor 1 (*IPF1*) gene encodes homeodomain transcription factor necessary for development and function of the pancreas (Jonsson *et al.*, 1994). Since originally thought that IPF1 is expressed exclusively in pancreas, it was therefore unexpected to find IPF1 among Nur77-responsive genes. Recently, however, a report was published on IPF1 expression and function in neurons (Schwartz *et al.*, 2000). Since Nur77 and its homologues induce differentiation when overexpressed in immature neuronal cells (Castro *et al.*, 2001) it is possible that IPF1 induction by ectopic expression of Nur77 in Jurkat T cells is reminiscent of Nur77 role in neurons. At present, however, there is no data available linking IPF1 expression to apoptosis.

4.5.3 *VIP* gene as Nur77 target.

Vasoactive intestinal peptide (VIP) is a 28-aa neuropeptide with many important immunomodulatory properties (Pozo *et al.*, 2000). It is secreted both by neurons and immune cells (Bellinger *et al.*, 1996). Recently, TCR signalling in T cells resulted in VIP secretion (Delgado and Ganea, 2001). In the thymus *VIP* mRNA can be detected in most thymocytes subpopulations with the exception of DN thymocytes (Delgado *et al.*, 1999). This is in agreement with the *VIP* mRNA expression profile in embryonic thymi obtained in this work. VIP has profound effects on thymocyte maturation *in vitro*. VIP significantly enhances conversion of $CD4^+CD8^+$ thymocyte-like cells to $CD4^+CD8^-$ T cells (Dorsam *et al.*, 2000). It has been therefore hypothesised that VIP could regulate the sensitivity to TCR signals and influence DP thymocyte maturation

in the thymus (Pozo *et al.*, 2000). While these data suggest that VIP could be a candidate mediator of Nur77 action in T cells, *VIP* mRNA was not elevated in embryonic thymi from FL-Nur77 tg animals.

4.5.4 KIAA0261 gene as Nur77 target.

Not much is known about the KIAA0261 protein (Wapl homologue). A gene with similarity to KIAA0261 cDNA was characterised in Drosophila and encodes large protein (1770 amino acids long) containing tract of acidic amino acids reminiscent of many chromatin-associated proteins (Verni *et al.*, 2000). Mutations in the Drosophila *wapl* gene result in late larval lethality associated with a unusual chromosome morphology. Further investigations lead the authors to propose that *wapl* gene is responsible for controlling heterochromatin organization in Drosophila melanogaster. There is so far no report on the function of KIAA0261 protein in mammalian cells.

4.5.5 Concluding remarks on novel putative Nur77 target genes characterised in Jurkat T cell system.

The potential Nur77 target genes *ENO1*, VIP and KIAA0261 protein are not induced by Dex in 293 cells stably expressing Nur77^{400}-GBD fusion protein. This result suggests that these genes respond to Nur77 in a cell-specific fashion. It is possible that Nur77 induces distinct mRNAs in different T cells so that genes characterised in the human Jurkat cells may not be Nur77 responsive in mouse thymocytes. Additional experiments will be necessary to address whether Nur77 induces the characterised genes in cell types distinct from Jurkat. An alternative explanation for the failure of Dex to induce these Nur77-responsive genes may be the inability of the Nur77^{400}-GBD fusion to access Nur77 binding sites embedded in chromatin. The latter possibility is, however, unlikely since Nur77^{400}-GBD fusion protein activated Nur77 reporter genes stably integrated into the chromosome (data not shown). *IPF1* and *VIP* transcripts were not detectable by RT-PCR in Jurkat cells and mouse *IPF1* mRNA also in mouse thymus. One of the reasons could be that designed primers simply did not work or that the expressed mRNA differed from the published sequence and did not include primer-binding sites. Further experiments with the use of reliable positive control templates for RT-PCR need to be performed to discriminate between these possibilities.

Nur77 overexpression in Jurkat T cells leads to upregulation of several mRNAs (see above). Nur77 overexpressed in Jurkat localised to the nucleus, suggesting a transcriptional mechanism of Nur77 action. It is, however, possible that Nur77 performs some nuclear but non-genomic function, which then leads to induction of described mRNAs. To discriminate between these possibilities a DNA-binding defective Nur77 mutant could be used. The overexpression of a such a Nur77 mutant should not result in the induction of described mRNAs if transcriptional mechanism is operating. This experiment needs to be performed in the future.

The identification of new putative Nur77 target genes has been described in this work. The existence of genes responding to Nur77 activation further strengthens a notion that Nur77 is a transcription factor. This findings raise, however, several important questions: what is the mechanism of induction of Nur77 targets? Is DNA binding necessary? Are these genes induced in a Jurkat T cell-specific fashion? Are any of these genes mediators in Nur77 induced apoptosis?

Even though these questions have not been addressed in this work, important progress was achieved in terms of generation of tools necessary to clear those points.

4.6 NF-κB in TCR mediated apoptosis - is it anti- or pro-apoptotic?

Several independent groups have addressed the role of NF-kB transcription factors in TCR-mediated apoptosis. Most of these studies focused rather on the overall phenotype resulting from NF-κB inhibition (or overexpression) and did not explore the underlying molecular mechanisms. The results obtained in this work are consistent with those reports, which noticed enhanced TCR-mediated apoptosis upon NF-κB inhibition (Dudley *et al.*, 1999). In one case, IKKγ-deficient Jurkat T cells show increased apoptosis after PMA plus ionomycin treatment (Rivera-Walsh *et al.*, 2000). It is questionable, however, whether this effect is due to direct TCR signalling. A similar mutant has recently been shown to be very sensitive to TNF induced cell death. Since PMA plus ionomycin could induce TNF transcription in normal Jurkat cells (Luo *et al.*, 1996) and at least in T cell hybridomas TNF is not NF-κB target gene (this work - data not shown), it remains possible that increased PMA plus ionomycin induced apoptosis is due to secreted TNF.

Some groups proposed positive role of NF-κB in TCR-mediated apoptosis in either hybridomas (Kasibhatla *et al.*, 1999) or transgenic thymocytes *in vivo* (Hettmann *et*

al., 1999). In another report no difference in TCR mediated apoptosis was observed in T cells from *nfkb1*$^{-/-}$, *c-rel*$^{-/-}$ or *RelA*$^{-/-}$ mice (Zheng *et al.*, 2001). It appears unlikely that removal of a single Rel/NF-κB family member results in a severe phenotype since TCR signalling induces multiple Rel/NF-κB proteins (Kahn-Perles *et al.*, 1997). On the other hand, naïve T cells from *c-rel*$^{-/-}$ *RelA*$^{-/-}$ double knockout and mIκBα-transgenic mice show greatly attenuated activation induced proliferation making it difficult to assay AICD ((Zheng *et al.*, 2001); Weih F., - unpublished observations). One group that succeeded in measuring AICD in T-cell blasts from mIκBα transgenic mice found increased apoptosis in response to TCR crosslinking (Dudley *et al.*, 1999). Thus, the role of NF-κB proteins in TCR-mediated apoptosis remains controversial.

In this work, inhibition of NF-κB in Do.11.10 hybridoma T cells by overexpression of non-degradable mIκBα molecule results in increased in sensitivity to TCR-mediated apoptosis. This is, however, a net effect since dissection of the mechanism of apoptosis revealed increased susceptibility to TNF-induced apoptosis and at the very same time a partial loss of the ability to undergo FasL-induced cell death. Thus, in contrast to previous reports where either anti- or pro-apoptotic role for NF-κB was proposed, data presented here argue for both anti- and pro-apoptotic function of NF-κB downstream of TCR.

4.7 NF-κB target genes in TCR-mediated apoptosis.

Recently TCR-induced expression of several anti-apoptotic NF-κB target genes has been investigated in Do.11.10 hybridoma cells (Zeller, 2001). TCR regulates *A20* and *c-IAP1* genes in NF-κB dependent fashion while most of other known NF-κB targets are either not induced by TCR or induced normally in the absence of NF-κB. Moreover, *A20* mRNA is induced by TCR in T cell blasts but not in naïve mature T cells (Zeller, 2001 and this work). These results show that at least in the context of AICD most of so-called "NF-κB target genes" are in fact only facultatively dependent on NF-κB. Thus, depending on differentiation status of the cell, target gene spectrum and consequently NF-κB function could vary from one cell type to another (e.g., immature thymocytes versus mature T cell blasts).

4.8 A20 replaces NF-κB function during AICD of T-cell hybridomas.

Previously was established that the A20 zinc finger protein replaces NF-κB function in TCR-induced apoptosis of Do.11.10 cells (Zeller, 2001). In this work NF-κB deficiency results in a decreased ability to undergo Fas dependent apoptosis and acquired sensitivity to secreted TNF. In principle, one could argue that the apparent switch from Fas-dependent to TNF-dependent cell death is a consequence of a competition between these death receptors for a limiting protein required to transmit the signal. Indeed, both Fas and TNF recruit FADD upon activation (Strasser *et al.*, 2000). In this scenario the observed "switch" is a simple consequence of TNF-induced cell death and normal Fas-dependent cell death should be measured once TNF-mediated cell death is blocked, for example by TNFR1-Fc. This is, however, not observed since NF-κB negative Do.11.10 cells reproducibly show less Fas-dependent apoptosis as compared to control cells when TNF signalling is inhibited. This observations lead to conclusion that NF-κB promotes Fas-dependent cell death which is further supported by the notion that *RelA*$^{-/-}$ fibroblasts show reduced apoptosis in response to Fas crosslinking (Ouaaz *et al.*, 1999).

Since A20 overexpression in NF-κB-negative hybridoma cells completely reverses the phenotype it is reasonable to postulate that A20 performs dual function. It inhibits TNF-mediated apoptosis and also promotes TCR-induced Fas-dependent apoptosis. The latter conclusion is based on indirect evidence from rescue experiment where A20 overexpression restores Fas-dependent AICD in the absence of NF-κB. In the future it will be necessary to substantiate this with direct demonstration of A20 involvement in Fas mediated cell death. The mechanism by which A20 inhibits TNF mediated apoptosis is not fully understood. A recent report demonstrates that A20 overexpression blocks recruitment of RIP1 and TRADD to TNFR1 in response to TNF (He and Ting, 2002). In Do.11.10 cells TNFR2 is the receptor primarily inducing apoptosis and A20 overexpression completely inhibits this PCD. Recently, RIP1 was reported to mediate induction of apoptosis by TNFR2 in T cells (Pimentel-Muinos and Seed, 1999). It is therefore likely that A20 blocks TNF signalling in Do.11.10 cells by inhibiting RIP1 recruitment to both TNFR1 and TNFR2. The mechanism by which A20 could promote Fas-mediated apoptosis is not clear. A20 does not have any effect on the expression of both Fas and FasL. Since A20 seems to affect TNF signalling very early in the signal transduction pathway and A20 co-precipitates with

activated TNFR1 (Zhang *et al.*, 2000) it would be interesting to see whether A20 is also recruited to Fas during AICD of T cell hybridomas. Clearly, this issue requires further investigation.

The observation that A20 is performing both anti- and pro-apoptotic roles in TCR-mediated apoptosis is important in the light of the recent findings, which indicate that some of the classical anti-apoptotic NF-κB target genes can also promote cell death. For example, c-IAP1 has been recently found to stimulate TNFR2-dependent degradation of the anti-apoptotic TRAF2 protein (Li *et al.*, 2002). In addition, c-FLIP protein has been reported to promote apoptosis by enhancing caspase-8 processing after Fas engagement. The pro-apoptotic function of c-FLIP operates at very low protein concentration (sub-stoichiometric amounts to caspase-8) and overexpression of c-FLIP, consistent with earlier reports, results in rescue from apoptosis. Taken together, these results argue that the propensity of a given gene (and consequently NF-κB as its regulator) to antagonise apoptosis may be a context-dependent phenomenon rather than an invariant feature.

4.9 Endogenous TNF induces Fas expression and sensitises to Fas-dependent cell death during AICD of Do.11.10 cells.

In this work endogenous TNF was found necessary for optimal AICD. This is an unexpected result in the light of a report, which investigated death receptor involvement in A1.1 hybridoma model of AICD (Wang *et al.*, 2001). These authors found exclusive requirement for Fas/FasL system in AICD. In fact results presented here argue that TNF acts to enhance Fas-mediated AICD in Do.11.10 hybridoma. It is possible that different hybridomas show different sensitivity towards Fas-induced apoptosis. In some hybridomas (e.g. Do.11.10), pre-treatment with recombinant TNF is required for effective Fas-mediated apoptosis (Elzey *et al.*, 2001). In accordance with that, CD8 T-cell blasts from *tnfr2*$^{-/-}$ knockout mice show defective Fas-mediated apoptosis (Teh *et al.*, 2000) and stimulation of naïve T cells with anti-TNFR2 agonistic Abs results in enhanced sensitivity to Fas crosslinking (Elzey *et al.*, 2001). Taken together, these results suggest a general positive influence of TNF (mediated by TNFR2) on the Fas pathway. It has previously been reported that TNF induces Fas upregulation in mouse embryonic fibroblasts (MEFs). NF-κB is critical for this process since *RelA*$^{-/-}$ MEFs do not induce Fas (Ouaaz *et al.*, 1999). These authors,

however, did not address whether similar requirements exist in T cells. In this work it has been demonstrated that NF-κB downstream of TNFRs is also critical for Fas induction in T cells. The absence of TNF-mediated Fas upregulation in NF-κB deficient cells correlates with reduced Fas-dependent apoptosis. The upregulation of Fas on T cells during AICD could result in sensitisation to apoptosis by increasing the probability of Fas engagement on the cell surface. This was, however, ruled out by experiments showing that Fas basal levels on T-cell hybridoma are sufficient for effective AICD. These results suggest that NF-κB promotes Fas-dependent cell death down-stream of Fas receptor possibly by induction of A20 expression.

4.10 TNFR2 is responsible for TNF signalling in Do.11.10 cells.

Most of the effects attributed to the action of TNF in Do.11.10 cells stimulated with αCD3 Abs are dependent on TNFR2 signalling. Indeed, there is growing evidence indicating that TNFR2 is the major TNF receptor in T cells, also for the induction of apoptosis (Zheng *et al.*, 1995)(Pimentel-Muinos and Seed, 1999). These data challenge other models, suggesting that TNFR1 is the exclusive mediator of TNF induced apoptosis (for review see, (Karin and Lin, 2002). TNFR2 seems to be responsible for induction of apoptosis in T cells despite the fact that it lacks a death domain. This raises the possibility that other TNFR superfamily members, which also lack a death domain and therefore have been originally excluded as potential apoptosis inducers, may turn out to participate in apoptosis regulation.

4.11 TCR activates *A20* gene expression - differential regulation of A20 mRNA and protein levels.

Results presented here (together with Zeller, 2001) demonstrate that TCR signalling of induces A20 expression in mature T cells. Clearly, NF-κB downstream of both TCR and TNFRs is necessary for full induction of *A20* mRNA. This is consistent with earlier reports, demonstrating functional NF-κB responsive elements in the *A20* promoter (Krikos *et al.*, 1992). The regulation of A20 expression by TCR at the protein level seems, however, quite unusual. In T cell hybridomas A20 protein is fully induced by TCR even in the presence of TNFR blocking reagents, which reduce the steady-state levels of *A20* mRNA and NF-κB activation. Furthermore, there is a

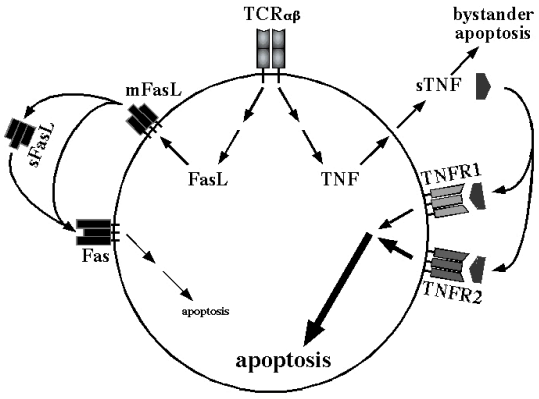
discrepancy between *A20* mRNA induction and protein levels. At 2 hr after initiation of TCR signalling, *A20* mRNA levels reach maximum whereas A20 protein levels are not induced at all. Steady-state levels of A20 protein overexpressed from retroviral vector also increase upon TCR signalling. This effect is not NF-κB dependent and is associated with increased A20 synthesis as demonstrated by pulse-chase experiments (not shown). Taken together, these results suggest that *A20* gene expression is controlled at the level of protein synthesis apart from transcriptional induction. The data presented here suggest a model in which both TCR and TNFR induce NF-κB and *A20* mRNA. In addition, both TCR and TNFR activate translation of *A20* mRNA in NF-κB independent fashion. Thus, the amount of A20 protein is not only limited by the levels of mRNA but also related to the regulated rate of translation. The translational regulation has also been described for another anti-apoptotic NF-κB gene, XIAP (Holcik *et al.*, 1999). It appears that cases where expression of a gene controlling apoptosis is regulated at multiple levels occur more often than previously appreciated.

A20 gene regulation at the level of translation could have several consequences for signalling in T cells. Firstly, high basal *A20* mRNA levels in naïve T cells enable a very fast appearance of A20 protein once signal to activate synthesis occurs. Secondly, some NF-κB inducing stimuli could fail to induce A20 protein despite the ability to induce *A20* mRNA upregulation. This would uncouple the signals resulting in NF-κB activation from the ones capable inducing A20 protein. Cells could exploit the latter possibility when NF-κB inducing stimulus (e.g. TCR) should not block TNF-mediated cell death, which is a mechanism of AICD in some primary cell models.

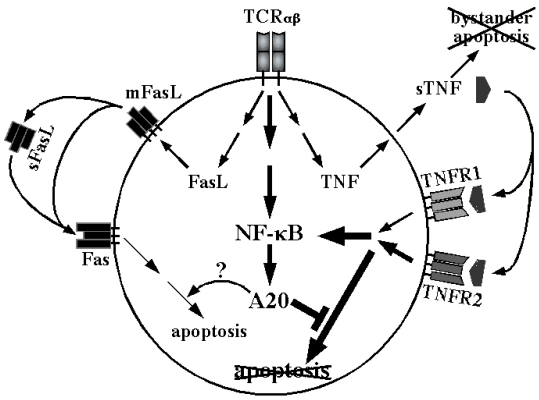
4.12 The role of NF-κB in TCR-mediated apoptosis of T cell hybridomas - the model.

The results presented in this work suggest a model in which NF-κB activated by both TCR and subsequently by secreted TNF induces transcription and synthesis of A20 protein. This is then responsible for inhibiting TNF-mediated cell death and at the very same time promotes FasL mediated apoptosis. Thus, A20 protein serves as both anti- and pro-apoptotic switch in TCR mediated apoptosis (Figure 21).

A current model involves AICD in regulation of both removal of activated T cells after an immune response as well as in balanced expansion of T cells during activation phase (Lenardo *et al.*, 1999). This is critical since the T cell compartment *in vivo* has limited capacity and a particular T cell clone should not dominate the immune response. Therefore, AICD operates in strictly antigen-dependent manner so that only TCR-stimulated T cells undergo PCD. In the absence of NF-κB, however, TNF secreted by TCR-stimulated cells kills NF-κB deficient cells *in trans*. In this so-called bystander cell death also non-activated cells die (Figure 21). For AICD this is a highly undesirable effect, disrupting its physiological function. In conclusion, NF-κB acting through *A20* ensures maintenance of clonal specificity during AICD.



AICD in the absence of NF-κB



AICD in the presence of NF-κB

FIGURE 21. Model of NF-κB function in AICD of T cell hybridomas (for details see text).

4.13 Nur77 and Rel/NF-κB families in TCR-mediated apoptosis – parallels and differences.

In this work the function of two different families of transcription factors in TCR-induced apoptosis have been studied. There are several parallels concerning the role of these proteins in AICD. Both Nur77 and Rel/NF-κB families are the primary targets of TCR signalling and probably regulate genes involved in TCR-mediated apoptosis. Their regulation by TCR seems to be independent, however, since Nur77 is induced normally in NF-κB-deficient hybridoma cells (data not shown). Nur77 inhibition leads to suppression of AICD of hybridomas (Woronicz *et al.*, 1994), whereas inhibition of NF-κB results in increased AICD ((Dudley *et al.*, 1999); Zeller, 2001 and this work). It is possible, however, that similarly to Rel/NF-κB, Nur77 regulates both pro- and anti-apoptotic genes in TCR-induced apoptosis and the observed phenotype after Nur77 inhibition is again only a net effect. The Nur77 target genes in TCR-induced apoptosis have not been characterised but the evidence presented in this work strongly suggests that they exist. A critical regulatory step in AICD of T cell hybridomas is the *FasL* gene induction. Majority of the transcription factors so far involved in AICD have been connected to regulation of the *FasL* promoter downstream of TCR. They include NFAT, EGR3 and c-myc (Rengarajan *et al.*, 2000)(Brunner *et al.*, 2000). It is therefore possible that Nur77 also regulates FasL gene transcription, which is further supported by the finding that *FasL* mRNA steady-state levels increase in thymi of FL-Nur77 tg mice (Weih *et al.*, 1996). Additional experiments will be necessary to clarify this issue. At the moment, there is no data available suggesting the existence of common target genes of Nur77 and Rel/NF-κB. Together with the notion that Nur77 and Rel/NF-κB are independently induced by TCR this suggests that these transcription factor families regulate distinct events in TCR-mediated apoptosis.

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