Growth inhibitory action of distinct sequences derived from the co-chaperone Bag-1L

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Zusammenfassung

Die Familie der Bag-1-Proteine (Bcl-2 assoziiertes Athanogen 1*)* besteht aus vier Mitgliedern: Bag-1L, Bag-1M, Bag-1 and Bag-1S. Die Proteine werden von derselben mRNA generiert, entstehen aber unter Verwendung alternativer Translationsinitiationsstellen. Bag1L, die längste Isoform, wird in einer großen Bandbreite von Tumoren überexprimiert, während es in benignem Gewebe gering nachweisbar ist, ein Befund, der auf eine Rolle des Proteins bei der Tumorprogression hindeutet. Die Auswirkung der erhöhten Expression von Bag1L in Tumorzellen wird unterschiedlich bewertet. Zum einen gilt die verstärkte Expression dieses Proteins ein Anzeichen für eine verbesserte Prognose, auf der anderen Seite wird Bag1L-Expression mit erhöhter Aggresivität des untersuchten Tumors assoziiert. Die Zielsetzung der vorliegenden Arbeit war es daher, die Funktion von Bag1-L bei der Entwicklung von malignen Prostatatumoren genauer zu untersuchen. Hierfür wurden Bag-1 defiziente Mäuse (Bag-$1^{-/-}$) mit Tieren eines Mausmodells für transgene Adenokarzinome der Mausprostata (TRAMP) verpaart. Die Analyse der aus dieser Verpaarung resultierenden Mäuse zeigte, dass sowohl das Tumorgewicht als auch der Verlauf der Erkrankung signifikant mit dem Expressionslevel von Bag1-L korrellierte. Überexpression von Bag1-L erhöhte die Fähigkeit der benignen Zelllinie BPH-1 Kolonien zu formen, zeigt aber einen gegenläufigen Effekt auf die malignen Zelllinie 22Rv.1. Die wachstumsinhibierende Funktion konnte auf einen im nachfolgenden Bag1- Peptid genannte 68 Aminosäuren umfassender Teilbereich von Bag-1L eingegrenzt werden. Die ektopische Expression des Bag1-Peptids reduzierte die Viabilität der malignen Zellen drastisch, während das Wachstum der benignen Zelllinie BPH-1 nicht signifikant beeinflusst wurde. Die Fähigkeit des Bag1-Peptids, das Wachstum von Prostatatumorzellen zu hemmen, konnte im Tierversuch unter Verwendung von Xenograf-Mausmodellen der humanen Zelllinien 22Rv.1 und LNCaP bestätigt werden. Überexpression des Bag1-Peptids in diesen Zelllinien veringert deren Fähigkeit Tumore zu bilden und erhöht die Apoptoserate in den Tumoren deutlich. Diese Wirkung des Peptids konnte mit seiner Interaktion mit den glukoseregulierten Proteinen GRP75 und GRP78 korelliert werden. Diese agieren während der Tumorentwicklung als molekulare

Chaparone. Immunfluoreszentfärbungen zeigten die Kollokalisation des Bag1-Peptids mit GRP75 und GRP78 im endoplasmatischen Retikulum und in der äußeren Membran der Mitochondrien. In Trunkierungsstudien konnte der N-terminale Bereich des Bag1-Peptids als der für die wachstumsinhibierende Wirkung verantwortliche identifiziert werden und in weiterführenden Studien schließlich auf 19 Aminosäuren eingegrenzt werden. Die vorliegende Arbeit beschreibt die Identifizierung und Charakterisierung einer kleinen Region des Co-Chaperones Bag1L als wachstumsinhibierendes Molekül, das für die Hemmung des Wachstums von Prostatatumorzellen eingesetzt werden kann.

Abstract

Bag-1 (Bcl-2 associated athanogene, member 1) is a family of proteins formed by four members (Bag-1L, Bag-1M, Bag-1 and Bag-1S) coded by the same mRNA via alternative translational initiation sites. The longest isoform of the family, Bag-1L, has been found overexpressed in a variety of cancers while it is almost undetectable in benign tissues, indicating its role in the transition of cells towards malignancy. However the exact function of Bag1-L in this process is controversial. On the one hand, high levels of this protein in tumour specimens are indicative of good prognosis and better patient outcome. On the other hand Bag-1L has been associated aggressive tumours. The aim of this work is therefore to clarify the role of Bag-1L in prostate cancer. For this purpose, transgenic adenocarcinoma of the mouse prostate (TRAMP) model was crossed with Bag-1 knock out mice (Bag-$1^{+/-}$). The resulting mice showed reduction in tumour weight and the level of expression of the Bag-1 proteins correlated with cancer formation. Moreover in clonogenic assay it was shown that Bag-1L overexpression increases colony formation in the benign cells BPH-1 while it has the opposite effect in the malignant cells 22Rv.1. A 68-amino acid sequence (defined as Bag-1 peptide) was identified to be responsible for the growth inhibitory function on the 22Rv.1 while it had no effect in the BPH-1. The ability of this peptide to inhibit prostate tumour cell growth was also demonstrated by establishment of xenografts with 22Rv.1 and LNCaP prostate tumour cells stably expressing the Bag-1 peptide. These cells showed a reduced ability to form tumours and increased apoptosis compared to the control. The growth inhibitory action of the peptide correlated with its ability to interact the glucose regulated proteins GRP75 and GRP78 that are molecular chaperones involved in cancer formation. Immunofluorescence experiments were used to show colocalisation of the peptide with the GRPs and localisation in the endoplasmic reticulum and the outer membrane of the mitochondria in tumour cells. The N-terminal part of the peptide was identified as the region responsible for its growth inhibitory action and was further narrowed down to the first N-terminal 19 amino acids. Thus in this work a small region derived from the co-chaperone Bag-1L has been identified as a growth inhibitory molecule that can be used to block growth of prostate tumour cells.

TABLE OF CONTENTS

TABLE OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AF-1	Activation function domain-1
Amp	amplitude
APS	Ammonium persulfate
AR	Androgen Receptor
ARE	Androgen responsive element
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
Bag	Bcl-2 associated athanogene
Bcl-2	B-cell leukemia/lymphoma 2
BiP	Binding protein
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	Bovine serum albumine
ºC	Degrees Celsius
CD	Circular Dichroism
cDNA	complementary DNA
CHIP	C-terminus of Hsc-70 interacting protein
CMV	cytomegalovirus
C-terminal	Carboxy- terminal
DAB	3'-3' diaminobenzidine
DBD	DNA binding domain
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	deoxynucleosides triphosphate
DTT	Dithiothreitol
dUTP	2'-deoxyuridine 5'-triphosphate
ECL	Enhancer of chemioluminescence
EDTA	Ethylenediamine Tetraacetic Acid
eg.	*Exempli gratia*, for example
eIF2a	elongation factor 2a
ER	Estrogen receptor
ER	Endoplasmic reticulum
et al.	*Et alii*, and others
FCS	Fetal calf serum
Fig.	Figure
FZK	Forschungszentrum Karlsruhe
g	gram
g	gravity (unit of relative centrifugal force)
GADD34	Growth Arrest and DNA Damage-Inducible Protein 34 kDa
GR	Glucocorticoid receptor
GRP	Glucose regulated protein
GST	Glutathion-S-transferase
h	hour
HA	hemagglutinin

HCl	Hydrochloric acid
Hip	Hsp70 interacting protein
Hop	Hsp70/Hsp90 organizing protein
HRP	Horseradish peroxidase
Hsc70	Heat shock cognate protein 70 KDa
Hsp	Heat shock protein
IF	immunofluorescence
IRES	Internal ribosomal entry site
ITG	Institute of Toxicology and Genetics
JCV	John Cunningham Virus
KCl	Potassium cloride
kDa	kilodalton
l	liter
LBD	Ligand binding domain
M	molar
µ	micro
m	milli
min	minute
MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	messenger RNA
mtHsp70	mitochondrial Hsp70
n	nano
NaCl	Sodium chloride
NMR	Nuclear magnetic resonance
NHR	Nuclear horomone receptor
NLS	Nuclear localisation signal
no	number
NP-40	Nonident P-40
N-terminal	Amino- terminal
OD	optical density
ON	overnight
PAGE	Polyacrylamide gel electrophoresis
PBS	Posphate buffer saline
PCBP1	Poly(rC) binding protein 1
PCD	programmed cell death
PCR	polymerase chain reaction
PFA	Paraformaldehyde
p	pico
PMSF	phenylmethanesulphonylfluoride
pro-HB-EGF	pro-heparin binding EGF-like growth factor
PTB	polypyrimidine tract binding protein
PVDF	Polyvinylidene difluoride
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rpm	race per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
s	second
SBD	Substrate binding domain
SD	standard deviation

SDS	Sodium dodecyl sulfate
SEM	standard error of the mean
SNT	supernatant
SPE	stimulated parametric emission
SV40	Simian Virus 40
TAE	Tri/acetate/EDTA elecrophoresis buffer
Tag	T antigen
TBS	Tris buffer saline
TCS	True confocal scanner
TdT	terminal deoxy nucleotidiltransferase
TEMED	tetramethylethylenediamine
TRAMP	Transgenic adenocarcinoma of the mouse prostate
Tris	Tris(hydroxymethyl)aminometane
TTBS	Tris buffer saline + Tween-20
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labelling
U	units
ULD	Ubiquitin-like domain
UPR	unfolded protein response
UV	ultraviolet
V	volt
WB	Western blot
VDR	Vitamin D receptor
w/o	without
v/v	volume on volume
v/w	volume on weight

1. INTRODUCTION

1.1 Molecular chaperones and carcinogenesis

Over the years organisms have developed systems to react to external insults which perturb normal cell homeostasis. One of the most conserved and studied of these systems is represented by the heat shock response, a mechanism first characterized as the reaction of cells to sudden increase of temperature (Ritossa, 1962). This kind of response allows cell survival in otherwise stressful conditions like exposure to heavy metals, lack of oxygen and nutrients, radiation and metabolic inhibitors. The characteristic feature of this system is the increased expression of a specific set of proteins termed the heat shock proteins or molecular chaperone (Amin *et al.*, 1988; Ritossa, 1962). The physiological role of this class of proteins is to prevent protein aggregation and misfolding during stress (Hightower, 1980), to regulate folding of nascent polypeptides (Frydman *et al.*, 1994) and to drive misfolded proteins to the proteasomal machinery for degradation (Hendrick and Hartl, 1993). In addition, the molecular chaperones play a role in gene expression, DNA replication, cell differentiation and proliferation, senescence, apoptosis and immortalization involving pathways crucial for embryonic development, cell homeostasis and cancer progression (Schmitt *et al.*, 2007)

One of the most representative subgroups of this class of proteins is the Hsp70 (Heat shock protein 70 kDa) family. This group of proteins consists of Hsc70 and Hsp70 that are the constitutively expressed and stress-induced isoforms of Hsp70 (Ritossa, 1962). Other members in this family are mtHsp70, also known as mortalin/GRP75 (Glucose Regulated Protein 75 kDa) (Bhattacharyya *et al.*, 1995) and BiP/GRP78 (Glucose Regulated Protein 78 kDa). Both GRP78 and GRP75 are induced by glucose depletion (Lee, 1992).

The Hsp70 molecular chaperones are generally organized in three distinct domains: an N-terminal ATPase domain, that covers one half of the protein and drives the hydrolysis of ATP necessary for the chaperone's activity (Bukau and Horwich, 1998); a central peptide/substrate binding domain through which the chaperones bind their client proteins (Flynn *et al.*, 1991; Rippmann *et al.*, 1991) and a C-terminal oligomerization domain that mediates the self interaction of the chaperones during

substrate binding (Palleros *et al.*, 1991). The action of these proteins is dependent on a cyclic series of events involving ATP hydrolysis. When bound to ATP, the molecular chaperones interact poorly with their substrate molecules (Steitz *et al.*, 1981). Binding affinity to the client proteins is increased during ATP hydrolysis. This is driven by the ATPase domain that triggers a conformational change throughout the protein upon ATP binding (Steitz *et al.*, 1981). The cycle of ATP hydrolysis ends with the binding of another molecule of ATP and the release of the client proteins (Steitz *et al.*, 1981).

Some members of the Hsp70 family are overexpressed in many tumours such as breast (Ciocca *et al.*, 1993), colon (Dundas *et al.*, 2005) and prostate tumours (Conford *et al.*, 2000) and are associated with poor prognosis and drug resistance neoplasia (Jäättelä, 1996). Intriguingly they are expressed on the surface of cells in the transition from the benign to the malignant state (Kaur *et al.*, 1998) providing the possibility to be used as biomarkers for discriminating between healthy and pathologic conditions.

A group of non-client proteins termed co-chaperones binds the molecular chaperones to modulate their activity. These proteins have functions ranging from catalysis of the nucleotide binding/hydrolysis, to the formation of multicomplexes and the choice of specific client proteins for the chaperones (Caplan, 2003). In addition they also have a chaperone activity themselves (Freeman and Yamamoto, 2002). One of the most characterized and studied groups of co-chaperones is the Bag family of proteins that serves as co-chaperones for Hsp70 proteins.

1.2 The Bag Family of Proteins

The Bag family of proteins consists of an evolutionary conserved group of proteins present in several species including yeasts, plants and mammals (Doukhanina *et al.*, 2006; Sondermann *et al.*, 2002; Sondermann *et al.*, 2001). The first gene of the Bag family was identified in a screen for interaction partners of Bcl-2 using mouse embryo expression cDNA library (Takayama *et al.*, 1995). This led to the identification of a gene that increased synergistically the anti-apoptotic action of Bcl-2 and was therefore named Bag-1, Bcl-2 associated athanogene (from the Greek: a-, anti- and thanaton, death) member 1.

In addition to Bag-1, five other members (Bag-2-6) were identified and these constituted the Bag family of proteins (Fig. 1). The Bag proteins are believed to

function as adaptors for the dynamic organization of complexes involved in several pathways, like apoptosis, cell proliferation, growth and stress response as well as events crucial for development, neurodegenerative diseases and cancer. Such a broad range of functions is covered by the presence of additional domains responsible for the specific action of every member of the family.

The Bag proteins share a common domain, the Bag domain, located in a single copy at their C-terminal region with the exception of Bag-5 that possesses five copies of this domain spread throughout the protein (Briknarova *et al.*, 2002). Through this domain the Bag family members interact with a wide variety of proteins including Bcl-2 (Takayama *et al.*, 1995), the molecular chaperone Hsp70 (Takayama *et al.*, 1999) and the E3 ubiquitin ligase CHIP (Carboxyl-terminus of Hsc70-interacting protein) (Arndt *et al.*, 2005). Nuclear magnetic resonance (NMR), X-ray crystallography and limited proteolysis studies identified the Bag domain as a 110-124 amino acid motif consisting of three antiparallel alpha helices of 30-40 amino acids each (Briknarova *et al.*, 2001; Brimmell *et al.*, 1999; Sondermann *et al.*, 2001). However the length of the Bag domain varies among the Bag family members, producing two distinct sub-groups. A "long" Bag domain present in Bag-1 family of proteins and a "short" Bag domain of Bag-3, Bag-4 and Bag-5 (Briknarova *et al.*, 2002). Alignment of the Bag domains of Bag-2 and Bag-6 showed a truncation of the second alpha helix of Bag-2, while in Bag-6 the region upstream the second helix contains several proline residues which most likely disorganizes the first helix (Briknarova *et al.*, 2002).

Bag-3 contains at the N-terminal region a WW domain, so called due to the presence of two conserved tryptophan (W) residues (Andre and Springael, 1994). Since the WW domain binds the proline-rich region XPPXY present in several proteins of the cytoskeleton, it is supposed that Bag-3 may mediate the chaperone action of Hsp70 with specific regulators of the cytoskeleton but this has not been fully investigate yet. Another domain present in Bag-3 is the PXXP domain that mediates the interaction of Bag-3 with proteins containing the SH3 domain. It has been reported that the PXXP domain of Bag-3 is important for the negative regulation of cell migration and adhesion by interfering with the interaction between the SH3 containing protein p^{130}Cas and the focal adesion protein FAK (Kassis *et al.*, 2006). Consistently it has been shown that Bag-3 PXXP domain interacts also with the SH3 domain of the latent PLC–γ (Doong *et al.*, 2000) a molecule involved as well in the regulation of cell

migration (Vidal *et al.*, 2001). These evidences indicate therefore that Bag-3 plays a role in cell motility through interactions mediated by its PXXP domain.

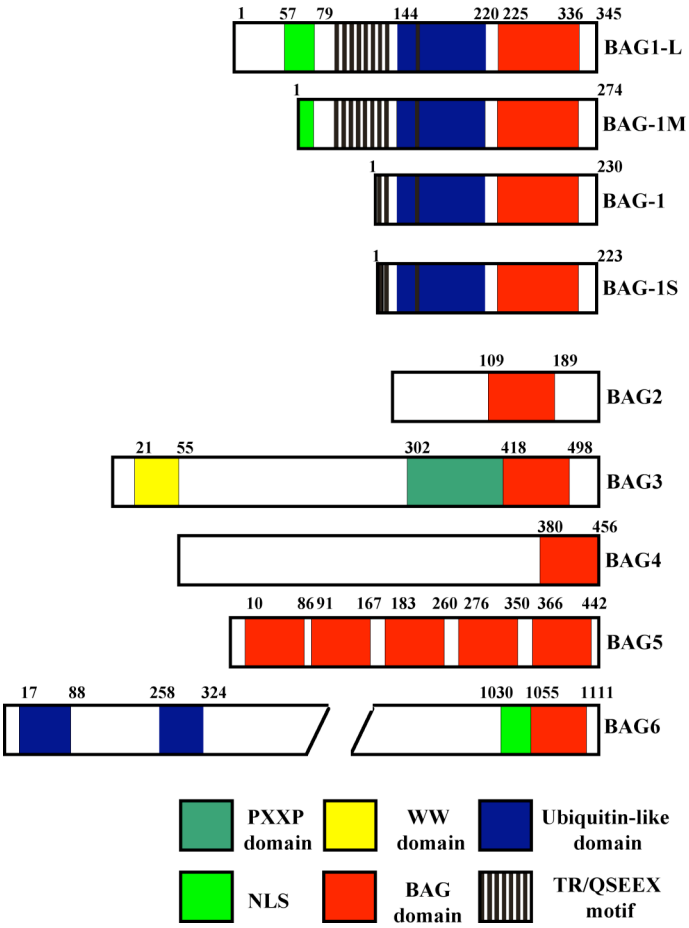

Fig. 1.1: The Bag-1 family.
The structure of human BAG-family proteins. In red is shown the BAG domain common to all the members of the family. In light green is represented the nuclear localization signal (NLS), in blue the ubiquitin-like domain, in yellow the WW domain, present only in BAG3 and in dark green the PXXP motif. The vertical bars represent the TXSEEX repeats present in the BAG-1 family members (adapted from Takayama and Reed, 2001).

A ubiquitin-like domain (ULD) is present in the central part of the Bag-1 proteins and in a double copy at the N-terminal part of Bag-6 (Manchen and Hubberstey, 2001). Ubiquitin is a 76 residues polypeptide binding covalently as a tag to proteins that are directed to the proteasome for degradation (Hershko *et al.*, 1979). In Bag-1, the ULD links the chaperone activity of Hsp70 to the degradation of unfolded substrates (Lüders, 2000). It interacts with the 20S core and 19S subunit of the proteasome in a complex involving Hsp70 and the E3 ubiquitin ligase CHIP where it facilitates the release of unfolded substrates to the proteasomal machinery (Demand *et al.*, 2001). The

two copies of ULD of Bag-6 do not have a clear function but it has been shown that deletion of both copies is lethal to the development of Xenopus embryos (Kikukawa *et al.*, 2005).

Specific motifs known as nuclear localization sequence (NLS) direct proteins to the nucleus. They consist of either a monopartite series of positively charged amino acids or a bipartite sequence formed by two of such series separated by a spacer sequence (Lange *et al.*, 2007). Some of the Bag family members possess a nuclear localization sequence for translocation in the nuclear compartment of the cell. Bag-1L contains at its N-terminal region a monopartite NLS and it is the only member of the family of Bag-1 proteins present exclusively into the nucleus (Knee *et al.*, 2001). A bipartite NLS common to all the members of the Bag-1 family occurs upstream of the Bag domain (between amino acids 219-234 of Bag-1L) (Zeiner and Gehring, 1995) but its function has not been well characterized. Bag-6 contains a NLS right upstream of the Bag domain and mutation of this sequence leads to nuclear exclusion of the protein (Manchen and Hubberstey, 2001).

A domain unique to the Bag-1 proteins is the TR/QSEEX motif present in nine copies in Bag-1L and M and three in Bag-1 and Bag-1-S (Townsend *et al.*, 2003a) The predicted structure of the repeats is an alpha helix with the acidic residues oriented toward one side. This structure represents the site of phosphorylation for some kinases (e.g. creatine kinase) (Takayama *et al.*, 1998) and it has indeed been found to be phosphorylated (Schneikert *et al.*, 2000) but its function is not known yet.

Among all the Bag proteins the best studied in different signal transduction pathways and in transcriptional regulation is the Bag-1 family of proteins.

1.3 The Bag-1 family of proteins

1.3.1 Isoforms and regulation of expression

In humans the Bag-1 family consists of four members (50 kDa Bag-1L, 46 kDa Bag-1M - otherwise known as RAP46/HAP46 - 36 kDa Bag-1 and a 29 kDa Bag-1S) encoded by the same mRNA via alternative translational initiation site (Yang *et al.*, 1998). The translation initiation site of the largest isoform, Bag-1L, consists of a non-canonical CUG, while three in-frame AUG downstream form the start codons for the

other isoforms (Yang *et al.*, 1998). All the Bag-1 isoforms share a common C-terminal region but with variable lengths of N-termini (Takayama *et al.*, 1998) (Fig.1.1). In mice, the isoforms produced are only the longest 50 kDa Bag-1L and the shorter 32 kDa Bag-1S (Packham *et al.*, 1997).

Translation of Bag-1S can also occur via an internal ribosomal entry site (IRES) that enables protein expression in a cap-independent manner providing constant levels of Bag-1 protein as a fast response to stress conditions (Dobbyn *et al.*, 2008). The action of the IRES requires two trans-acting factors: poly(rC) binding protein 1 (PCBP1) and polypyrimidine tract binding protein (PTB) (Takahashi *et al.*, 2001). These proteins accumulate in the cytoplasm in response to chemotoxic stress induced by vincristine that leads to increased translation of Bag-1S. In general the expression levels and the cellular localization of the different Bag-1 isoforms can vary considerabily for different pathological conditions (Takayama *et al.*, 1998).

1.3.2 The Bag-1 proteins are multifunctional

The Bag-1 family members interact with several proteins involved in apoptosis, proliferation, stress response and regulation of transcription. For this reason these proteins are considered multifunctional. Some of their actions are isoform specific and/or cell type specific. The main interaction partners and their actions are summarized in Table 1.1.

1.3.2.1 Regulation of Hsp70 activity and stress response

The most studied (and the strongest) interaction partner of the Bag-1 proteins is Hsp70. This protein uses the ATPase domain at its N-terminus to bind to helices 2 and 3 of the Bag domain in a stoichiometry of 1:1 and with a dissociation constant K_d of 1-3 μM (Sondermann *et al.*, 2001). In this respect Bag-1 serves as a nucleotide exchange factor of Hsp70 and plays a role in the chaperone activity of this protein by allowing the release of folded substrates. This action of Bag-1 is competitively inhibited by another co-chaperone, Hip (Hsp70 inhibitor protein), and stimulated by Hsp40 (Nollen *et al.*, 2001).

Isoform	Interaction partner	Action	Reference
All Bag-1 proteins	Bcl-2	Inhibition of apoptosis	(Takayama *et al.*, 1995)
All Bag-1 proteins	Hsp-70	Regulation of chaperone activity	(Takayama *et al.*, 1997)
Bag-1-L/M	GR	Reduction of DNA binding	(Kullmann *et al.*, 1997)
Bag-1L	ER	Increase of transcriptional activity	(Cutress *et al.*, 2003)
Bag-1	RAR	Inhibition of transcriptional activity	(Liu *et al.*, 1998)
Bag-1-L	AR	Increase of transcriptional activity	(Froesch *et al.*, 1998)
Bag-1-L	VDR	Activation Inhibition Metabolism	(Guzey *et al.*, 2000) (Witcher *et al.*, 2001) (Lee *et al.*, 2007)
Bag-1; Bag-1-S	Raf-1	Proliferation	(Wang *et al.*, 1996)
All Bag-1 proteins	GADD34	Inhibition of UPR	(Hung *et al.*, 2003)

GR= Glucocorticoid receptor; RAR= Retinoic acid receptor; AR= Androgen receptor; VDR= Vitamin D receptor; UPR= Unfolded protein response

Table 1.1: Interaction partners and activity of the Bag-1 proteins
The Bag-1 proteins exert multiple functions through the interaction with different proteins. The table lists the main interaction partners and the action of the Bag-1 proteins.

In stressful conditions, a mechanism that protects the cells from the lethal action of heavy metal exposure, heat shock, hypoxia, etc. is induced. In this context, Bag-1 proteins provide a pro-survival advantage to the cell through their interaction with Hsp70. For instance, overexpression of Bag-1 isoforms increases survival of MCF-7 breast cancer cells after heat shock and point mutations in the Bag domain that destroy its binding to Hsp70 fail to rescue the cell (Townsend *et al.*, 2003b). A further confirmation that the pro-survival action of Bag-1 is mediated by its interaction with Hsp70 comes from studies that show that overexpression of Bag-1 but not of a deletion mutant lacking the region of interaction with Hsp70, in rat nigral CSM14.1 and human neuroblastoma SHSY-5Y cells, induces resistance to staurosporine and thapsigargin treatment (Liman *et al.*, 2005).

The protective function of the Bag-1 proteins is also dependent on the subcellular localization. For example cytoplasmic Bag-1S protects primary cardiac mouse myocytes from simulated ischemia/reperfusion (Townsend *et al.*, 2004) while direction of Bag-1S to the nucleus via the fusion to a nuclear localization signal abrogated its cytoprotective action. The protective action of Bag-1 has also been confirmed in an *in vivo* mouse model of stroke (Kermer *et al.*, 2003) where gene transfer of Bag-1

increased survival of neurons upon glutamate treatment and decreased mortality after stroke injury (Kermer *et al.*, 2003).

The attenuation of stress response by the Bag-1 proteins is mechanistically described as interference in the function of the stress protein GADD34 (Growth Arrest and DNA Damage-Inducible Protein 34 kDa). Exposure to stressful conditions induces protein aggregation and misfolding resulting in cell toxicity. Activation of a response termed unfolded protein response (UPR) represents the reaction of cells to stress leading to reduction of protein aggregation and attenuation of transcription (Kaufman, 1999). During UPR, GADD34 interacts with the protein phosphatase PP1 and attenuates the translational elongation through dephosphorylation of the elongation factor 2α (eIF2α) (Connor *et al.*, 2001) resulting in shutoff of protein synthesis. Bag-1 proteins interact with GADD34 during stress response in a complex including also Hsp70 and PP1 resulting in the inhibition of GADD34 effect on protein synthesis and in inhibition of stress-induced apoptosis (Hung *et al.*, 2003).

1.3.2.2 Regulation of proliferation and apoptosis

Bag-1 proteins were first identified as interactors of Bcl-2 leading to synergistic inhibition of apoptosis when the two genes were co-expressed in the lymphoid Jurkat cell line (Takayama *et al.*, 1995). Since then several mechanisms have been proposed to explain the action of Bag-1 in cell proliferation and apoptosis.

First, Bag-1 is reputed to regulate cell proliferation and cell growth. through its interaction with the serine/threonine kinase Raf-1 (Wang *et al.*, 1996). Raf-1 phosphorylates and activates the MAP kinase cascade leading to enhanced cell proliferation (Weidong *et al.*, 1993). It is thought that Bag-1 can increase cell growth through its interaction with this protein. In addition it has been shown that Bag-1 concentrates Raf-1 at the proximity of the mitochondrial membrane where it interacts with Bcl-2. In this situation, Raf-1 can phosphorylate proteins to which it is otherwise inaccessible such as the pro-apoptotic protein Bad which is then dissociated from Bcl-X_L leading to cell survival (Wang *et al.*, 1997). Bag-1 proteins also interact with B-Raf in a complex with Akt and Hsp70 which leads to the phoshorylation of Bad at serine 136 and the inhibition of apoptosis. Disruption of this complex results in early lethality

in hematopoietic and neuronal cells in a Bag-1 knock-out mouse model (Götz *et al.*, 2005).

Second, Bag-1 is reputed to bind to the membrane form of the heparin binding EGF-like growth factor (pro-HB-EGF) (Lin *et al.*, 2001) to decrease etoposide-induced apoptosis in LNCaP and PC3 metastatses-derived prostate cancer cells. The interaction of the two proteins occurs through the cytoplasmic tail of pro-HB-EGF and a region on Bag-1 including the ULD but lacking the Bag domain.

1.3.2.3 Regulation of transcription

Bag1 proteins can regulate transcription either directly, by functioning as transcription factors, or indirectly, by influencing the activity of other transcription factors like the nuclear hormone receptors (NHR).

1.3.2.3.1 Bag-1 proteins as transcription factors

The genes that are activated directly by the Bag-1 proteins are mainly viral genes. For instance Bag-1M, but not Bag-1L and Bag-1S, can stimulate the transcriptional activity of the Cytomegalovirus (CMV) early region promoter requiring both the N- and the C-terminal regions (Takahashi *et al.*, 2001). Moreover Bag-1S can function as transcription factor stimulating the transcription of the John Cunningham Virus (JCV) early promoter (Devireddy *et al.*, 2000). The mechanism by which the Bag-1 proteins regulate the viral gene expression is not understood but it is likely to be through discreet binding sites of the protein to DNA. It has been reported that Bag-1M is able to bind DNA through the first 10 amino acids at the N-terminal region (Zeiner *et al.*, 1999). These consist of two clusters of three lysine (from 2 to 4) and three arginins (from 6 to 8) included in the nuclear localization sequence present only in the isoforms Bag-1M and -L (Zeiner *et al.*, 1999).

1.3.2.3.2 Bag-1 proteins and the nuclear hormone receptors

Bag-1 proteins have also been reported to bind nuclear receptors and to influence their transcriptional activity. Nuclear hormone receptors are transcription factors

structurally organized in distinct domains: an the N-terminal activation function domain (AF-1) followed by a DNA binding domain, a hinge region, a ligand binding domain (LBD) and a variable C-terminal domain according to the subtype of receptor (Kumar and Thompson, 1999).

In all the cases analysed it was found that the C-terminal region of the Bag-1 proteins binds the nuclear receptors (Froesch *et al.*, 1998; Guzey *et al.*, 2000; Kullmann *et al.*, 1997; Liu *et al.*, 1998). Since this region also binds Hsp70 (Takayama *et al.*, 1997) this indicates that the mediation of Hsp70 in the regulation of nuclear receptor action by the Bag-1 proteins is required. The following examples are some of the reported specific interactions and modulation of nuclear receptor actions by Bag-1 proteins (see also Table 1.1 for a summary).

1. Bag-1-M interacts with the GR and inhibits, together with Hsp70, dexamethasone-induced activation of transcription by the receptor (Kullmann *et al.*, 1997; Schneikert *et al.*, 2000). It has also been shown that the binding of Bag1-M to the GR destroys the interaction of the receptor with its responsive elements on the DNA (Kullmann *et al.*, 1997; Schmidt *et al.*, 2003).

2. The longest isoform Bag-1-L binds the androgen receptor (AR) to enhance its activation upon hormone binding (Froesch *et al.*, 1998). The binding to the receptor takes place through an head-to-tail interaction between the Bag domain and the AF-1 domain of the AR on one hand, and the N-terminal region of Bag-1-L and the ligand binding domain of the AR on the other hand (Shatkina *et al.*, 2003) Bag-1L also binds chromatin on the androgen responsive elements (AREs) playing a role in the recruitment of the receptor to its response element (Shatkina *et al.*, 2003).

3. Bag-1L interacts and regulates the action of the Vitamin D_3 receptor (VDR) (Guzey *et al.*, 2000). This effect seems to be cell type specific since in glioblastoma cells Bag-1L inhibits VDR activation upon treatment with 1,25-dihydroxyvitamin D_3 (Witcher *et al.*, 2001). Conversely it has been reported that in oral keratinocytes (Lee *et al.*, 2007) and in the prostate cancer bone metastasis-derived cell line PC3 (Guzey *et al.*, 2000) Bag-1L enhances VDR transactivation. Bag-1L acts on VDR-dependent transcription also by enhancing the synthesis of the 24-hydroxylase, an enzyme important for the catabolism of vitamin D_3, influencing therefore the levels of the ligand and its negative feedback loop regulation (Lee *et al.*, 2007).

1.3.3 Bag-1 in cancer

In addition to its effect in transcription, studies of overexpression of Bag-1 proteins *in vitro* and *in vivo* led to the suggestion that Bag-1 levels could be used as prognostic marker for patient outcome. On the other hand, changes in levels of Bag-1 can also affect tumour progression.

1.3.3.1 Bag-1 as prognostic marker

Overexpression of Bag-1 has been detected in patient specimen of colorectal tumour (Clemo *et al.*, 2008), laryngeal cancer (Yamauchi *et al.*, 2001), cervical tumour (Yang *et al.*, 1999) and follicular thyroid carcinoma (Ito *et al.*, 2003). In breast cancer nuclear expression of Bag-1 has been associated with poor prognosis (Tang *et al.*, 1999) while reduced nuclear and increased cytoplasmic levels have been related to invasive tumours (Krajewski *et al.*, 1999). Intense staining for Bag-1 proteins has also been detected in the nucleus and the cytoplasm of prostate cells in early stage prostate cancer patient associated with more aggressive tumours (Krajewska *et al.*, 2006). In addition it has been shown that Bag-1 gene is amplified and overexpressed in hormone-refractory prostate cancer (Mäki *et al.*, 2007). Intriguingly increased levels of Bag-1 can be also predictive of better patient outcome and overall survival when detected in early stage breast cancer (Millar *et al.*, 2008; Nadler *et al.*, 2008; Turner *et al.*, 2001) and lung carcinoma (Rorke *et al.*, 2001).

1.3.3.2 Bag-1 in tumour progression

From *in vitro* experiments it has been shown that Bag-1 proteins can increase cancer cell migration implicating a possible role in metastasis. For example Bag-1 overexpression increased motility of MKN74 cells derived from well differentiated gastric cancer (Naishiro *et al.*, 1999). In addition stable transfection of these cells with Bag-1 and injection into the peritoneal cavity increased metastatic lesion formation (Yawata *et al.*, 1998). Similarly an increase in metastatic potential upon Bag-1 gene transfer has also been observed in B16 melanoma cells injected into immunodeficient mice (Takaoka *et al.*, 1997). The influence of Bag-1 on metastases has been

demonstrated also in a lung-metastasis mouse model where reduction of Bag-1 gene level resulted in decreased number of lung metastatic foci (Götz *et al.*, 2004).

The overexpression of Bag-1 proteins in cancer is directly correlated with the anti-apoptotic action of these proteins resulting in a survival advantage for the cancer cells. In some cases it has been described that the four isoforms of the Bag-1 family have distinct anti-apoptotic function in tumour cells. For example Bag1-L and Bag-1M are shown to be the only members of the family that increase resistance to anti-cancer drug treatment in cervical carcinoma cells (Chen *et al.*, 2002) and in breast cancer cell line MCF-7 while the other isoforms are uneffective (Liu *et al.*, 2009).

From *in vivo*, *in vitro* and clinical studies it is clear that the role of Bag-1 in cancer is still controversial and strongly dependent on factors such as cell type, stage of the neoplasia and relative abundancy of the isoforms of the family.

1.4 AIM

Bag-1 family of proteins consists of four members, Bag-1L (p50), Bag-1M (p46), Bag-1 (p32) and Bag-1S (p29) originating from the same mRNA through different translational initiation sites by a leaky mechanism. These proteins regulate diverse cellular processes in physiological and pathophysiological situations. In cancer their role is controversial since in some cases they are associated with good prognosis and better patient outcome when overexpressed and in other cases their increased levels are indicative of aggressive and invasive tumours. One Bag-1 isoform associated with such reported dual function is Bag-1L.

The aims of this study are three-fold. First, to determine whether changes in Bag-1L levels can influence prostate cancer cell growth. Second, to identify specific sequences on Bag-1L that regulate prostate cancer cell growth. Third, to investigate whether these sequences can be used as tools in prostate cancer therapy.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and consumables

Name	Source
Agarose	Peqlab, Erlangen
Ampicillin	Roth, Karlsruhe
Ammonium Persulfate (APS)	Roth, Karlsruhe
Bovine Serum Albumine (BSA)	PAA Laboratories GmbH, Pasching
Bacto-Agar	Otto Nordwald GmbH, Hamburg
Bacto-petri dishes	Greiner Labortechnik, Nürtingen
Bacto-Trypton	Roth, Karlsruhe
Bacto-yeast extract	Roth, Karlsruhe
Bromophenolblue	Sigma–Aldrich Chemie, Steinheim
n-buthanol	Roth, Karlsruhe
Chloroform	Merck, Darmstadt
Crystal violet	Lighting Powder Company, INC.,
Dimethylsuloxide (DMSO)	Fluka, Neu Ulm
Dithiothreitol (DTT)	Gibco, Invitrogen, Karlsruhe
DNA Marker 1 Kb	PeqLab, Erlangen
DNA Marker 100 bp	PeqLab, Erlangen
Dulbecco's Modified Eagle Medium (DMEM)	Gibco, Invitrogen, Karlsruhe
ECL™ Western Blot Detection Reagents	Amersham Pharmacia Biotech, Freiburg
Ethylenediamine Tetraacetic Acid (EDTA)	Roth, Karlsruhe
Ethanol (EtOH)	Roth, Karlsruhe
Ethidium Bromide	Roth, Karlsruhe
Fibronectin	Sigma Aldrich, Taufkirchen

FBS (Fetal Bovine Serum)	Gibco, Invitrogen, Karlsruhe
G418 (Geneticin®)	Sigma Aldrich, Taufkirchen
Glycylglycerine	Roth, Karlsruhe
Glycine	Roth, Karlsruhe
Glycerol	Roth, Karlsruhe
Glucose	Roth, Karlsruhe
Glutamine	Sigma Aldrich, Taufkirchen
Hydrogen Chloride (HCl)	Roth, Karlsruhe
Isopropanol	Roth, Karlsruhe
Magnesium Chloride	Roth, Karlsruhe
Magnesium Sulfate	Roth, Karlsruhe
Methanol (MeOH)	Roth, Karlsruhe
β-mercaptoethanol	Roth, Karlsruhe
Milk powder	Saliter, Obergünzburg
Nonident P-40 (NP40)	Boehringer, Mannheim
Phosphate Buffered Saline w/o $CaCl_2$ and $MgCl_2$ 1X and 10X	Gibco, Invitrogen, Karlsruhe
Phenol	Roth, Karlsruhe
PMSF (phenyl methanesulphonyl fluoride)	Sigma Aldrich, Taufkirchen
Potassium Chloride	Merck, Darmstadt
Poly-L-lysine	Sigma Aldrich, Taufkirchen
Protein Marker	PeqLab Erlangen
Rotiphorese® Gel30: Acrylamide/ bis-acrylamide (30%/0,8%)	Roth, Karlsruhe
Rotisol	Roth, Karlsruhe
RPMI medium 1640	Gibco, Invitrogen, Karlsruhe
Sodium Acetate	Roth, Karlsruhe
Sodium Chloride	Roth, Karlsruhe
Sodium Dodecyl Sulphate (SDS)	Roth, Karlsruhe
Sodium Hydroxide	Roth, Karlsruhe
Sodium N-lauryl sarcosinate (Sarkosyl)	Sigma Aldrich, Taufkirchen
Tetramethyl ethylen diamine (TEMED)	Roth, Karlsruhe
Tris-base	Roth, Karlsruhe

Tris-HCl	Roth, Karlsruhe
Triton-X-100	Sigma Aldrich, Taufkirchen
Trypsin 0,025%	Difco, Detroit
Trypsin 0,025% with EDTA	Gibco, Invitrogen, Karlsruhe
Triton-X-100	Biorad, Munich
Tween 20	Roth, Karlsruhe

2.1.2 Bacteria and eukariotic cell lines

2.1.2.1 Bacteria

Name	Genotype
E. Coli DH5 α (for standard cloning)	F-, end A1, hsd R17 (r_k^-, m_k^+), sup E44, thi-1, λ-, rec A1, gyr A96, relA1
E. Coli BL21 (for the expression of the GST-fused proteins)	F-, ompT, $hsdS_B$ (r_B^-, m_B^-), dcm, gal, DE3, pLysS (Cm^r),

2.1.2.2 Eukariotic cell lines

All the cells grew adherently in monolayers at 37 °C with 5% CO_2 on Cellstar® Petri dishes (Greiner Bio-one, Frickenhausen, Germany).

Name	Source and description
22Rv.1	Human prostate carcinoma cell line derived from a human prostate carcinoma xenograft (CW22R) that was serially propagated in nude mice after castration-induced regression and relapse of the parental, androgen dependent CWR22 xenograft.
LNCaP	Human cancer cell line derived from a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year-old Caucasian male with confirmed diagnosis of metastatic prostate carcinoma.
BPH-1	Human epithelial cells from a 68-year-old man with benign prostate hyperplasia immortalized with SV40 large T antigen. They express cytokeratins 8, 18 indicative of a luminal specific origin and 19, lost during neoplastic progression, but not 14 and 7, overexpressed in carcinoma.

2.1.3 Oligonucleotides for cloning experiments

Specific primers were used to amplify portions of genes of interest using Polimerase Chain Reaction technique. The oligonucleotides were designed in order to have specific and unique restriction sites to clone the fragment of interest into the desired vector.

For cloning of the Bag-1 derived peptides into pcDNA3.1-HA the restriction site for the endonuclease BamHI was designed in every forward primer while XbaI site was designed for the reverse primers.

For cloning GRP5, GRP78 and their deletion mutants into pGEX4T.1 the restriction site for BamHI was designed in every forward primer and the restriction site for XhoI for every reverse.

The following primers were used for cloning the Bag-1 peptide (202-269), the N-terminal peptide (202-241), the C-terminal peptide (241-269), the ΔN-peptide (220-269), the 19-mer peptide (202-220) and the 22-mer peptide (220-241) into pcDNA3.1-HA (the direction is always 5' – 3').

Name	**Sequence** *(restriction site underlined)*
202_forward_BamHI	TCG GAT CCC CAA GAA GAA GCG GAA GGT CCG AAC ACC GTT GTC AGC ACT TGG
220_forward_BamHI	TCG GAT CCC CAA GAA GAA GCG GAA GGT CCG AAA AAA GAA CAG TCC ACA G
241_forward_BamHI	TCG GAT CCC CAA GAA GAA GCG GAA GGT CCG AGA GAA GAT AGC TGA CCA GC
269_reverse_XbaI	TCT CTA GAT CAT TCA GCT TGC AAA TCC TTG GG
241_reverse_XbaI	TCT CTA GAT CAC TCC ACA GAC TTC TCC
220_reverse_XbaI	TCT CTA GAT CAT TTC CCA ATT AAC ATG ACC CGG C

For cloning into pGEX4T.1 GRP75 (1-679), GRP75ΔATPase (434-679), GRP75-ATPase domain (1-433), GRP75 substrate binding domain (434-588), GRP75 Oligomerization domain (589-679), GRP78 (1-651), GRP78ΔATPase (408-651), GRP78-ATPase domain (1-407), GRP78 substrate binding domain (408-567), GRP78 Oligomerization domain (568-651) the following primers were used (the direction is always 5' – 3'):

Name	**Sequence** *(restriction site underlined)*
GRP75_1_forward_BamHI	CCG GAT CCA TGA TAA GTG CCA GCC G
GRP75_434_forward_BamHI	CCG GAT CCG ATG TGC TGC TCC TTG ATG TC
GRP75_589_forward_BamHI	CCG GAT CCA TTC ACG ACA CAG AAA CC
GRP75_433_reverse_XhoI	CGC TCG AGT CAC GTG ACA TCG CCG GCC
GRP75_588_reverse_XhoI	CGC TCG AGT CAG ATT CCT TCA GCC
GRP75_679_reverse_XhoI	CGC TCG AGT CAC TGT TTT TCC TCC TTT TGA TC
GRP78_1_forward_BamHI	CCG GAT CCA TGA AGC TCT CCC TGG TGG CCG CG
GRP78_408_forward_BamHI	CCG GAT CCC AAG ATA CAG GTG ACC TGG
GRP78_568_forward_BamHI	CCG GAT CCG CCT ATT CTC TAA AGA ATC AG
GRP78_407_reverse_XhoI	CGC TCG AGT CAA TCA CCA GAG AGC ACA CC
GRP78_567_reverse_XhoI	CGCTCGAGTCAATAGCTTTCCAACTC
GRP78_651_reverse_XhoI	CGC TCG AGT CAC AAC TCA TCT TTT TCT GC

2.1.4 Antibodies

Name	Description	Experimental conditions	Producer	Application
Anti-HA	Mouse monoclonal antibody	1:100 in TBS 1X + 10% Milk, ON, 4ºC		WB
Anti-HA (clone 3F-10)	Rat monoclonal antibody	1:1000 in blocking buffer, 1 h, RT	Roche Diagn.	IF
Anti-GRP78 (N-20)	Goat polyclonal antibody recognizing GRP78 of rat, mouse and human origin	1:1000 in TBS 1X + 10% BSA, ON, 4ºC	Santa Cruz Biotech.	WB
Anti-GRP78	Rabbit polyclonal antibody recognizing GRP78 of human, mouse, rat, chinese hamster and pig origin	1:500 in blocking buffer (4% goat serum in PBS 1X), 1 h, RT	Abcam	IF
Anti-GRP75 (H-155)	Rabbit polyclonal antibody recognizing GRP75 of rat, mouse and human origin	1:1000 in TBS 1X + 10% BSA, ON, 4ºC	Santa Cruz Biotech.	WB
		1:250 in blocking buffer (4% goat serum in PBS 1X), 1 h, RT		IF
Anti-Bag1 (FL-274)	Rabbit polyclonal antibody recognizing all the Bag-1 proteins	1:1000 in TBS 1X + 5% Milk, 4 h, RT	Sigma	WB

WB= western blot; IF= immunofluorescence; TBS= Tris buffered saline; BSA= bovine serum albumine; RT= room temperature; ON= overnight

2.1.5 Secondary antibodies

All secondary antibodies HRP-conjugated were purchased from DAKO Diagnostic GmBH (Hamburg, Germany). All secondary fluorescently-labelled antibodies were purchased from Jackson Immuno Research (USA).

2.1.6 Restriction enzymes

All restriction enzymes were purchased from New England Biolabs (Beverly, USA), Promega (Mannheim, Germany) and Invitrogen GmbH (Karlsruhe, Germany).

2.1.7 Plasmids

The following plasmids made in the vector pcDNA3.1-HA (made by Dr. S. Mink) from the vector pcDNA3.1 (Invitrogen, Karlsruhe, Germany) were used for stable or transient transfections into mammalian cells..

Name	Description
pcDNA3.1-Bag-1L	Expression plasmid with the cDNA of the protein Bag1-L. The starting codon CUG is mutated into ATG
pcDNA3.1-HA-Bag-1LΔ202-269	Expression plasmid with the cDNA of the protein Bag1-L lacking the fragment between position 202 and 269. The starting codon CUG is mutated into ATG
pcDNA3.1-HA-Bag-1 peptide	Expression plasmid with the cDNA of the protein Bag1-L form position 202 to 269
pcDNA3.1-HA-ΔN peptide	Expression plasmid with the cDNA of the protein Bag1-L form position 220 to 269
pcDNA3.1-HA N-terminal peptide	Expression plasmid with the cDNA of the protein Bag1-L form position 202 to 241

pcDNA3.1-HA C-terminal peptide	Expression plasmid with the cDNA of the protein Bag1-L from position 241 to 269
pcDNA3.1-HA-19-mer peptide	Expression plasmid with the cDNA of the protein Bag1-L from position 202 to 220
pcDNA3.1-HA-22-mer peptide	Expression plasmid with the cDNA of the protein Bag1-L from position 220 to 241

Plasmids for production and purification of GST-fused proteins. The following plasmids were made in the vector pGEX4T.1 (GE Healthcare, Frieburg, Germany).

Name	**Description**
pGEX4T.1 GRP75	Encodes fort he full length GRP75
pGEX4T.1 GRP75ΔATPase	Encodes for a deletion mutant of GRP75 covering its sequence from position 434 to 679
pGEX4T.1 GRP75 ATPase domain	Encodes for a deletion mutant of GRP75 covering its sequence from position 1 to 433
pGEX4T.1 GRP75 substrate binding domain	Encodes for a deletion mutant of GRP75 covering its sequence from position 434 to 588
pGEX4T.1 GRP75 oligomerization domain	Encodes for a deletion mutant of GRP75 covering its sequence from position 589 to 679
pGEX4T.1 GRP78	Encodes for the full length GRP78
pGEX4T.1 GRP78ΔATPase	Encodes for a deletion mutant of GRP78 covering its sequence from position 408 to 651
pGEX4T.1 GRP78 ATPase domain	Encodes for a deletion mutant of GRP75 covering its sequence from position 434 to 679
pGEX4T.1 GRP78 substrate binding domain	Encodes for a deletion mutant of GRP75 covering its sequence from position 408 to 567
pGEX4T.1 GRP75 oligomerization domain	Encodes for a deletion mutant of GRP75 covering its sequence from position 568 to 651

2.2 Methods

2.2.1 Cloning methods

2.2.1.1 Polymerase chain reaction (PCR)

All Polymerase chain reactions were carried out in a Thermal Cycler machine (GeneAmp® PCR System 2700, Applied Biosystem). The reaction volume was usually of 20-50 µl containing 10 ng of plasmid template or 100 ng of genomic cDNA template. The PCR reaction solution also contained 100-200 µM deoxynucleosides triphosphate (dNTPs), 10 pmol of each primer, reaction buffer (containing a final concentration of 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% Triton X-100 and 0.1 mg/ml bovine serum albumine) and 0.25-1 unit (U) of Pfu proof-reading DNA polymerase (from the archaeon Pyrococcus furiosius). The Pfu was stored in storage buffer (20 mM Tris-HCl, pH 8.2, 1 mM DTT, 0.1% Tween 20 and 50% glycerol) at -20ºC. Cycle number and reaction conditions were determined empirically for each fragment of DNA to amplify.

For cloning the Bag-1 peptide, the N-terminal peptide, the C-terminal peptide, the 19-mer peptide and the 22-mer peptide the following programme was used:

1. 94ºC, 1’
2. 94ºC, 1‘
3. 55ºC, 1’ 30”
4. 72ºC, 1’
5. Hold at 4ºC

The steps 2-4 were repeated for 30 cycles.

For cloning GRP75ΔATPase, GRP75-substrate binding domain, GRP75-oligomerization domain, GRP78ΔATPase, GRP78-substrate binding domain and GRP78-oligomerization domain the following programme was used:

1. 94ºC, 3’
2. 94ºC, 30”
3. 60ºC, 1’
4. 72ºC, 6’
5. Hold at 4ºC

The steps 2-4 were repeated for 30 cycles.

For cloning GRP75-ATPase domain and GRP78-ATPase domain the following programme was used:

1. 94ºC, 5’
2. 94ºC, 20”
3. 74ºC, 1’ 30”
4. 72ºC, 6’
5. 94ºC, 20”
6. 72ºC, 1’
7. 72ºC, 6’
8. 94ºC, 20”
9. 70ºC, 30”
10. 72ºC, 5’
11. Hold at 4ºC

The steps 2-4 were repeated for 5 cycles, the steps 5-7 for 10 cycles and the steps 8-10 for 30 cycles.

For cloning GRP75 and GRP78 the following programme was used:

1. 94ºC, 3’
2. 94ºC, 30”
3. 60ºC, 1’ 30”
4. 72ºC, 5’
5. Hold at 4ºC

The steps 2-4 were repeated for 30 cycles.

For testing mycoplasma contaminations in cell lines 100 µl of supernatant was boiled at 95ºC and 2 µl were used for a PCR reaction. For this PCR the Taq DNA polymerase enzyme (Promega, Mannheim, Germany) was used with the following programme:

1. 94ºC, 2'
2. 94ºC, 30"
3. 60ºC, 30"
4. 72ºC, 30"
5. Hold at 4ºC

The steps 2-4 were repeated for 35 cycles.

2.2.1.2 Separation of nucleic acids by agarose gel electrophoresis

DNA separation was performed on a horizontal agarose gel ranging from 0.8 to 2% of concentration according to the size of the fragments to separate. The desired amount of agarose was dissolved in TAE buffer 1X (0.04 M Tris pH 7.2, 0.02 sodium acetate, 1mM EDTA). To dissolve the agarose the solution was boiled and then cooled down to an approximate temperature of 40ºC. After addition of ethidium bromide to have a final concentration of 0.4 mg/ml, the solution was poured into a horizontal gel chamber and a comb was placed over the chamber to allow the formation of slots where the samples could be placed. For the electrophoretic run the chamber was filled with TAE buffer 1X and the samples were mixed with DNA sample buffer (5mM EDTA, 50% glycerol, 0,01g bromophenolblue) and loaded onto the gel. The standard ladder used was either the peqGOLD 1 kb DNA-Leiter (0.5 mg DNA/ml, for fragments ranging from 250 to 10000 bp) or the peqGOLD 100 bp DNA-Leiter (for fragments from 80 to 1031 bp). The electrophoresis was carried at 80-120 V and the separation was visualized under a UV light source. Since agarose gel contains ethidium bromide nitril-based gloves were use for handling.

2.2.1.3 DNA fragment extraction from agarose gel

For extracting distinct DNA fragments from agarose gel the peqGOLD gel extraction kit (peqLab Biotechnology GmbH, Erlangen, Germany) was used. When the run was completed the distinct band was visualized under a source of UV light of mild intensity and removed with a scalpel. The agarose was dissolved at 55ºC in XP2 Binding Buffer. Thereafter the solution was added to a HiBind® DNA spin column and centrifuged at 10000 rpm (Eppendorf centrifuge 5417R) 1 min. The column was washed once with XP2 Binding Buffer and twice with SPW Wash Buffer (completed with ethanol 80% final concentration) by centrifugation at 10000 rpm (Eppendorf centrifuge 5417R), 1 min, 4ºC. Residual ethanol was remove from the column by one additional centrifugation at 10000 rpm (Eppendorf centrifuge 5417R) for 1 min. 30 µl of elution buffer were then added in the centre of the column and collected into an eppendorf tube by centrifugation at 10000 rpm (Eppendorf centrifuge 5417R) for 1 min. The presence of the DNA fragment was confirmed by gel electrophoresis.

2.2.1.4 Ligation of DNA fragments

All ligations were performed using 1 U of the enzyme T4 DNA ligase (Fermentas, St Leon-Rot - Germany). For insertion of a specific DNA fragment into a vector the ratio insert:vector was usually 1:3 or 1:6. The reaction was carried in presence of T4 DNA ligase buffer (containing a final concentration of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA) in a final volume of 20 µl for 1 hour at 22ºC or for 4 hours at 16ºC or ON at 4ºC. Every ligation experiment was performed incubating the empty vector digested with the same restriction enzymes as control. The reaction was stopped by inactivation of the enzyme at 65ºC for 10 min.

2.2.1.5 Transformation of plasmid DNA into bacteria

For transformation typically 50 µl of chemically competent bacteria DH5α were incubated with 10 µl of the ligation mix or 1 µl of the purified plasmid DNA for 20 min on ice. Thereafter the cells were thermically shocked at 37ºC for 5 min or 42ºC for 2 min

and then incubated on ice for 2 min. The transformed bacteria were then allowed to grow in a final volume of 500 µl of LB 1X without amipicillin for 30 min at 37ºC on a shaker. In the meantime LB 1X agarose plates with ampicillin were pre-warmed at RT. Finally 100 µl of bacteria were plated and incubated ON at 37ºC.

2.2.1.6 Small-scale purification of plasmid DNA

For mini-preparation of DNA 3 ml of transformed bacteria were incubated in LB 1X with ampicillin ON at 37ºC on a shaker at 220 rpm. Thereafter 1 ml of the bacteria culture was transferred into an eppendorf tube and centrifuged at 12000xg for 30 s at 4ºC (centrifuge Eppendorf 5417 R). The purification was performed using the solutions P1, P2 and P3 from the Qiagen Plasmid Maxi Kit® (Qiagen, Hilden - Germany). The SNT was removed, the pellet was resuspended in 100 µl of solution P1 with RNase A (10 mM EDTA, 50 mM Tris-HCl pH 8.0, 400 mg/ml RNase A) and incubated 5 min at RT. Alkaline lysis was performed by adding 200 µl of solution P2 (200mM NaOH, 1% SDS) and incubating for 5 min on ice. Thereafter addition of 200 µl of buffer P3 (3M Na Acetate, pH 4.8) followed by vortexing and incubation again for 5 min on ice neutralized the pH. To separate the lysate from cells debris the solution was then centrifuged at 12000 rpm for 15 min at 4ºC (Eppendorf centrifuge 5417R). The SNT was transferred into a new eppendorf tube and a phenol:chloroform extraction was performed. Thereafter the DNA was precipitated by adding 1 ml of ice-cold ethanol to 400 µl of aqueous solution. The reaction was carried at -80ºC for 30 min. The DNA was collected by centrifugation at 12000 rpm at 4ºC for 15 min (Eppendorf centrifuge 5417R). After discarding the SNT the DNA pellet was washed from residual salts with 200 µl of ethanol 80%. After centrifugation at 12000 rpm for 2 min at 4ºC (Eppendorf centrifuge 5417R) the SNT was discarded and the residual ethanol allowed to evaporate. The DNA was resuspended in 30 µl of double-distilled water and dissolved by incubation at 50ºC for 10 min.

2.2.1.7 Phenol:Chloroform extraction of plasmid DNA

For extraction of undesired protein content from DNA plasmid preparation phenol (Carl Roth, Karlsruhe, Germany) and chloroform:isoamyl alcohol 24:1 (Sigma Aldrich, Taufkirchen, Germany) were mixed in a ratio 1:1. An equal volume of this solution was added to the bacteria lysate and vortexed. The two phases were separated by centrifugation at 12000 rpm for 2 min at 4ºC. The upper aqueous phase containing DNA was transferred into a new reaction tube while the lower one was discarded according to the safety rules.

2.2.1.8 High-scale purification of plasmid DNA

For high-scale purification of plasmid DNA the Qiagen Plasmid Maxi kit was used. Bacteria were cultured the day before the purification in 300 ml of LB 1X with ampicillin at 37ºC with shaking at 220 rpm. The day of the experiment bacteria were collected by centrifugation at 4000xg for 5 min at 4ºC in a fixed angle rotor (centrifuge Hermle ZK 401) and resuspended in 10 ml of buffer P1 containing RNase A. After incubation for 10 min at RT alkaline lysis was performed by adding 10 ml of buffer P2 and vortexing. The reaction was allowed to proceed for 10 min on ice. Thereafter the pH was neutralized by 10 ml of buffer P3 and the whole mixture was gently inverted. To separate the lysate from membrane debris the suspension was centrifuged at 6000xg, 15 min at 4ºC (centrifuge Hermle ZK 401). After centrifugation the SNT was poured into a Qiagen Tip 500 column pre-equilibrated with 15 ml of buffer QBT (700 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol (v/v), 0.15% Triton X-100 (v/v)). The column contained a resin able to bind DNA and to purify it from the lysate. Thereafter the column was washed twice with 30 ml of buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol (v/v)). The DNA was eluted from the resin by addition of 15 ml of buffer QF (125 mM NaCl, 50 mM Tris-HCl pH 8.5, 15% isopropanol (v/v)) and collected in a corex glass tube. To precipitate the DNA 11 ml of isopropanol were added to the flow-through and the mixture obtained was gently inverted and incubated on ice for 15 min. To collect the precipitated DNA the solution was then centrifuged at 7500xg for 15 min at 4ºC (centrifuge Beckman Coulter). The SNT was discarded and the pellet was resuspended with 1 ml of Ethanol 80% and collected in an eppendorf tube. After

centrifugation at 12000 rpm for 2 min at 4ºC (Eppendorf centrifuge 5417R) the SNT was removed and the pellet dried at 50ºC. The DNA was dissolved in 200-300 µl of double distilled water and quantified.

2.2.1.9 Quantification of plasmid DNA

To quantify the amount of DNA the optical density (OD) at 260, 280 and 230 nm was measured with the NanoDrop® and the software ND-1000 (version 3.1.2). An OD_{260}=1 corresponds to 50 µg/ml of double-stranded DNA. A ratio OD_{260}/OD_{280}=1.8 indicates a nucleic acid preparation relatively free from protein contamination. A ratio OD_{260}/OD_{230} above 1.6 indicates a preparation free of organic chemicals and solvents.

2.2.2 Cell culture and transfection methods

2.2.2.1 Cell culture

All mammalian cells were cultured in standard conditions of 37ºC, 5% of CO_2 and 95% of humidity in an incubator (Forma Scientific Labortech GmbH, Göttinghen, Germany). All cell lines were grown in sterile Cellstar® Petri dishes (Greiner Bio-One, Frickenhausen, Germany) of different formats depending on the experimental conditions. Cells were grown to a confluence of 80-90%, then the culture medium was removed, the cells were washed once with PBS 1X at room temperature and incubated with warm trypsin for 5 minutes (10 min for the cell line BPH-1) at 37ºC. Fresh medium was then added to the plate and the cells were seeded at a lower concentration in a new Petri dish. The human primary prostate cancer cell line 22Rv.1 and the human lymphonode methastasis LNCaP cell line were cultured in RPMI 1640 enriched with 10% FBS. Culturing the LNCaP cell line required a pre-coating of the Petri dishes with Poly-L-Lysine (Sigma Aldrich, Taufkirchen, Germany) for at least 1 hour at 37ºC to increase the adherence to the culture plate.

The human cell line BPH-1 derived from the prostate epithelium affected by benign prostatic hyperplasia was cultured in RPMI 1640 enriched with 10% FBS and Glutamine (Invitrogen, Karlsruhe, Germany).

2.2.2.2 Transfection with FuGene®

For transfection with FuGene® cells were subcultured the day before so they could reach 70-80% of confluency the day of the experiment. For a 10 cm culture dish 30 µl of FuGene® were diluted in 1 ml of pre-warmed serum-free medium and incubated at room temperature for 5 mins. Thereafter 10 µg of plasmidic DNA were added and the final solution was inverted and incubated for 20 min at room temperature. Finally the solution was dropwised into the Petri dish and distributed evenly by gentle swirling.

2.2.2.3 Colony forming assay

For colony forming assay 22Rv.1 cells and BPH-1 cells were seeded at 70-80% of confluence and transfected with 10 µg of plasmidic DNA with FuGene®. The following day the cells were washed with 5 ml of PBS 1X and 1 ml of trypsin was added to the Petri dish. After 5 minutes of incubation at 37ºC, cells were directly resuspended in fresh culture medium, counted with a hemocytometer and seeded in triplicate in a 6 well plate. For 22 Rv.1 $3x10^4$ cells were seeded, while for BPH-1 $3x10^3$ cells were seeded. After 48 hours the medium was removed and fresh medium with the antibiotic G418 was added to start the selection. Since the vector contained a gene for the resistance to the antibiotic, only the transfected cells were able to grow and form colonies. When the size of the colonies was visible, cells were washed with PBS 1X and fixed with methanol for 20 minutes at room temperature on a shaker. Thereafter methanol was removed and the colonies were stained with 0,05% Crystal Violet for 20 minutes at room temperature on a shaker. The plates were finally washed with tap water and the colonies counted. The value of the vector was set as 100%. The experiment was repeated at least three times with three different plasmid preparations.

2.2.2.4 Cell viability assay

For evaluation of viability the CellTiter-Blue® Cell Viability Assay kit (Promega, Mannheim, Germany) was used. The assay consists in estimating the amount of viable cells based on their ability to convert a non-fluorescent dye (resazurin) into a fluorescent product (resorufin). 22Rv.1 stably overexpressing the Bag-1 peptide or the empty vector control were seeded into two 96 well plate (4500 cells/well) in triplicate. After 2-3 hours 20 µl of dye were added to each well containing 100 µl of culture medium for 4 hours at 37ºC.

Fluorescence was measured as the ratio between the value of intensity at the wavelenght of excitation (560 nm) and emission (590 nm) using the plate-reading fluorometer FluoStar Optima (2001, BMG Labtechnology, software version 1.10-0). Cell proliferation was determined from the difference between the fluorescence intensity obtained at day2 and the value obtained at the day of seeding (day0).

2.2.2.5 Immunofluorescence

For immunofluorescence a 4-well glass slide Lab-Tek®II Chamber Slide™ System (VWR International, Bruchsal, Germany) was pre-coated with approximately 100 µl of a solution of 1µg/µl of Fibronectin (Sigma Aldrich, Taufkirchen, Germany) and incubated for 1 hour at 37ºC. Thereafter each well was washed once with PBS 1X and $1x10^5$ cells were seeded in each well. The following day the medium was removed, the cells washed with PBS 1X once and fixed with 4% PFA for 10 min at room temperature. After fixation, PFA was removed, cells washed twice with PBS 1X and incubated with permeabilization solution (Triton X-100 0,1% in PBS 1X) for 10 min at room temperature. Then cells were washed twice with PBS 1X and incubated with blocking solution (4% goat serum in PBS 1X) for 15 min at room temperature and subsequentially incubated with primary antibody diluted in blocking buffer for 1 hour at room temperature. Cells were then washed twice with PBS 1X and secondary antibody diluted in blocking buffer was applied for 15' at room temperature. Finally cells were washed twice with PBS 1X and once with water, dried and a glass coverslip (Erie Scientific, Portsmouth, US) was mounted using polyvinilalcohol (PVA) as mounting medium. Samples were then analysed with a Leica TCS SPE confocal microscope (Software:

Leica Application Suite Advance Fluorescence – 2.0.1 build 2043 – Leica Microsystems, Wetzlar, Germany). For staining the mitochondria, before the fixation living cells were incubated 20 min at 37ºC with medium containing 300 nM final concentration of the Mitotracker® Mitochondrion-Selective Probes Deep Red FM (Invitrogen, Karlsruhe, Germany). For staining of the endoplasmic reticulum the SelectFX® Alexa Fluor® 488 Endoplasmic Reiculum Labeling kit (Invitrogen, Karlsruhe, Germany) was used. This kit provided a specific antibody recognizing the PDI protein present in the ER. The anti-PDI antibody was used at a dilution of 1:1000 in PBS 1X containing 4% goat serum.

2.2.3 *In vivo* animal experiment

All the studies carried out using animals were performed according to protocols approved by the animal care and use committee of Baden-Württemberg.

2.2.3.1 Transgenic adenocarcinoma of the mouse prostate (TRAMP)

2.2.3.1.1 Tumour harvesting and protein analysis

For tumour weight determination 25 week-old TRAMP mice wild type or eterozygous for Bag-1 gene (Bag-$1^{+/-}$) were sacrificed and prostate and seminal vescicles were weighed using a scale. The samples were stored at -80˚C or fixed in 4% PFA for 16 hours and then stored in an aqueous solution containing 50% ethanol. For the analysis of Bag-1 expression 50 µg of prostate and seminal vesicles lysates quantified with the Bradford assay were separated on a 15% polyacrylamide-SDS gel, transferred to a nitrocellulose membrane and probed with rabbit-anti-Bag-1 antibody (Sigma, FL-274). Thereafter the membrane was stripped and re-probed with an anti-β-actin antibody to determine the levels of β-actin as loading control. The Bag-1 and β-actin protein amount were quantified using the programme ImageJ®. Bag-1 relative protein level was calculated according to the formula:

Relative Bag-1 level= Bag-1 band intensity/ β-actin band intensity

Each value was associated to the respective weight and the correlation was calculated using the application Microsof Excel® (Microsoft Corporation, US).

2.2.3.1.2 Survival experiment

For survival experiment mice were sacrificed by neck dislocation either when the tumour size reached the maximum limit allowed or when the animals showed clear signs of imminent death. For calculation of the survival curve the programme GraphPad Prism® was used. For the experiment 60 wild type and 50 $Bag1^{+/-}$ TRAMP mice were used.

2.2.3.2 Xenograft model of prostate cancer cells

2.2.3.2.1 Injection of 22Rv.1 stable clones into athymic nude mice

Before the injection every cell line was checked for mycoplasma infection using the VenorGem® mycoplasma kit (Sigma, Taufkirchen, Germany).

For generation of xenograft models cells were washed with PBS 1X and trypsinized incubating with trypsin 0,025% + EDTA for 5 minutes (10 for LNCaP cells) at 37˚C. After detachment cells were resuspended in fresh culture medium and counted with a hemocytometer. The amount of cells needed was collected into a new falcon tube, washed twice with PBS 1X and resuspended in the appropriate volume of PBS 1X. LNCaP were resuspended in a solution of 50% Matrigel® (BD Bioscience, Heidelberg, Germany) in PBS 1X. Since Matrigel is solid at room temperature, it was stored for maximum three months at -20ºC and on ice at the moment of use. $5x10^6$ 22Rv.1 or LNCaP prostate cancer cell lines were subcoutaneously injected into 6-8 week-old athymic nude mice in both flanks in 100 µl of PBS 1X. Tumour size was measured with a calliper every week for a maximum of 9 weeks for the injection of stable clones overexpressing the Bag-1 peptide and 5 weeks for the ones expressing the N-terminal, the C-terminal and the ΔN-peptide. Tumour size was assessed measuring three perpendicular diameters according to the formula: V=(1/6) [π] (d1d2d3), where π is a

matematic constant and d1, d2 and d3 represent the three spatial dimensions (width, depth and height).

Mice were sacrificed by neck dislocation either because the tumour reached the maximum size (around 1000 cubic mm) or because the time limit of the experiment was reached.

2.2.3.2.2 Tumour harvesting

For harvesting tumour tissue mice were sacrificed by neck dislocation and the tumour was removed using forceps and scissors. Thereafter the tumour was weighed using a scale. Part of the tissue was stored in a tissue embedding cassette (Labonord, Mönchengladbach, Germany) for immunohistochemical analysis.

2.2.4 Immunohistochemistry

2.2.4.1 Tissue processing and embedding

Tumour tissues were fixed in a solution of 4% PFA (for 1 L of solution pH 7.4: 892 ml of double distilled water, 108 ml of Formalin 37%, 11.86 g f Na_2HPO_4, 9.07 g KH_2PO_4) for 16 hours at RT. Thereafter the fixative solution was removed and the samples were kept in Ethanol 50% for long-term storage. For processing the samples a full-automatic tissue processor Hypercenter XP was used (Thermo Fischer Scientific, Dreich, Germany). The first part of the process dehydrates the tissues through washing steps with solutions containing increasing amount of ethanol followed by a washing step with xylene to remove the alcohol. Finally tissues were kept in liquid paraffin before the embedding process. For dehydrating the tissues the following programme was used:

1. 50% ethanol, 15’ immersion, 1h 15’ drain
2. 70% ethanol, 30’ immersion, 1h 15’ drain
3. 95% ethanol, 45’ immersion, 15’ drain
4. 95% ethanol, 45’ immersion, 15’ drain
5. 95% ethanol, 45’ immersion, 2h drain

6. 100% ethanol, 45’ immersion, 15’ drain
7. 100% ethanol, 45’ immersion, 15’ drain
8. 100% ethanol, 45’ immersion, 2h drain
9. xylene, 1 h immersion, 15’ drain
10. xylene, 1 h immersion, 2h drain
11. paraffin, 1 h immersion, 15’ drain
12. paraffin, 1 h immersion, 2h drain

After processing tumour samples were paraffin-embedded using a Leica EG 1660 embedding machine (Leica Microsystems, Wetzlar, Germany). Paraffin embedded tissues were kept at 4ºC to allow solidification of the paraffin matrix.

2.2.4.2 Cutting of paraffin embedded tissue blocks

Paraffin embedded blocks were cooled on ice before cutting into slices. To cut the block into 5 µm-thick slices a Leica RM2155 microtome was used (Leica Microsystems, Wetzlar, Germany). After cutting the slice was placed into a water-bath (Medax Nagel GmbH, Kiel, Germany) set at 50ºC (10ºC below the melting temperature of the paraffin) to remove all the wrinkles. Thereafter the slice was lifted onto a XTRA^{TM} Adhesive Microslide 26x76x1 mm (Surgipath, Richmond, US). After briefly drying on the side of the water bath, the section was placed in a Heraeus oven (Weiss-Gallenkamp, Loughborough, United Kingdom) at 60ºC, 1 h to dry out.

2.2.4.3 Immunohistochemical analysis of apoptotic cells

For staining apoptotic cells in paraffin embedded tumour slides the ApopTag® Peroxidase *in situ* apoptosis detection kit was used (Millipore, Schwalbach, Germany). 5 µm-thick slides were subjected to several washes to remove the paraffin and re-hydrate the section. The procedure was as following: 3 times in xylene for 5’, 2 times in ethanol for 5’, once in ethanol 95% for 3’ and once in ethanol 70% for 3’. Finally tissue sections were washed with PBS 1X for 5’. Since fixation forms protein cross-links that mask the

antigenic sites in the tissue, slices were treated with Protein kinase (20µg/ml in water) to break the protein cross-links, therefore unmasking the antigens and epitopes and enhancing the staining. After two washes of 2' each with double distilled water, the endogenous peroxidase was quenched by treating the sections with a solution of 3% H_2O_2 in PBS 1X for 5'. Thereafter the slides were washed two times for 5' with PBS 1X and approximately 100 µl of equilibration buffer were applied to the section. The sections were then treated with terminal deoxynucleotidil transferase (TdT) enzyme diluted in reaction buffer (working strength TdT enzyme) for one hour at 37ºC. The reaction was stopped with working strength stop/wash buffer for 10 min at room temperature. The slides were then washed with PBS 1X 3 times and anti-digoxygenin conjugate antibody was applied for 30 min at room temperature. The excess of antibody was removed by washing 4 times with PBS 1X and the brown staining was developed by using the peroxidase substrate kit – DAB (Vector Laboratories, Burlingame, US). To prepare 5 ml of substrate solution 2 drops of buffer stock solution, 4 drops of 3'-3' diaminobenzidine stock solution and 2 drops of H_2O_2 were added to 5 ml of double distilled water. The solution was incubated for 2 min at room temperature. Thereafter the sections were washed 3 times for 1 min and 1 time for 5 min with double distilled water. To visualize the nuclei the sections were counterstained by incubation for 2 min at room temperature with hematoxylin (Labonord, Mönchengladbach, Germany). After rinsing with fluent water for 2 min, the sections were dehydrated by one wash with n-butanol for 2 min and three washes with xylene for 2 min. A glass coverslip was mounted on top of the slide with the non-aqueous mounting medium Coverquick (Labonord, Mönchengladbach, Germany). For microscopy analysis and image acquisition an Axioscop Zeiss microscope (Carl Zeiss Microimaging, Heidelberg, Germany) and the software Axiovision (Release 4.5 12/05) were used.

2.2.5 Protein methods

2.2.5.1 Preparation of protein lysates from cells

Typically for protein extraction cells in a 10 cm dish were washed with 5 ml of ice-cold PBS 1X and scraped in 1 ml of PBS 1X. The resuspended cells were collected into

an eppendorf tube and centrifuged at 4000 rpm 5 min at 4ºC (Eppendorf centrifuge 5417R). The SNT was then aspirated and cells were resuspended in 100 µl of lysis buffer (Tris pH 8.0 50 mM, NaCl 150 mM, EDTA pH 8.0 5 mM, NP40 1%, protease inhibitor cocktail 1:100). The extract was thereafter sonified at Amp 60, 10 pulses and centrifuged at 12000 rpm, 5 min, 4ºC (Eppendorf centrifuge 5417R). Thereafter protein concentration was quantified wit the Bradford method. After quantification the lysate was diluted in 2X Sample Buffer (Tris pH 6.8 100 mM, SDS 4%, 2% β-mercaptoethanol, glycerol 10%, bromo phenol blue 0.2%) and boiled 5 min at 95ºC to allow complete denaturation.

2.2.5.2 Preparation of protein lysates from tissues

For protein extraction from mouse tissue the samples were thaw out on ice and resuspended in lysis buffer (Tris pH 8.0 50 mM, NaCl 150 mM, EDTA pH 8.0 5 mM, NP40 1%, SDS 0.1%, PMSF 1 mM, protease inhibitor cocktail 1:100). To have an efficient extraction the tissue was mechanically destroyed using pestle and mortar. After incubation for 30 min on ice the lysate was sonified at Amp 60, 10 pulse and quantified with the Bradford assay. Thereafter an equal amount of 2X Sample buffer was added to the lysate and the solution was boiled for 5 min at 95ºC.

2.2.5.3 Quantification of protein extracts

For quantifying the protein content the Bradford assay was used. Typically 5 µl of protein extract were diluted in 1 ml of Bradford (Bio-rad, Munich, Germany) solution 1X. 200 µl of the solution were added into a 96 well plate and the intensity of the signal was defined by reading at an optical density of 595 nm using a EL_X 808 UI Ultra Microplate Reader (software KC4 v 3.01). The signal background was determined by diluting 5 µl of lysis buffer into 1 ml of Bradford solution 1X. A standard curve using defined amount of BSA was used to calculate the final protein content using the formula:

y= ax+b,

y= amount of protein (µg)
a= slope of the curve
x= optical density (595 nm)
b= origin on y axis

2.2.5.4 Separation of proteins by SDS PAGE (polyacrylamide gel electrophoresis)

The Penguin Doppelgelsystem P9DS apparatus (PeqLab, Erlangen, Germany) was used to cast the polyacrylamide gel. Typically 10% polyacrylamide gel was used (for 30 ml of final volume, 11.9 ml of double distilled water, 10 ml of 30% Acrylamide mix, 7.5 ml of Tris 1.5 M pH 8.8, 300 µl of 10% SDS, 300 µl of APS, 12 µl of TEMED). The resolving gel was poured and overlaid with Rotisol 70%. After polymerization the Rotisol was washed away with distilled water and the separating gel was covered with stacking gel (for 10 ml of solution 6.8 ml of double distilled water, 1.7 ml of 30% Acrylamide mix, 1.25 ml of Tris 1 M pH 6.8, 100 µl of 10% SDS, 100 µl of APS, 10 µl of TEMED). A comb was placed over to allow the formation of slots where the samples could be placed. The gel was then fixed in the running chamber and 1X Laemmli buffer (for 1L of 10X solution: 30.26 g of Tris base, 144.13 g of Glycin, 50 ml of SDS 20% in 1 L of double distilled water) was poured over. The desired amount of cell lysate diluted in sample buffer was then loaded onto the SDS gel. Samples were run at 80V in the stacking gel and 150V in the separating gel for 3-4 hours or at 30V ON.

2.2.5.5 Western blotting

For western blotting analysis proteins were transferred onto an Immobilion^{TM} poly vinylidene fluoride (PVDF) membrane (Millipore, Schwalbach, Germany), pre-soaked in methanol. The transfer was performed at 35 V ON at 4ºC. Thereafter western blot analysis was performed. To reduce unspecific binding of the antibody the membrane was incubated with blocking solution for 1 hr at RT. For detecting the protein of interest

specific primary antibody was incubated in blocking solution for 4 hr at RT or ON at 4ºC (see table of antibodies for detailed information). Thereafter the membrane was washed three times with TBS 1X (for 1L of 10X stock solution pH 7.6: 80 g of NaCl ; 24.2 g of Tris base in deionized water) + 0,1% Tween 20 (TTBS 1X or TBST 1X) 10 min each. After washing the membrane was incubated with the secondary antibody for 1 hr at RT and washed four times with TTBS 1X 10 min each. Detection of proteins was finally performed using the enhancer of chemioluminescence (ECL) western blot detection reagent (Amersham, Braunschweig, Germany). The signal was developed using films from ECL Hyperfilm (Amersham, Braunschweig, Germany) or Fuji Super RX 18x24 film (Ernst Christiansen GmbH, Planegg, Germany).

2.2.5.6 Membrane stripping

To use more than once the membranes for Western blot analysis the filters were incubated with stripping solution (3,125 of Tris·HCl 1 M pH 6.8, 5 ml of 20% SDS, 400 µl of β-mercaptoethanol in a final volume of 50 ml) at 50ºC for 20 min with shaking. The membranes were then washed 5 times 10 min each with TTBS 1X.

2.2.6 GST-pull down experiment

2.2.6.1 Preparation of GST-fused proteins

For preparation and purification of GST-fused proteins BL21 bacteria transformed with the plasmid encoding for the desired GST-fused protein were incubated ON in 20 ml LB 1X + ampicillin. The following day the bacteria culture was expanded to 1 L of LB 2X and allowed to grow till OD_{600}=0.5-0.8 measured with a spectrophotometer Biophotometer (Eppendorf, Wesseling-Berzdorf, Germany). Thereafter to induce the production of the recombinant protein isopropilthiogalactoside (IPTG) was added to a final concentration of 1 mM and incubated for 3 hours at 37ºC on a shaker. At the end of the induction cells were collected by centrifugation at 4000xg, 5 min at 4ºC in a fixed angle rotor (centrifuge Hermle ZK 401) and the pellet were frozen at -80ºC. Bacterial pellets were resuspended in resuspension buffer (2 mM EDTA, 2 mM PMSF in PBS

1X). Thereafter to allow cell lysis 1 mg/ml of lysozyme (Sigma Aldrich, Taufkirchen, Germany) was added to the bacteria and incubated for 10 min on ice. To have a complete protein extraction 1% Triton-X-100 was added to the solution ad incubated at 4ºC on a rotor. For the purification of the proteins GST-GRP75 and GST-GRP78 1% of sodium N-Laurylsarcosinate (or Sarkosyl) was added and incubated for 30 min at 4ºC on a rotor. Thereafter the solution was sonified at Amp 60, 10 pulses and centrifuged at 7000xg, 15 min at 4ºC (centrifuge Beckman Coulter). In the meantime approximately 1 ml of glutathione-sepharose 4B slurry beads (GE-Healthcare, Freiburg, Germany) were washed twice with PBS 1X. The GST-fused proteins present in the supernatant were then added to the glutathione beads and incubated at least for 1 hour at 4ºC on a rotor. Thereafter they were washed with PBS 1X by centrifugation at 2000 rpm, 2 min, RT (Eppendorf centrifuge 5417R) twice to remove all the unbound proteins. The recombinant proteins bound to the beads were then resuspended in a solution of PBS 1X containing DTT 1 mM and protease inhibitors diluted 1:100 and kept at 4ºC for short-term storage (maximum 24 hours). For long-term storage, proteins were eluted from the beads by incubation with 500 µl of reduced glutathione (0.5 M in 50 mM Tris pH 8.0) for 10 min at RT. Typically 6 fractions were collected and the protein amount was quantified by polyacrylamide gel electrophoresis and subsequent staining with Coomassie® blue. Defined amount of BSA (Promega, Mannheim, Germany) were used as standard to determine the concentration of protein present in each fraction. The most abundant fractions were pooled together, requantified and stored at -80ºC.

2.2.6.2 Incubation of GST-fused proteins with cell lysate

For GST-pull down 22Rv.1 cells stably overexpressing the Bag-1 peptide (clone P29) were cultured. The day of the experiment the medium was aspirated and cells were scraped in approximately 5 ml of ice cold PBS 1X. After centrifugation at 2000xg, 2 min at 4ºC (centrifuge Beckman Coulter) the cell pellet was resuspended in 500 µl of lysis buffer (Na_2HPO_4 pH 7.4 10 mM, EDTA 1 mM, KCl 150 mM, glycerol 15% in double distilled water). The resuspended cells were subjected to 3-5 cycles of freeze-thaw by immersion in liquid nitrogen followed by incubation at 37ºC. Thereafter the lysate was sonified at Amp 50, 10 pulses and centrifuged at 12000xg, 15', 4ºC (centrifuge Beckman

Coulter). The supernatant was collected in a new eppendorf and quantified using the Bradford assay.

In parallel GST-fused proteins bound to glutathione sepharose beads were quantified by loading onto a polyacrylamide gel together with defined amounts of BSA. At the end of the run protein amount was determined by staining the gel with Coomassie® blue.

Approximately 400 µg of cell extract were incubated with 50 µg of GST-fused protein in LBST buffer (20 mM Hepes·KOH pH 7.9, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1mM DTT, protease inhibitors 1:100, 0.05% NP40, 1.5% Triton-X-100) in a final volume of 200 µl, ON at 4ºC on a rotor. The following day the sample were washed 4 times with LBST buffer by centrifugation at 2000 rpm, 30", 4ºC (Eppendorf centrifuge 5417R). The beads were resuspended in 60 µl of 2X sample buffer and boiled at 95ºC for 5 min. Finally the samples were centrifuged at 12000 rpm, 5 min, 4ºC (Eppendorf centrifuge 5417R). 20 µl of each sample were loaded onto a polyacrylamide gel and subjected to electrophoretic separation. The proteins were blotted on a PVDF membrane and western blot analysis was performed. To check if equal amount of recombinant protein were loaded at the end of the experiment the membrane was stained with Coomassie® blue.

2.2.6.3 Staining with Coomassie® blue

For staining with Coomassie® blue the polyacrylamide gel was washed twice with distilled water for 10 minutes at room temperature on a shaker to remove the salts that could interfere with the staining. Thereafter the gel was incubated 1 h at RT on a shaker with SimplyBlue™ Safe stain (Invitrogen, Karlsruhe, Germany). Finally the gel was washed several times with distilled water to remove the excess of staining and stored at 4ºC. The same procedure was applied to PVDF membranes.

3. RESULTS

3.1 Bag-1 levels and tumour formation

Increased level of expression Bag-1 has been correlated with aggressive and hormone refractory prostate tumour (Krajewska *et al.*, 2006; Mäki *et al.*, 2007) but no clear evidence that Bag-1 can indeed influence tumour formation has been provided so far. To study the impact of Bag-1 gene level on tumour formation the transgenic adenocarcinoma of the mouse prostate (TRAMP) model was crossed with a Bag-1 knock out mouse (Bag-$1^{+/-}$). Bag-$1^{+/-}$ mice have a deficiency for one allele of the Bag-1 gene resulting in decreased expression of the Bag-1 isoforms Bag-1L and Bag-1S. Animals homozygous for Bag-1 showed embryonic lethality at stage 13.5 (Götz *et al.*, 2004) and therefore could not be used for the purpose of this study.

The TRAMP is a mouse model used to study prostate cancer development. TRAMP mice carry a transgene composed of the SV40 early genes T and t antigens (Tag) fused to a fragment (-426/+28 bp) of the rat probasin promoter (Hurwitz *et al.*, 2001). This model is based on the observation that transgenes expressing the SV40 antigens can indeed lead to tumour formation (Brinster *et al.*, 1984). Since the rat probasin promoter that controls the expression of the SV40 antigen is specifically expressed in the prostate epithelium in a androgen regulation manner (Greenberg *et al.*, 1994; Johnson *et al.*, 2000), increased levels of hormone during puberty leads to specific expression of the SV40 Tag into the mouse prostate epithelium (Greenberg *et al.*, 1995). Typically at the 10^{th} week of age, TRAMP mice develop prostatic intraepithelial hyperplasia (PIN, a pre-neoplastic lesion in the prostate leading to cancer). At the 22^{nd} week of age, prostate cancer expanding into the seminal vesicles (Gingrich *et al.*, 1999) is detected (Greenberg *et al.*, 1995). Between the 24^{th} and the 30^{th} week of age, the mice develop a poorly differentiated prostate cancer (Greenberg *et al.*, 1995). On the base of these changes, the animals were sacrificed at the 25^{th} week of age and prostate tumour and seminal vesicles were harvested and weighed. TRAMP/Bag-$1^{+/-}$ showed a reduction of 23.3% in the weight of tumour and seminal vesicles compared to the wild type, indicating that Bag-1 plays a role in tumour development (Fig. 3.1A).

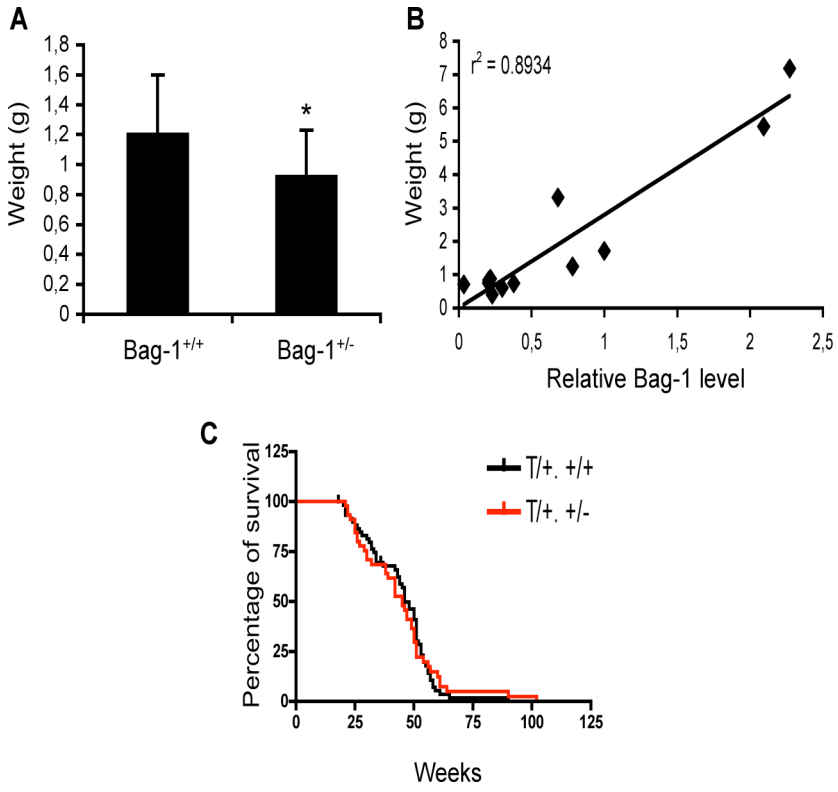

Fig. 3.1: Bag1 gene level influences prostate cancer development in TRAMP mouse model
TRAMP model was generated in B57/BL6 mice and crossed with B57/BL6 mice heterozygous for Bag-1 gene. A) Bag-1 haploinsufficiency impairs tumour development. 25-week old TRAMP mice T/+; +/+ (n=17) and their Bag1+/- haploinsufficient littermates T/+; +/- (n=16) were sacrificed and tumour and seminal vesicles were weighed. The results are showed as mean value ± SEM (* $p<0.05$). B) Bag-1 protein level correlates with tumour weight. Protein extracts were obtained from representative tumour and seminal vesicles of TRAMP wild type (n=4) and TRAMP/Bag-$1^{+/-}$ (n=4) mice and loaded onto a polyacrylamide gel. Western blot analysis using an anti-Bag-1 antibody was performed to determine the level of expression of Bag-1 proteins and an anti-β-actin antibody for normalization. C) Bag-1 gene level reduction does not affect overall survival. Kaplan-Meier curve shows no difference in the survival between wild type (T/+;+/+, black line, n=60) and Bag-$1^{+/-}$ (T/+;+/-, red line, n=50) TRAMP mice (p= 0.9669).

Moreover for a direct evidence of Bag-1 proteins expression contributing to tumour growth, cell extracts from representative prostate and seminal vesicles of TRAMP/Bag-$1^{+/+}$ and TRAMP/Bag-$1^{+/-}$ mice were loaded onto a polyacrylamide gel. Bag-1 protein level was analyzed by immunoblot using an anti-Bag-1 antibody and normalized to β-actin. Since the level of expression of Bag-1L is very low in mouse cells, Bag-1S level was used in this study. The relative intensity of the bands corresponding to Bag-1S was quantified using the software ImageJ^{TM} and plotted against the weight of tumours and seminal vesicles. The curve obtained from these two values showed an r squared of 0.89 indicating that Bag-1 protein expression strongly correlated with tumour weight (Fig. 3.1B). In addition it was determined if reduced Bag-1 level would influence the overall survival of the mice. Kaplan-Meier analysis showed comparable survival curves of

TRAMP wild type and TRAMP/Bag-$1^{+/-}$ mice (Fig. 3.1C). Thus although reduction of Bag-1 level slightly reduced tumour size it did not influence the overall survival of the mice.

3.2 Effect of Bag1-L overexpression in benign and cancer cells

The TRAMP experiment showed that Bag-1 expression is important for tumour formation consistent with previous studies on overexpression of Bag-1 protein in prostate cancer (Krajewska *et al.*, 2006). Reports that Bag-1L is overexpressed (Mäki *et al.*, 2007) or downregulated (Hague *et al.*, 2002) in cancer indicates a dual function of this protein. In addition increase in Bag-1L expression has been reported to correlate with malignant cell transformation (Takayama *et al.*, 1998). In order to analyze mechanistically the role of Bag-1L in prostate cancer development, two cell lines derived from two different stages of the human prostate were used. On the one hand BPH-1 cell line derived from a patient with benign prostatic hyperplasia and on the other hand 22Rv.1 cells obtained from a primary prostate cancer. These two cell lines showed different levels of Bag-1L as determined by western blot experiment. In this assay equal amounts of protein extract were loaded onto a polyacrylamide gel and subjected to electrophoretic separation and transferred on a PVDF membrane. Hybridization of the membrane with an anti-Bag-1L specific antibody showed an undetectable level of the protein in the benign cell line BPH-1 (Fig. 3.2A lane 1) compared to the cancer cell line 22Rv.1 (Fig. 3.2B lane 2). β-actin levels were used as loading control.

To assess the effect of increased level of Bag-1L on cell growth in the benign and malignant cells, colony forming assay was used. This assay is based on the ability of cells to grow into colony when seeded at a low density (Franken et al., 2006). Quantification of the number of colonies generated from a given population of cells gives a direct estimation of the degree of cell growth. For the purpose of this study, 22v.1 and BPH-1 cells were transfected with an expression vector containing the cDNA of Bag-1L and the gene for resistance to the antibiotic Geneticin (also called G418). The cells were then diluted to allow colony formation and the transfected cells were selected by G418 treatment. Overexpression of Bag-1L in BPH-1 produced an increase in colony formation compared to cells transfected with empty vector indicating a positive effect of Bag-1L on cell growth (Fig. 3.2B, black bars). Intriguingly the same experiment in the

22Rv.1 produced the opposite result, since it showed a significant reduction in colonies (Fig. 3.2B, white bars). These results suggest that Bag-1L has a dual function in the regulation of cell growth and that this effect is cell type specific.

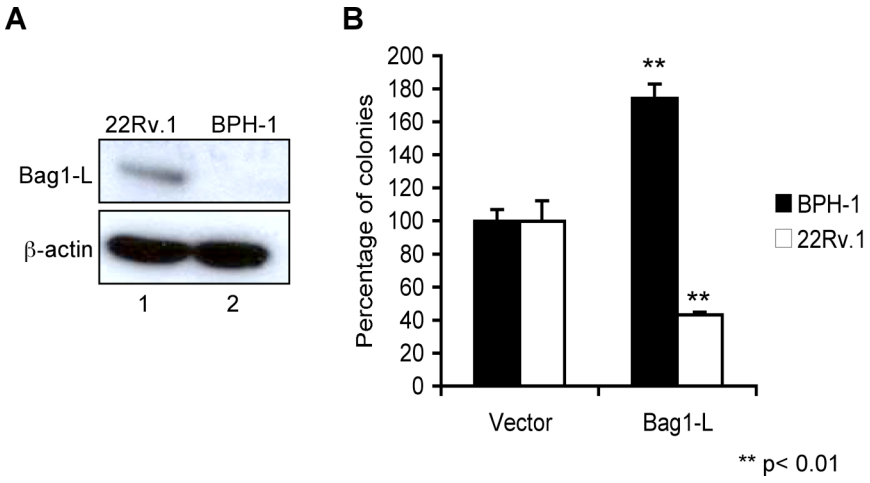

Fig. 3.2: Effect of Bag-1L on growth of benign and malignant prostatic cells
A) BPH-1 and 22Rv.1 cell extracts were loaded on a polyacrylamide gel. After transfer to a PVDF membrane an anti-Bag-1L antibody was used to detect Bag-1L levels and an anti-β-actin antibody for normalization. B) Bag-1L has opposite effects in bening and malignant cells. BPH-1 and 22Rv.1 were transfected and selected with G418. The colonies formed were stained with crystal violet and quantified setting the value of the vector as 100%. The results are shown as the mean value of at least three independent experiments carried out with three different plasmid preparations ±SD (** $p<0.01$). Open bars represent the result of the 22Rv.1 and the filled bars are the result of the BPH-1 cells.

3.3 Identifcation of a growth-inhibitory Bag-1L-based peptide

The fact that Bag-1L reduces the survival of 22Rv.1 prostate cancer cells when overexpressed indicates that sequences in this protein must contribute to this effect. One of the best studied functions of Bag-1L is its ability to bind chromatin (Shatkina et al., 2003). In chromatin immunoprecipitation assay carried out by Dr. L. Shatkina (unpublished data) a region of 68 amino acids encompassing amino acids 202 to 269 of the Bag1-L protein, when deleted, abrogated the binding to chromatin. This sequence includes part of the ubiquitin-like domain and part of the Bag domain (approximately one and a half helical turns).

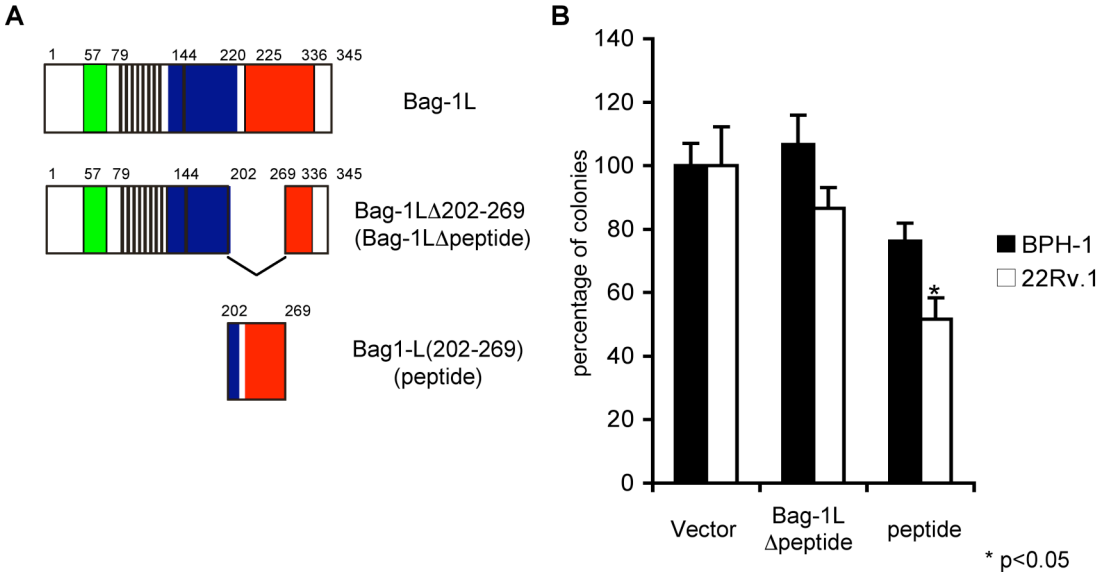

Fig. 3.3: A region of 68 amino acids of Bag-1L is important for the growth inhibitory effect on the cancer cells

A) Schematic diagramme of Bag1-L, Bag-1LΔ202-269 (or Bag-1LΔpeptide) and Bag-1L(202-269) (or Bag1-L peptide). The domains of Bag-1L in the diagramme are: the nuclear localization sequence (in green), the ubiquitin-like domain (in blue) and the Bag domain (in red). The numbers indicate the position of the amino acids. B) Bag-1L peptide reduces cell growth in prostate cancer cell 22Rv.1. For clonogenic assay BPH-1 and 22Rv.1 cells were transfected with constructs encoding the indicated peptides and selected with G418. The results are shown as the mean value of at least three independent experiments carried out with three different plasmid preparations ±SEM (* $p<0.05$). The value of the vector is set as 100%. Filled bars represent BPH-1 cells and open bars the 22Rv.1 cells.

Schematic diagrammes of Bag1-L, the identified deletion mutant Bag1-LΔ202-269 (otherwise known as Bag1-LΔpeptide) and Bag1-L(202-269) (or Bag-1L peptide) are showed in Fig. 3.3A.

These three Bag-1L constructs were stably transfected into the 22Rv.1 cancer and the BPH-1 benign cells and subjected to a clonogenic assay. In this colony forming assay, overexpression of Bag-1LΔpeptide in BPH-1 cells showed an impairement of the growth increase observed with the wild type Bag-1L in Fig. 3.2B, indicating a loss of function of this protein. Similarly the growth inhibitory function of the wild type Bag-1L in 22Rv.1 was abrogated when the Bag-1LΔpeptide was expressed. Overexpression of the Bag-1L peptide in the BPH-1 cells did not show any significant effect on colony formation compared to the vector control. Intriguingly expression of the peptide in 22Rv.1 cells reduced the colony formation, indicating that this sequence contained the information for the growth-inibitory effect (Fig. 3.2B). In addition, since this sequence did not show any effect on the BPH-1 cells, it seems that its action is cancer cell specific.

Since the region covered by the peptide is common to all the isoforms of the Bag-1 family, in the next paragraphs the peptide will be named as Bag-1 peptide.

3.4 Stable expression of Bag-1 peptide reduces proliferation *in vitro*

The growth inhibitory action of the Bag-1 peptide and the specificity towards cancer cells deserved a deeper analysis. In order to focus on this aspect, 22Rv.1 cells were transfected with an empty expression vector containing G418 resistance gene as control or the plasmid containing the hemagglutinin (HA) tagged Bag-1 peptide sequence. After transfection, the cells were selected with G418 and single clones were isolated and expanded. The level of expression of the peptide was determined by western blot analysis on cell extracts using an antibody recognizing the HA tag and β-actin for the control of equal loading. Stable clones with different level of expression of the peptide (Fig. 3.4B, lanes 4-8) or the empty expression vector (Fig. 3.4B, lanes 1-3) were selected. To determine whether the expression of the peptide influenced cell proliferation, Cell titer blue® proliferation assay was performed. This assay is based on the ability of cells to metabolize a dye (resoazurin) into the fluorescent dye (resorufin). Measuring the ratio between the wavelength of excitation (560 nm) and emission (590 nm) of fluorescence intensity gives a direct estimation of the number of cells. Cell proliferation was determined from the difference between the fluorescence intensity obtained on the second day (day2) over the value obtained at the day of seeding the cells (day0). The graph in Fig. 3.4A shows that all the stable clones expressing the peptide exhibited a decreased proliferation compared to the control cell clones independent of the amount of peptide expressed. This result confirms the growth inhibitory action of the peptide in the prostate cancer cell line 22Rv.1.

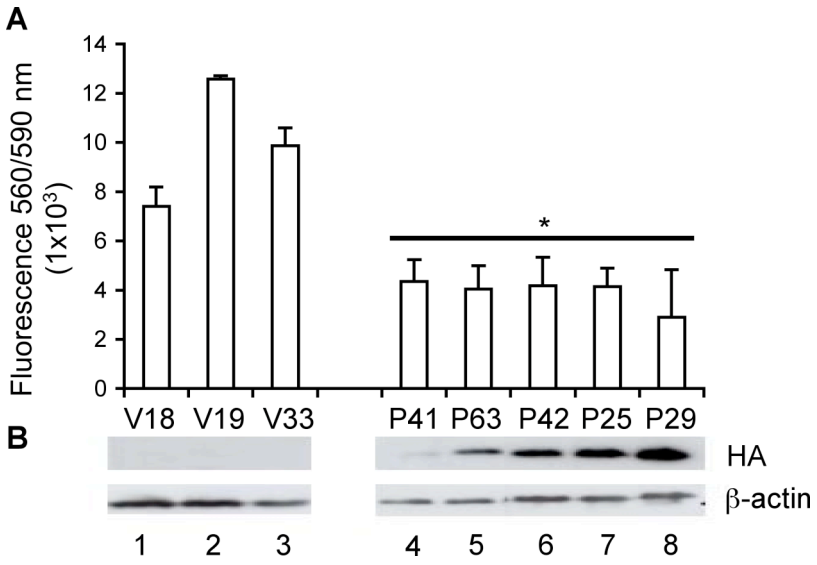

Fig. 3.4: Stable clones of 22Rv.1 cells show reduced cell viability *in vitro*

Bag-1 peptide stably expressed in 22Rv.1 cells decreases cell proliferation. A) Cell titer blue® proliferation experiment with stable clones transfected with the empty vector control or with the Bag-1 peptide sequence. Stable clones were seeded in a 96-well plate and the cell number in each well was estimated by measuring the ratio between the wavelength of excitation and emission (560/590 nm). The assay was performed over two days of duration and the fluorescence was measured with a Fluostar Optima™ plate reader (BMG). Values are represented as the mean of at least four independent experiments ±SD (* $p<0.05$). B) Level of Bag-1 peptide in the 22Rv.1 clones. Cellular extracts of stable clones were subjected to Western blot analysis. An anti-HA antibody was used to detect the peptide and an anit-β-actin antibody for β-actin as an indication of equal loading control.

3.5 Bag-1 peptide reduces tumour growth and weight *in vivo*

The Bag-1 peptide showed anti-growth properties in clonogenic and proliferation assay. Another possibility to study its effect is the *in vivo* tumour mouse xenograft model (Kerbel, 2003).

The animals used for this model are the athymic nude mice that are phenotypically hairless and do not have the thymus gland (Pantelouris, 1968). These mice do not have a functional immune system and since they lack the thymus, which is the source of T-cells, they can accept grafts from other organisms like rats or humans without any risk of rejection (Spang-Thomsen and Visfeldt, 1976).

To determine whether the peptide affects tumour growth, stable 22Rv.1 cell clones expressing the Bag-1 peptide were injected subcutaneously into both flanks of the mice. The clones were divided into three groups: the vector control group (V18, V19 and V33), clones expressing weak levels of peptide (P41 and P63) and clones expressing high level of peptide (P25, P29 and P42). Tumour growth was estimated by measuring the size

once a week with a caliper and calculating the volume using the formula V= 1/6xπ(d1d2d3), where V is the volume (in cubic mm), π is a mathematical constant and d1d2d3 the three dimensions measured (depth, width and height). Nine weeks after the injection, the animals were sacrificed by neck dislocation and the tumours were harvested and weighed. Alternatively mice were sacrificed when the maximum limit (around 1000 cubic millimiters) allowed by the animal care committee was reached.

The group of tumours formed by cells with the vector control reached the maximum size in a time range of 4 to 8 weeks (Fig. 3.5A). Mice bearing tumours expressing low levels of peptide were sacrificed between the 6th and the 7th week following the injection. This shift in termination time did not correspond to a significant difference in the tumour size (Fig. 3.5B). On the contrary, the stable clones expressing high levels of peptide showed a significant decrease in tumour size compared to the vector control and the weak peptide groups without reaching the maximum limit within the time period of nine weeks (Fig. 3.5C).

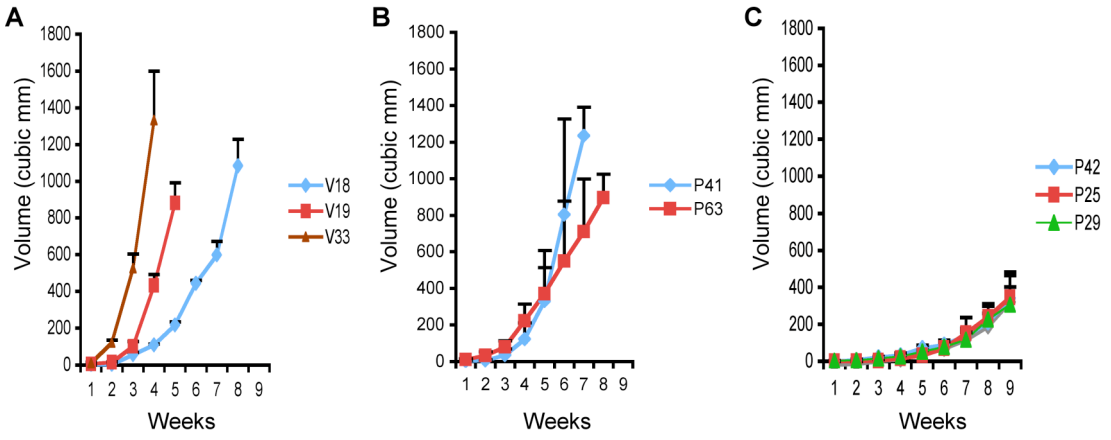

Fig. 3.5: Bag1 peptide reduces 22Rv.1 prostate tumour cell growth *in vivo*

Growth curves of xenograft models of 22Rv.1 stable clones. Six-week old athymic nude mice were injected subcutaneously into each flank with 5x10^6 cells of each stable clone. To follow the growth, tumours were measured every week up to nine weeks using a caliper and the formula V= 1/6π (d1d2d3), where V is the volume, π is a mathematical constant and d1d2d3 the three measurements of the three dimensions. Mice bearing tumours above the maximum size limit were sacrificed. The graphs represent the growth of 22Rv.1 cells stably expressing the empty vector as control (A), weak levels of peptide (B) and high levels of peptide (C). Curves are presented as the mean value of 5-10 tumours ±SD.

In order to demonstrate that the growth-inhibitory effect of the peptide is not restricted to a particular cell type, stable clones were generated in LNCaP cell line derived from a prostate cancer lymph node metastasis. Two clones containing the empty vector (V69 and V82) and two clones expressing the peptide (P12 and P35) were injected into the flank of the athymic nude mice. The clones transfected with the empty vector reached the maximum size of 1000 cubic millimiters within 8 weeks. Tumour growth was accompanied by loss of weight of the animals and for this reason the experiment carried out with the clone V69 had to be terminated 6 weeks after the injection (Fig. 3.6A). Injection of clones overexpressing the peptide hardly grew over the period of 8 weeks confirming that indeed the sequence identified affects prostate cancer cell growth (Fig 3.6B). The growth of tumours expressing the clone P35 was monitored only up to 6 weeks because the tumours became necrotic.

In addition to measuring the sizes, the weights of the tumours were also recorded after sacrificing the mice.

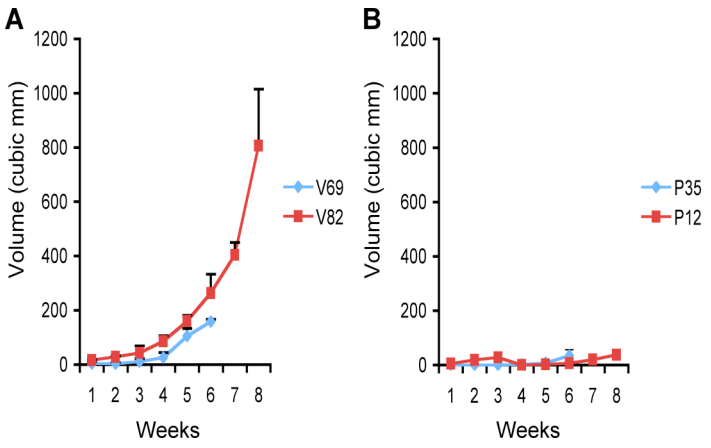

Fig. 3.6: Bag-1 peptide reduces LNCaP cell growth *in vivo*
Growth curves of xenograft models of LNCaP cells. Six-weeks old athymic nude mice were injected subcutaneously with $5x10^6$ cells mixed 1:1 with matrigel®. The curves represent the growth of the tumours over the time range of 8 weeks. Stable clones expressing empty vector as control and the peptide are shown in panel A and B. The results are shown as the mean value of 5-10 tumours ±SD.

A summary of the tumour weights of tumours formed by the 22Rv.1 and LNCaP cell clones and corresponding termination time for each clone injected is showed in table 3.1. In 22Rv.1 cell xenografts no significant differences were observed between the group of the clones transfected with the empty vector and the low expressing peptide. Consistent with the measurements of the tumour sizes, an approximately 4-fold

difference was observed between the high expressing peptide clones and the vector control group. Tumours derived from the injection of clones generated in LNCaP cells showed a striking 10-fold reduction in the weight over the control group.

22Rv.1 cells			
	Clone no	**Tumor weight (g)**	**Termination time (weeks)**
Vector clones	V33	1.24±0.61	4
	V19	1.36±0.54	4-5
	V18	1.73±0.48	7-8
Weak peptide clones	P41	1.09±0.41	6-7
	P63	1.27±0.29	6-7
Strong peptide clones	P25	0.31±0.14	7-9
	P42	0.38±0.20	9
	P29	0.42±0.17	9

LNCaP cells			
	Clone no	**Tumor weight (g)**	**Termination time (weeks)**
Vector clones	V82	0.65±0.29	8
	V69	0.22±0,03	6
Peptide clones	P35	0.05±0.01	6
	P12	0.04±0.03	8

Table 3.1: Bag-1 peptide reduces tumour weight in xenograft models of 22Rv.1 and LNCaP cells

Tables of tumour weights and termination times for xenograft models of 22Rv.1 and LNCaP stable clones. When tumour reached the maximum size or nine weeks after injection, animals were sacrificed and tumours harvested and weighed. The numbers (in grams) represent the mean value of at least five tumours ±SD.

To determine the reason for the reduced volume and weight of the tumours derived from cells expressing the Bag-1 peptide, histological staining for apoptosis was carried out. Apoptosis is the process of programmed cell death (PCD) occurring for the natural turnover of cells in tissues. In cancer, this process is inhibited resulting in uncontrolled cell division. To detect apoptosis, the terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay was used. The principle of the assay is based on the observation that during apoptosis DNA is nicked by DNase. The enzyme deoxynucleotidyl transferase can recognize the nicks and add dUTPs at the 3'-OH terminus of the DNA. Addition of digoxygenin conjugated-UTP allows for specific recognition of cells undergoing apoptosis (Gavrieli *et al.*, 1992). For this purpose, tumour tissues derived from the 22Rv.1 stable clones where paraffin-embedded and cut into 5-µm-thick sections for immunohistochemical analysis.

Apoptotic cells were detected using an anti-digoxygenin antibody conjugated to a peroxidase. Addition of the substrate 3',3'-diaminobenzidine (DAB) and hydrogen peroxide produces a brown-coloured signal. Nuclei of normal cells were stained blue-

purple with hemtoxylin which binds to the negatively charged DNA. In Fig. 3.7 representative sections of xenografts established from 22Rv.1 stable clones are showed. Tumour tissue derived from clones expressing the empty vector showed a prevalent blue staining indicating the absence of apoptosis. Histological analysis of the sections obtained from clones expressing low levels of peptide showed a slight increase in the staining of apoptotic cells. Conversely when sections of xenografts generated from high expressing peptide clones were analysed, almost the whole surface was stained with apoptotic cells accompanied by some diffused brownish staining indicative of necrosis.

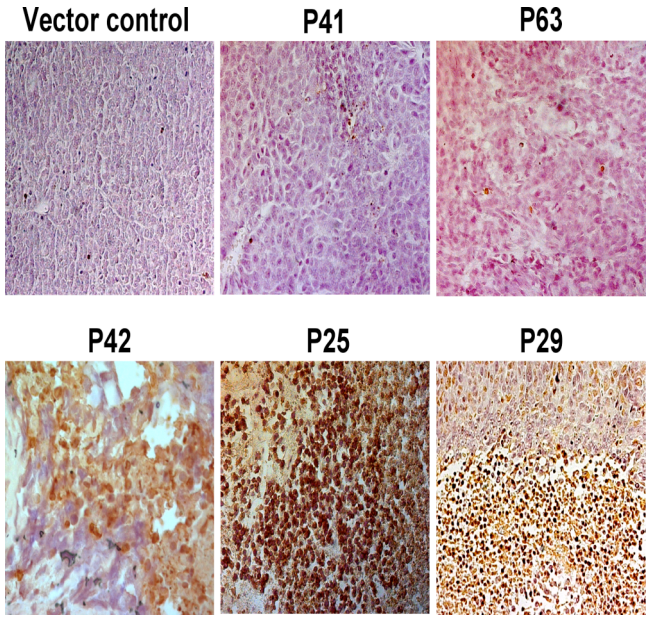

Fig. 3.7: Xenografts of stable clones expressing Bag-1 peptide show increased apoptosis
Paraffin-embedded sections of tumours generated from subcutaneous injection of 22Rv.1 stable clones into athymic nude mice (20X magnification). Tumour tissues were harvested at the moment of sacrifice and fixed in 4% paraformaldheyde. Thereafter the specimens were embedded in paraffin, cut into 5 µm-thick slices and subjected to immunohistochemical analysis. Nuclei were stained in blue-purple with hematoxylin while apoptotic cells were detected using the TUNEL assay and stained brown. Representative sections of xenografts obtained from each 22Rv.1 stable clone were acquired with an Axioscop microscope (Zeiss).

3.6 The Bag-1 peptide interacts with distinct sequences of GRP75 and GRP78

In this work a peptide derived from Bag-1 has been shown to inhibit prostate cancer cell growth but its mechanism of action is unknown. One of the first steps towards understanding its mode of action is to identify the cellular targets through which it acts. To accomplish this, interaction partners of the peptide were investigated. The 68 amino acid peptide was fused to glutathione-S-transferase to generate a recombinant protein for GST-pull down assay. Addition of 22Rv.1 and LNCaP cell lysate to the GST-fused peptide led to identification of glucose-regulated protein 75 (GRP75) and 78 (GRP78) as the interaction parner of the peptide.

GRP75 (also known as mortalin or mtHsp70) belongs to the family of the heat shock proteins (Hsps) and is localised mainly in the mitochondria (Bhattacharyya *et al.*, 1995). GRP78 (also called BiP) is mainly in the endoplasmic reticulum and is involved in the regulation of the unfolded protein response (UPR) (Lee, 2001). Both proteins are overexpressed in a large number of cancers (Daneshmand *et al.*, 2007; Wadhwa *et al.*, 2006).

In order to better understand the function of the peptide, its binding site on the two target proteins was investigated. GRP75 and 78 share 47% of amino acid identity (Bhattacharyya *et al.*, 1995) and consist of three domains: an ATPase domain that drives ATP hydrolysis, a substrate binding domain (SBD) that recognizes the client protein and an oligomerization domain that regulates the interaction of the GRPs with themselves (Bhattacharyya *et al.*, 1995). To show which domain is bound by the Bag-1 peptide, GST-pull down assay was performed using glutathione-S-transferase fused to GRP75, GRP78 and several truncations. These consisted of the ATPase domain, the substrate binding domain, the oligomerization domain and the full length protein lacking the ATPase domain (ΔATPase). A schematic representation of the GST-fused GRP75 and 78 and their deletion mutants is shown in Fig. 3.8A. The GST-fused proteins were pre-adsorbed on glutathione-sepharose beads and then incubated with cell extract of 22Rv.1 stably expressing the Bag-1 peptide. As negative control the cell lysate was incubated with the glutathione-S-transferase. At the end of the incubation the samples were washed and loaded onto a polyacrylamide gel for electrophoretic separation. After transfer onto a PVDF membrane, western blot analysis was carried using an anti-HA antibody to detect

the Bag-1 peptide bound to the GST-fused proteins. To verify equal protein loading, the membranes were stained with Coomassie® blue.

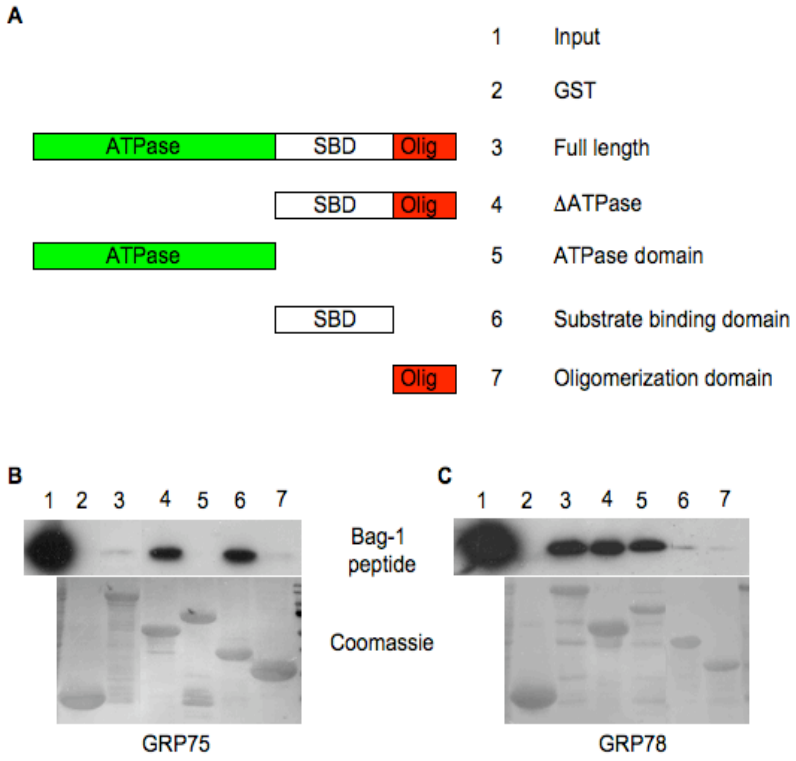

Fig. 3.8: Interaction of the Bag-1 peptide with GRP75 and GRP78
GST-pull down experiment with GRP75 and 78 and their deletion mutants. A) Schematic diagramme of GRP75 and GRP78 and their deletion mutants. The ATPase domain is depicted green, the peptide binding domain white and the oligomerization domain red. B) GST-pull down assays were performed incubating 50 µg of GST-fused GRP75 and its deletion mutants with 400 µg of cell lysate of 22Rv.1 stably overexpressing the Bag-1 peptide. Prior to the incubation with the cell lysate, the GST-fused proteins were bound to gutathion sepharose beads 4B. After washing, the samples were boiled, loaded onto a polyacrylamide gel and transferred onto a PVDF membrane. Western blot analysis was performed using an anti-HA antibody to detect the peptide bound to the GST-fused protein. The membrane was stained Comassie® blue for equal loading control. C) GST-pull down assay performed incubating 50 µg of GST-fused GRP78 and its truncations with 400 µg of lysate from 22Rv.1 cells stably overexpressing the Bag-1 peptide.

Incubation of the cell lysate with GST-fused GRP75 and its truncations showed a weak interaction of the full length protein with the Bag-1 peptide (Fig. 3.8B lane 3) while deletion of the ATPase domain increased the binding (Fig. 3.8B, lane 4). The ATPase and the oligomerization domain did not show significant binding (Fig. 3.8B, lane 5 and 7), but the substrate binding domain bound the Bag-1 peptide (Fig. 3.8B, lane

6) to the same extent as the GRP75ΔATPase protein indicating that the interaction is mediated by the substrate binding site.

Studies with GST-fused GRP78 and its deletion mutants incubated with the cell extract obtained from the 22Rv.1 stably expressing the Bag-1 peptide produced different results. The full length GRP78 protein bound the peptide (Fig.3.8C, lane 3) as well as GRP78ΔATPase (Fig.3.8C, lane 4) even if the extent is reduced considering the loading control. Unlike the case of the studies with GRP75, the ATPase domain showed interaction (Fig. 3.8C, lane 5) and the substrate binding domain and the oligomerization domain (Fig. 3.8C, lane 6 and 7) showed almost undetectable binding indicating that GRP78 interacts with the peptide mainly through its ATPase domain. These results show that the Bag-1 peptide recognizes specific sequences of distinct domains on GRP75 and GRP78.

3.7 The Bag-1 peptide is colocalised with GRP75 and GRP78

If the Bag-1 peptide binds to GRP75 and 78 as shown in GST-pull down experiment, these proteins should colocalise in cultured cells. For this reason 22Rv.1 cells expressing the peptide were fixed with 4% paraformaldehyde and subjected to immunofluorescence assay (Fig. 3.9). For detecting the peptide an anti-HA antibody was used while GRP78 and GRP75 were identified by the use of specific antibodies.

Laser confocal microscopy analysis showed that the peptide (green staining) was equally distributed in the nucleus and cytoplasm of the cell. In the cytoplasm it was identified in punctuated staining indicating the targeting of subcellular compartment(s) (Fig. 3.9A and D). GRP75 (red staining) occurred mainly with a punctuated distribution in the cytpolasm (Fig. 3.9B). Staining with the peptide showed a partial colocalisation of the two proteins around the punctuated structures (Fig. 3.10C). GRP78 (red staining) was visible mainly in the cytoplasm (Fig. 3.9E). Co-staining of the peptide and GRP78 showed an intense colocalisation (orange signal) in the cytoplasm of the cells (Fig. 3.9F) indicating that the peptide mainly co-localises with this protein.

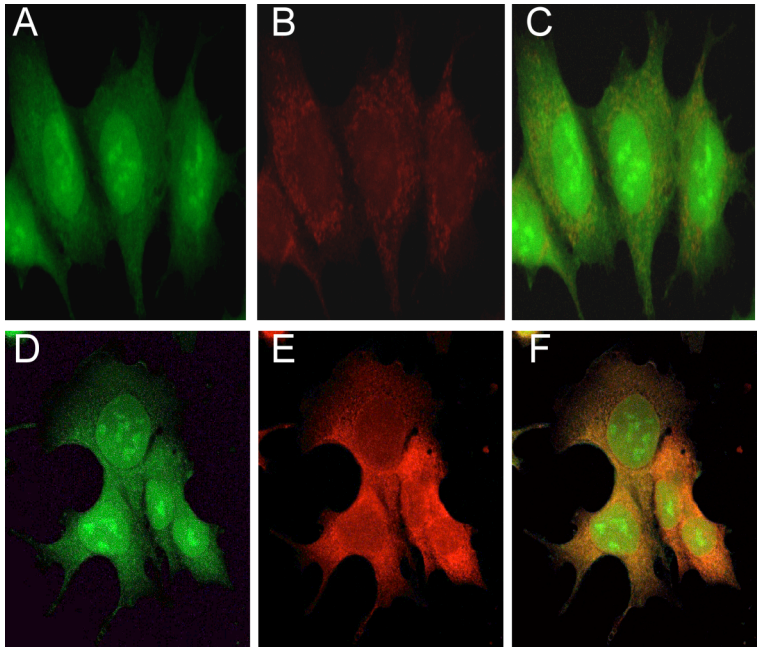

Fig. 3.9: The Bag-1 peptide is colocalised with GRP75 and GRP78
Confocal microscopy analysis of 22Rv.1 cells stably expressing the Bag-1 peptide (63X magnification). For immunofluorescence assay 22Rv.1 cells were fixed with paraformaldehyde on a coverslip and stained with an anti-HA antibody to detect the peptide (green, A and D). GRP75 (B) and GRP78 (E) were detected with anti-GRP75 and 78 specific antibodies and are represented in red. The merge of the two channels (E and F) produces an orange colour when the two proteins colocalise. Images were acquired with a Leica TCS SPE confocal microscope (Leica Microsystems).

3.8 The Bag-1 peptide is localised in the endoplasmic reticulum but not in the mitochondria

Confocal microscopy analysis showed that the Bag-1 peptide colocalised with both targets in the cytoplasm in punctuated structures. Since GRP75 and GRP78 are preferentially in the mitochondria and the ER respectively and both these structures have a dotted distribution in the cytoplasm, the localization of the peptide in these two subcellular compartments was investigated. For this purpose immunofluorescence assay was performed using 22Rv.1 cells stably transfected with the Bag-1 peptide (Fig. 3.10). Mitochondria were detected with the Mitotracker® mitochondrion selective probe (Fig. 3.10A, II) that accumulates selectively into the mitochondria where it is converted into a fluorescent dye. For tracking the ER an antibody recognizing the ER-associated protein disulfide isomerase (PDI), one of the most abundant proteins in the ER (Lyles and Gilbert, 1991) was used (Fig. 3.10A, V). Merging of the images of the peptide (Fig. 3.10A, I) and the mitochondria (red staining, Fig. 3.10A, II) showed a yellow staining

indicating that the peptide was rather localising at the outer membrane of the mitochondria (Fig. 3.10A, III). The merge of the channel of the peptide (Fig. 3.10A, IV) with the channel of the ER (Fig. 3.10A, V) showed an orange-yellowish colour indicating colocalisation of the peptide with the PDI (Fig. 3.10A, VI).

In order to quantify the amount of peptide localizing in the mitochondria and in the ER, the IMARIS® software was used with three representative confocal images for each staining. Only 15% of colocalisation of the peptide with the mitochondria was observed, while the peptide and the ER colocalised in almost half of the surfaces taken into account for the study (Fig. 3.10B) indicating that the peptide preferentially localizes in the endoplasmic reticulum.

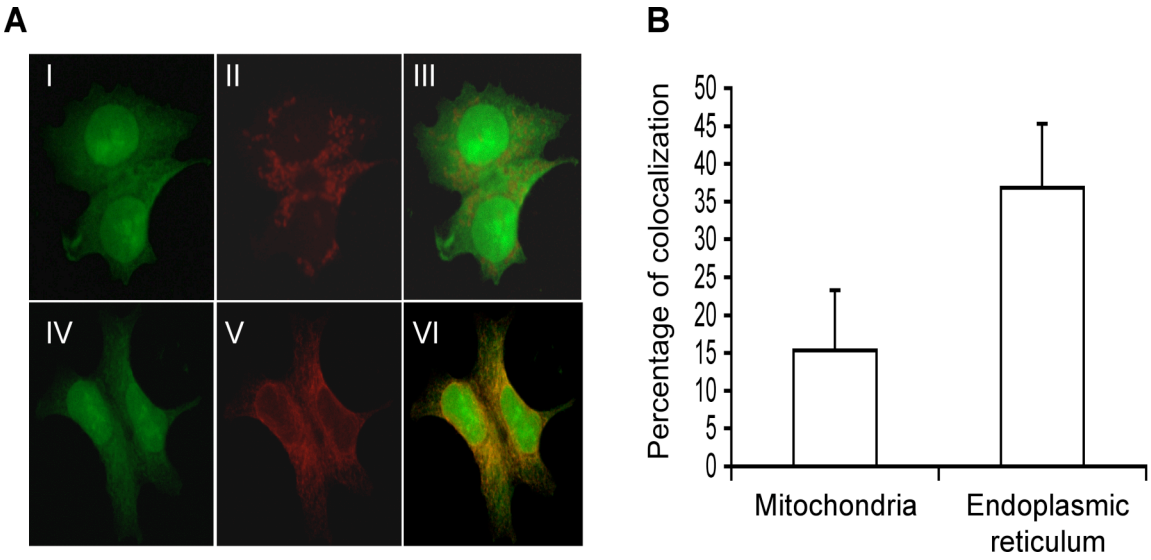

Fig. 3.10: Subcellular localisation of the Bag-1 peptide

Immunofluorescence analysis on 22Rv.stably expressing the Bag-1 peptide to determine its subcellular localisation. Confocal images of 22Rv.1 stably expressing the Bag-1 peptide fixed with 4% paraformaldehyde (63X magnification). The Bag-1 peptide was detected with an anti-HA antibody and is represented in green (I and IV). For staining of the mitochondria, the Mitotracker® dye was used (II) while for the endoplasmic reticulum the ER-tracker® kit was used (V). Both the mitochondria and the endoplasmic reticulum are represented in red. Merge of the channels shows the area of colocalisation (III and VI). Images were acquired with a Leica TCS SPE confocal microscope (Leica Microsystems). B) Quantification of the percentage of colocalisation of the Bag-1 peptide in the mitochondria (left bar) and the endoplasmic reticulum (right bar). The calculation was performed using the IMARIS® software (v 6.3.1, Bitplane). The values represent the average of three independent experiment ±SD. Images were acquired with a Leica TCS SPE confocal microscope.

3.9 The N-terminal region of the Bag-1 peptide is sufficient for growth inhibition

The region of 68 amino acids identified from Bag-1L included a portion of the ubiquitin-like domain at the N-terminal region and a part of the Bag domain at the C-terminal region. With the help of secondary structure predictors (PSIPRED and PROFSEC) it was shown that the peptide consists of a β-sheet and a loop derived from the ubiquitin-like domain followed by an α-helix structure representing the Bag domain (W. Wenzel, unpublished data). In order to determine which of these regions of the peptide is responsible for its growth-inhibitory properties, several deletion mutants of the Bag-1 peptide were generated. These consisted of the N-terminal part of the peptide encompassing the first 40 amino acids (from position 202 to 241) and covering the whole portion of the ubiquitin-like domain plus a small sequence of the Bag domain. This mutant was termed N-terminal peptide. The last 29 amino acids (from position 241 to 269) covering the C-terminal end of the peptide was named C-terminal peptide. In addition, the first 18 amino acids representing the ubiquitin-like domain were removed generating a peptide of 50 amino acids (from position 220 to 269) defined as ΔN-peptide. A schematic representation of the peptides used for this study is shown in Fig. 3.11A.

To determine which of the described sequences retained the ability of inhibiting cell growth, they were cloned into an expression vector carrying the gene for G418 resistance and transfected into the 22Rv.1 prostate cancer and the BPH-1 bening prostate cells for colony forming assay (Fig. 3.11B).

Overexpression of the C-terminal peptide did not significantly reduce the colony number both in 22Rv.1 and in BPH-1 cells. Similar results were obtained when the ΔN-peptide was overexpressed, indicating that the mutant truncated at the N-terminal region of the peptide did not contribute to the growth inhibitory function. In contrast, when the N-terminal peptide was expressed in the cells it reduced colony formation in the 22Rv.1 to the same extent as the Bag-1 peptide. However expression in the BPH-1 cells did not have a significant effect. These results indicate that the first 40 amino acids at the N-terminal region of the Bag-1 peptide is sufficient to inhibit cell growth and that this action is specific for cancer cells since the BPH-1 cells are not affected. Consistent to

this result, GST-pull down assay, using the described peptides fused to the GST and 22Rv.1 cell lysate, showed that the sequences that did not inhibit cancer cell growth (the C-terminal peptide and the ΔN-peptide) did not bind GRP75 and 78 while the N-terminal peptide bound the two proteins (unpublished data from K. Jehle, a diploma student in our laboratory).

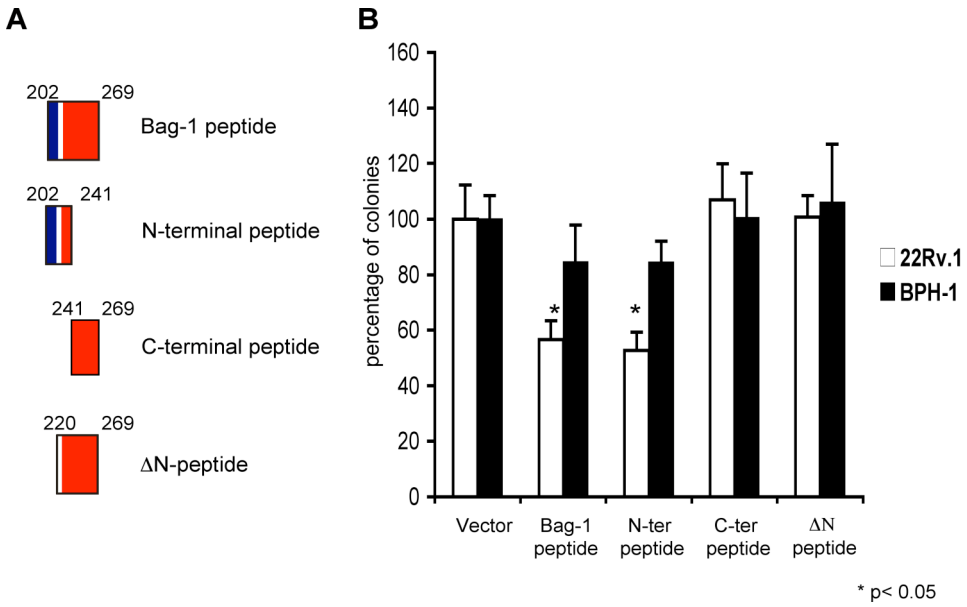

Fig. 3.11: A region of 40 amino acids at the N-terminal part of the Bag-1 peptide is sufficient to reduce colony formation

A) Schematic representation of the deletion mutants of the Bag-1 peptide. The domains depicted blue and red are the ubiquitin-like domain and the Bag domain respectively. B) Clonogenic assay with constructs expressing deletion mutants of the Bag-1 peptide in BPH-1 and 22Rv.1 cells. Cells transfected with these constructs were selected with medium containing G418. Results are shown as the mean value ±SEM of at least three independent experiments using three different plasmid preparations (*p <0.05). The values obtained with the empty vector were set as 100%. Open bars represent the result of the 22Rv.1 and the filled bars are the result of the BPH-1 cells.

To confirm the effect of the identified peptide in an *in vivo* tumour model, the 22Rv.1 cells were transfected with constructs carrying the respective peptides and the G418 resistance gene. After antibiotic selection single clones were picked, expanded and again subcutaneously injected into the flanks of athymic mice to establish xenograft models. Tumour sizes were measured weekly over a period of five weeks (Fig. 3.12).

Injection of stable clones expressing the C-terminal region of the peptide produced tumour sizes comparable to the vector controls indicating that this sequence does not have any effect on the growth profile of the xenografts (Fig. 3.12A). Similar results were obtained when single clones expressing the Bag-1 peptide lacking the first 18 amino

acids were analysed (Fig. 3.12B). On the other hand xenografts of stable clones expressing the N-terminal region of the peptide could not be established indicating that this sequence is sufficient and necessary for the growth inhibitory action of the Bag-1 peptide (Fig. 3.12C).

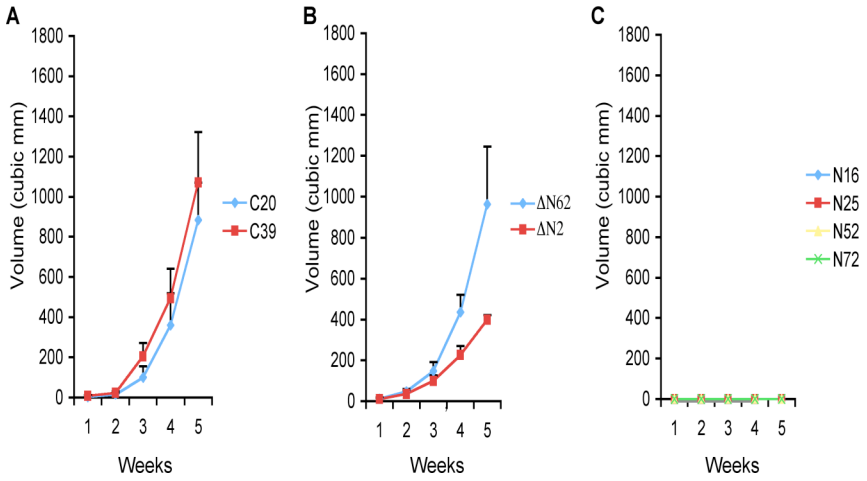

Fig. 3.12: Bag1 N-terminal peptide reduces prostate tumour growth *in vivo*.
Growth curves of stable clones expressing Bag-1 peptide deletion mutants. Six-week old athymic nude mice were subcutaneously injected with $5x10^6$ cells of stable clones expressing the C-terminal peptide (A), the ΔN-peptide (B) and the N-terminal peptide (C). Tumours were measured every week for five weeks using a caliper. Results are presented as the mean value of 5-10 tumours ±SD.

Comparison of the growth curves of the Bag-1 peptide expressing clones (Fig. 3.5C) and the clones containing the N-terminal peptide (Fig. 3.12C) shows that the two peptides identified have similar efficacy of action.

3.10 A sequence of 19 amino acids at the N-terminal region of the Bag-1 peptide is sufficient to reduce cell growth

The colony forming assay and the *in vivo* xenograft models shown in section 3.11 demonstrated that deletion of the region covering a portion of the ubiquitin-like domain (between amino acids 202 and 220) in the Bag-1 peptide abrogated its growth inhibitory action and that expression of 40 amino acids from the N-terminal region was sufficient to inhibit tumour growth. Since the N-terminal peptide (202-241) still contained sequences

derived from two domains (the ubiquitin-like domain and the Bag domain) it was further divided into a fragment of 19 amino acids (from position 202 to 220) including only the ubiquitin-like domain portion and a fragment of 22 amino acids (from position 220 to 241) including the Bag domain and 5 upstream amino acids (Fig. 3.13A). In order to study the effect of these sequences on cell growth, the peptides were cloned into an expression vector and colony forming assay was performed in 22Rv.1 and BPH-1 cell. When the 19-mer peptide (202-220) was transfected in 22Rv.1 the colony formation was reduced to the same extent as the N-terminal peptide indicating that this sequence is able to reproduce the effect of the N-terminal peptide. Overexpression of the same construct into BPH-1 cells did not reduce colony formation indicating that this peptide exerts a cell type specific action (Fig. 3.13B). When the 22-mer peptide (220-241) was expressed, it did not show any effect on colony formation in the two cell lines analysed (Fig. 3.13B).

The Bag-1 peptide and its truncation showed a specificity of action towards the cancer cell line 22Rv.1 while it did not affect the growth of the benign cell line BPH-1. If these sequences exert indeed their growth inhibitory action through the binding to GRP75 and GRP78, their specific effect on cancer cell lines could be explained by differences in the level of expression of the two target proteins in the cell lines analysed. Thus extracts from 22Rv.1 and BPH-1 cells were run onto a polyacrylamide gel and subjected to electrophoretic separation to check the relative levels of GRP75 and GRP78. After transfer onto a PVDF membrane, Western blot experiment was performed using anti-GRP75 and GRP78 antibodies for detecting GRP75 and GRP78 and an anti-β-actin antibody to determine the level of β-actin which was used for equal loading control. The 22Rv.1 cancer cell line (Fig. 3.13C, lane 1) showed a higher level of expression of GRP75 and GRP78 compared to the benign cell line BPH-1 (Fig. 3.13C, lane 2) suggesting that the reduced level of expression of these target proteins may explain the differential effect of the Bag-1 peptides towards growth of these cells.

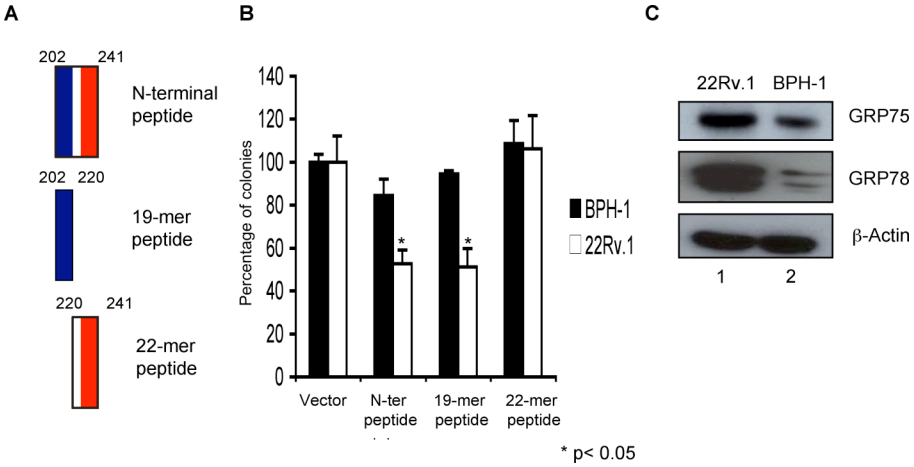

Fig. 3.13: A sequence of 19 amino acids of Bag1-L reduces cancer cell growth.

A) Schematic diagramme of the deletion mutants derived from the N-terminal region of the Bag1 peptide. B) Colony forming assay performed in 22Rv.1 and BPH-1 cells. Results are shown as the mean value of at least three independent experiments using three different plasmid preparations (*$p<0.05$). Vector values are set as 100%. Open bars represent the result of the 22Rv.1 and the filled bars are the result of the BPH-1 cells. C) Level of expression of GRP75 and GRP78 in 22Rv.1 and BPH-1 cells. Extracts from 22Rv.1 and BPH-1 cells were loaded on a polyacrylamide gel and transferred to a PVDF membrane. Western blot analysis was performed with anti-GRP75 or anti-GRP78 antibody to detect GRP75 or GRP78 and an anti-β-actin antibody to detect β-actin used as loading control.

4. DISCUSSION

Bag-1 (Bcl-2 associated athanogene, member 1) is a family of proteins formed by four members (Bag-1L, Bag-1M, Bag-1 and Bag-1S) encoded by the same mRNA via alternative translational initiation site (Yang *et al.*, 1998). The longest isoform of the family, Bag-1L, has been found overexpressed in a variety of cancers while it is almost undetectable in benign tissues, indicating that this protein plays a role in the transition of cells towards malignancy (Takayama *et al.*, 1998). However reports on Bag1-L function in cancer have so far led to controversial results. On the one hand, detection of high levels of this protein in tumour specimens is indicative of good prognosis and better patient outcome (Millar *et al.*, 2008). On the other hand, it has been demonstrated that Bag-1L inhibits apoptosis in cancer cells and is associated with aggressive tumours (Kikuchi *et al.*, 2002; Krajewska *et al.*, 2006; Takayama *et al.*, 1995).

In this work, to determine whether changes in the levels of Bag-1L could affect prostate cancer cell growth, the transgenic adenocarcinoma of the mouse prostate (TRAMP) model was crossed with a Bag-1 knock out mice (Bag-$1^{+/-}$). The resulting progeny showed a correlation of Bag-1 levels with size of the prostate tumours. In addition, the effect of Bag-1L overexpression was compared between benign and malignant prostate cells and the results showed that this protein reduces prostate cancer cell growth. The region responsible for this action was identified as a 68 amino acid long peptide. This fragment is localised in the endoplasmic reticulum and binds distinct domains of the glucose regulated proteins GRP75 and GRP78. The first 19 amino acids at the N-termianl part of the peptide were further identified as the region responsible for the growth inhibitory effect.

4.1 Bag-1L in prostate cancer: a protector or a killer?

An increase in Bag-1 level has been found in patients with prostate cancer (Krajewska *et al.*, 2006; Mäki *et al.*, 2007) suggesting that this protein plays a role in the development of this tumour. However there is no clear evidence that the level of Bag-1L

is related to the development of prostate tumour. In this work it was shown in a TRAMP (transgenic adenocarcinoma of the mouse prostate) model that the level of Bag-1 gene expression correlated with the size of the tumour. Although significant, the reduction (about 23,3% in the TRAMP/Bag-$1^{+/-}$ mice) was expected to be higher when compared with another work where crossing Bag-1 heterozygous mice with a model of lung metastasis clearly reduced the number of metastatic foci (Götz *et al.*, 2004). One of the reasons of the weaker effect of the reduction of Bag-1 levels on tumour formation in the TRAMP could be due to the nature of the model used. In the TRAMP model a transgene consisting of the fusion of the rat probasin promoter with the SV40 T antigen is responsible for the tumour formation. It is likely that the T antigen may interfere with the function of Bag-1. This could explain why the knock down of Bag-1 did not influence the overall survival of the TRAMP mice when survival studies were carried out comparing the TRAMP wild type with the TRAMP/Bag-$1^{+/-}$ mice.

A comparative study to determine the level of expression of the Bag-1 proteins in benign and malignant tissues showed that the longest isoform of the family, Bag-1L, is seldom expressed in normal tissues while is found in high levels in cancer (Takayama *et al.*, 1998). This observation suggests that Bag-1L levels are critical for tumorogenesis and therefore in this work the role of this isoform was studied in prostate cancer. Overexpression of Bag-1L in benign prostate cell line BPH-1 increased colony formation ability in clonogenic assay, an observation in line with studies reporting that this protein is important for the transition of cells from normal to neoplastic condition (Takayama *et al.*, 1998). On the contrary increased expression of Bag-1L in the cancer cell line 22Rv.1 led to a significant decrease of cell growth, indicating that the sequence of this protein contains the information for inhibiting cell growth. Thus the Bag-1L protein could exert both positive and negative effect on cell growth. It remains to explain why the same protein has opposite effect in two cell lines both of the same tissue of origin. One possibility is the pathophysiological stage of the prostate cells at the time of the experiment. In fact it is important to note that the 22Rv.1 cells are derived from a primary prostate cancer and express Bag-1L while the BPH-1 are derived from benign prostatic hyperplasia and do not express the protein. The inhibitory effect and the stimulatory action of Bag-1L in these two cell lines would suggest that a threshold for Bag-1L determines the protective or the lethal function of the protein.

Deletion of a region of 68 amino acids has been shown in this work to abolish the inhibitory effect of Bag-1L. When the sequence was expressed on its own, it also

decreased tumour growth. This observation is consistent with the finding that overexpression of a large 174 amino acid peptide, corresponding to Bag-1L lacking the first N-terminal 124 the last 47 C-terminal amino acids, exerted a dominant negative effect reducing the tumour size of xenograft models of the breast cancer cell line ZR-75-1 (Kudoh *et al.*, 2002). In the work presented here the growth inhibitory region has been narrowed down to 68 amino acids and finally to the region included between the positions 202-220. Thus this sequence of 19 amino acids is included in the larger sequence of 174 amino acids that exerted the tumour growth inhibitory effect in the breast cancer xenograft. This would suggest that the effect of the peptide described in this work would not only be limited to prostate cancer but might also cover a wider range of malignant cell lines.

Bag-1 proteins are known to promote cell proliferation and inhibit apoptosis (Liu *et al.*, 2009; Townsend *et al.*, 2003a; Yang *et al.*, 1999). This effect is common to all the isoforms of the family and deletions of sequences at the N-terminal region of the longest isoform, Bag-1L, do not affect the anti-apoptotic property. However deletion of the C-terminal region converts these proteins into tumour cell growth inhibitors (Kudoh *et al.*, 2002). This therefore suggests that the C-terminal Bag domain plays a major role in the regulation of the action of these proteins. This could occur through the following mechanisms. First, the C-terminal sequences could hide the fragment responsible for the growth inhibitory action making it unaccessible. Thus, deletion of the C-terminus provides the Bag-1 proteins with growth inhibitory properties. Second, removal of the C-terminal region could cause a conformational change that converts the proteins into cell growth inhibitors. Third, deletion of the C-terminal Hsp70 binding domain abrogates the strong binding of the Bag-1 proteins to Hsp70 (Takayama *et al.*, 1997) making them available for other protein-protein interactions. These three hypothesis do not exclude each other and it could well be that the mechanism by which the Bag-1 proteins are converted into cell growth inhibitors is a combination of these options. In this connection it is important to note that the the 68 amino acid Bag-1 peptide identified in this work also lacks the C-terminal Bag domain.

It is amazing how such a relatively small peptide could influence the growth of cancer cells. One striking feature of this small peptide is that it covers part of the ubiquitin-like domain (ULD). This domain has been found in a growing number of proteins and contains an hydrophobic core sequence with an isoleucine at the centre which can be conjugated to a ubiquitin moiety that drives protein degradation

(Hochstrasser, 2000). It could be hypothesized that the peptide binds to proteins vital for the cancer cells to degrade them. However, even though sequence alignment study has shown that the wild type Bag-1L also contains the core sequence recognized by the ubiquitin (Grabbe and Dikic, 2009), this region is not included in the peptides identified in this work. Alignment of the 68 amino acid peptide identified in this work with the whole human genome using the BLAST programme did not retrieve any result other than the Bag-1 proteins, indicating that the sequence of the peptide is unique.

4.2 Interaction of the Bag-1 peptide with GRP75 and GRP78

Immunofluorescence assay carried out in this work showed that the Bag-1 peptide co-localises with glucose regulated proteins 75 and 78 (GRP75 and GRP78) that have been identified in our laboratory as interacting partners of the peptide. The GRP75 and 78 belong to the family of the Hsp70 proteins and share an identity of 43% and 57% respectively with Hsp70 (Bhattacharyya *et al.*, 1995). The fact that the Bag-1 peptide binds to GRP75 and 78 but not to Hsp70 (D. Maddalo and K. Jehle, unpublished data) shows that the action is mediated by specific binding to the target proteins.

The GRPs function as chaperones controlling the conformation of nascent polypeptides (Bukau, 2006). GRP75 and 78 as well as Hsp70 are organized in three distinct domains: an ATPase domain that drives the hydrolysis of ATP necessary for the enzymatic activity of these proteins, a substrate binding domain (SBD) that recognizes the client proteins and an oligomerization domain that modulates the interaction of these proteins with themselves. In spite of the high homology, in studies carried out by a diploma student in our laboratory it has been shown that the Bag-1 peptide does not bind Hsp70 indicating that it is specific in the choice of its substrate. A “kettle pot” model has been proposed for the structural organization of GRP75 and GRP78 domains (Kaul *et al.*, 2007), where the ATPase domain represents the handle that, upon ATP binding, drives a conformational change that opens the lid (the oligomerization domain) allowing the entrance of the substrate protein into the pot, represented by the SBD. At the end of the process the folded substrate is released by the opening of the lid. In this work it has been shown that the peptide can recognize distinct domains on its target proteins. According to the above described model, the binding of the peptide to the SBD of GRP75 would block the entrance of the client proteins into the pot interfering with the

chaperone activity of GRP75. The finding that the binding of the Bag-1 peptide to the SBD or the deletion mutant lacking the ATPase domain of GRP75 is stronger compared to the full length GRP75 protein could be due to a structural obstruction of the binding site of the peptide by the ATPase domain.

Binding of the Bag-1 peptide to the domains of GRP78 produced different results. The Bag-1 peptide binds to the ATPase domain of GRP78 which could impair the enzymatic activity of the protein and possibly interfere with its chaperone activity. The fact that the Bag-1 peptide binds only to the ATPase domain of GRP78 suggests that the interaction takes place through a region not conserved in other chaperones. The ATPase domain of GRP78 is based on the model of Hsp70 that consists of 382 amino acids and is organised in the domain I (from position 3 to 188), covering the first half of the domain, and a second region consisting of the domains IIA (129-288) and IIB (307-382) (Flaherty *et al.*, 1990; Osipiuk *et al.*, 1999). Alignment of the sequences of GRP78, GRP75, Hsp70 and Hsc70 reveals that the less conserved regions are a small fragment between position 96 and 104 (in domain I) and a larger portion between position 235 and 320 (at the edge of the domains IIA and IIB) on the ATPase domain of Hsp70 (Bhattacharyya *et al.*, 1995). Thus it is likely that the Bag-1 peptide binds to these non-conserved regions in the ATPase binding domain of GRP78. It has been observed that a molecule, (-)-Epigallocatechin gallate, binds the ATPase domain of GRP78, on a region covering the domain IIA and IIB and induces apoptosis by inhibiting the interaction between GRP78 and Caspase-7 (Ermakova *et al.*, 2006). A possibility therefore exists that the Bag-1 peptide exerts its growth inhibitory function in a similar way. The Bag-1 peptide can also bind the mutant of GRP78 lacking the ATPase domain, even if to a lesser extent. This binding could represent an additional way the peptide affects the action of GRP78 by binding to its C-terminal region.

Further mechanism of action of the Bag-1 peptide through its interaction with GRP75 and 78 can be envisaged. GRP75, for example, exerts a positive effect on cell growth by sequestering p53 in the cytoplasm to inhibit its nuclear pro-apoptotic activity in cancer cells (Wadhwa *et al.*, 1998). MKT-077, a rhodacyanine dye analogue, that binds GRP75 to abrogate its interaction with p53, has been shown to induce apoptosis in cancer cells. A possibility therefore exists that the Bag-1 peptide functions similarly. However, the effect of the binding of the Bag-1 peptide to GRP75 does not seem to interfere with p53 since no accumulation of this protein in the cytoplasm has been observed when the peptide is overexpressed (K. Jehle unpublished data). The finding

that the peptide binds two proteins and is localised in the endoplasmic reticulum and possibly on the mitochondrial outer membrane could give alternative hints to its mechanism of action. The fact that the Bag-1 peptide binds both GRP75 and 78 would mean that it integrates two independent events, a mitochondrial and an ER function to optimally inhibit cell growth. One possibility would be that the peptide interferes with the unfolded protein response (UPR). The UPR is a pathway that represents the reaction of cells to stress inducing agents otherwise lethal like hypoxia and hypoglycemia (Kaufman, 1999). Upon stress the ER and the mitochindria cross-talk (Le Bras *et al.*, 2006) and this process is regulated by GRP78 (Lee, 1992). In addition it has been shown that during the UPR GRP78 translocates from the ER to the mitochondria (Sun *et al.*, 2006) and that the interaction between these two organelles is mediated by GRP75 (Hayashi *et al.*, 2009). Therefore a peptide which binds to both GRP75 and 78 would probably interfere with the UPR and promote cell death, as observed in the tumour tissues derived from the clones expressing the peptide.

Considering that the level of expression of GRP75 and 78 differs between normal and malignant cells, it could be explained why the peptide has a different impact on the 22Rv.1 and BPH-1 cells showing the possibility to discriminate between healthy and malignant cells.

4.3 The Bag-1 peptide is unstructured

The 68 amino acids peptide identified in this work as a growth inhibitory peptide covers a portion of two domains of the wild type Bag-1L protein. The sequence covers part of the ubiquitin-like domain at the N-terminal and part of the Bag domain at the C-terminal region. Circular dichroism studies and NMR analysis of the peptide showed that except for a 25% of alpha helical folding the sequence does not have any particular secondary or tertiary structure (Dr. C. Muhle, unpublished work). This finding is consistent with a structural prediction of the peptide carried out by Dr. Wolfgang Wenzel from the Institute of Nanotechnology at the Forschungszentrum Karlsruhe.

In this work the 68 amino acid peptide was further narrowed down to 40 amino acids and in this case the circular dichroism analysis carried out by Dr. C. Muhle showed that it is completely unstructured. The observation that the peptide is not structured

could lead to the speculation that the binding to the GRPs is driven by its misfolded organization rather than through a specific sequence.

In the last few years several proteins lacking an intrinsic structure under physiological conditions have been identified. These proteins have a high structural flexibility which allows them to bind several targets (Wright and Dyson, 1999). They gain their active conformation only when in the proximity of the protein to which they bind (Uversky and Narizhneva, 1998; Wright and Dyson, 1999). Examples are the cyclin dependent kinase (Cdk) $p21^{Waf1/Cip1/Sdi1}$ that regulates cell cycle progression by inhibiting Cdk2 (Kriwacki *et al.*, 1996) or the C-terminal activation domain of the transcription factor c-fos (Campbell *et al.*, 2000). It has been observed that the lack of folding could be due to electrostatic repulsion of the amino acidic residues contained in the sequence of these proteins resulting in a combination of low overall hydrophobicity and large net charge (Uversky *et al.*, 2000). The 68 amino acid sequence of the Bag-1 peptide is in line with these observations since it does not contain any aromatic residue except for a phenylalanine at position 60 and is rich in charged residues. Unstructured peptides that regulate cell growth are usually protected from degradation by substitution of the L-amino acids by their D-enantiomers, not present in nature and thereby less susceptible to proteolysis. An example is represented by a sequence derived from the orphan receptor Nur77 which binds the unfolded loop of Bcl-2 (Feifel *et al.*, 1998; Kolluri *et al.*, 2008) and inhibits cancer cell growth. Such substitutions could also be applied to the Bag-1 peptide to maintain or increase its stability.

4.4 Potential applications of the peptide

In the work presented a peptide derived from Bag-1L that binds the molecular chaperones GRP75 and GRP78 has been described. Intriguingly these two molecules are overexpressed on the membrane surface of malignant cells (Shin *et al.*, 2003) providing the possibility to discriminate between healthy and neoplastic cells. Moreover it has been shown that GRP78 is glycosylated and that this modification drives its expression on the cell surface (Rauschert, 2008). From a study of Dr A. Neeb in our laboratory it was possible to demonstrate that the Bag-1 peptide binds specifically to the glycosylated form of GRP78 indicating the potential ability of the sequence identified in this work to recognize cancer cells when administered exogenously.

In the last decade tremendous efforts have been made to design small particles in the size range of nanometers (nanoparticles) able to recognize a specific target in order to deliver drugs and/or pro-drugs specifically to tumours to reduce as much as possible side effects and toxicity in patients (Taton, 2002). One possible way of targeting these particles to the tumour is to functionalise them with peptides able to recognize molecules exposed on the surface of the target cells. In this contest the ability of the Bag-1 peptides described here to specifically bind cancer cell markers could be exploited to decorate nanoparicles for targeting cancer cells to trigger apoptosis.

A 13 amino acid cyclic peptide, Pep42, that binds GRP78 on the surface of tumour cells has been described. This peptide penetrates and delivers the anti-cancer drugs hematoporphyrin and a pro-apoptotic peptide to tumour cells (Liu *et al.*, 2007). In the work presented here, since the Bag-1 peptides have by themselves a growth inhibitory property, it would not even be necessary to conjugate them with other anti-cancer drugs.

The Bag-1 peptide described here could also be used for diagnostic purposes by conjugation to quantum dots to identify and visualize tumour cells expressing GRP75 and/or 78 on their surface. Similar studies have been done with streptavidin-conjugated quantum dots for *in vivo* cell imaging (Kaul *et al.*, 2003). The peptides described in this work (Devireddy *et al.*, 2000)have the great potential of recognizing GRP75 and GRP78 and exerting an growth inhibitory effect on cancer cells, making possible to achieve both tracking malignant cells and reducing their growth.

4.5 Conclusions

In this work a 68 amino acid peptide derived from the co-chaperone Bag-1L with growth inhibitory properties has been identified. The finding that this region can bind specific domains of the glucose regulated proteins GRP75 and GRP78 and the studies reporting overexpression of these proteins in cancer but not in normal cells suggests a big potential of the peptide in discriminating between normal and pathological stages.

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(2009 – submitted)

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Bag-1 peptide that inhibits prostate cancer - European Patent 08013552.8-2405 (pending)

Oral presentations

Distinct peptides derived from the co-chaperone Bag1-L inhibit prostate cancer cell growth
3rd Cancure Meeting
June 2009, Amsterdam – The Netherlands

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Poster presentations

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Bag-1L: an essential regulator of androgen receptor action in prostate cancer

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