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Characterization and subcellular localization of the human BAT3 protein.

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CHARACTERIZATION AND SUBCELLULAR LOCALIZATION OF THE HUMAN BAT3 PROTEIN

by

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ABSTRACT

Human BAT3 is a 120kDa protein containing a large number of proline, glycine and charged amino acids. Located within the amino-terminus is a region displaying 35% homology to ubiquitin. The functional significance of BAT3 in biological systems remains unknown. However, human BAT3 has recently been shown to share 57% amino acid identity with the *Xenopus* Scythe protein, a regulator of Reaper-induced apoptosis. Due to this high degree of conservation, BAT3 may play a role in mammalian cell death. We have investigated the subcellular localization of human BAT3 by generating a fusion protein between the hemagglutinin (HA) epitope-tag and the full-length BAT3 protein. Using immunofluorescence microscopy and confocal imaging, we have found that full-length BAT3 is predominantly expressed in the nucleus, however a small percentage (10%) of cells exhibit cytoplasmic localization. We have constructed a series of deletion mutants fused to the HA-epitope tag and have over-expressed these proteins in HeLa cells. Immunofluorescent detection reveals that the expression of the amino terminal 656 amino acid residues results in strict cytoplasmic localization of BAT3. In contrast, expression of the carboxyl-terminal 313 amino acid residues displays strict nuclear localization. Site directed mutagenesis has defined a nuclear localization sequence (AKRRKT) within this region (amino acids 1048-1053). Expression of the NLS mutant results in a predominant cytoplasmic localization, suggesting that the function of BAT3 lies primarily within the nucleus. Cell viability assays further support this notion, as cells over-expressing the C-terminal NLS mutant showed a reduced ability to initiate cell death as compared to the wildtype protein. The apparent cell death induced by the over-expression of this domain of BAT3, does not lead to cytochrome c release or DNA

fragmentation, suggesting that cell death is not due to the induction of apoptosis. Yeast two-hybrid screening using an amino-terminal domain of BAT3 has identified Chap1 as a BAT3 interacting protein. Chap1 is the human homolog of the yeast Dsk2 protein, which has been shown to be involved in regulating the G_2/M transition of the cell cycle. These results suggest that BAT3 is involved in the regulation of both cell cycle and cell death events.

To my Parents and Brother

Acknowledgements

When I reflect back upon the many years I have spent at the University of Windsor, I can't help but be overwhelmed by how far I have come. The level of growth and maturity that I have obtained today brings me great satisfaction. What I will miss most about my time spent in Windsor, are the many great people I have come to know, as I have learned much about life from my time spent with them. It is only appropriate that I take the time to thank those who have touched me the most.

First and foremost, Dr. Andrew Hubberstey for giving me the opportunity to conduct this research, an opportunity that others were not willing to give. I thank you for sharing with me your superior knowledge and admire your tremendous patience and unselfish dedication to the growth and development of others.

Importantly, I would like to thank my parents for the tremendous financial support that they have provided for me throughout the years. Perhaps worth more than all of the money, has been the incredible emotional support provided by my parents and brother. Without you, I would not be who or where I am today.

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List of Abbreviations

AIF- Apoptosis Inducing Factor

Apaf-1- Apoptosis protease activating factor

BAT- HLA-B-Associated Transcript

Bcl- B cell lymphoma

BH- Bcl-2 homology

BIR- Baculoviral IAP Repeat

CARD- Caspase-activation recruitment domain

CAP- Cyclase-associated protein

Ced- C. elegans death

DD- Death Domain

DED- Death Effector Domain

DRP1- Dsk2 Related Protein

Dsk-2- Diatom Spindle Kinesin-2

FADD- Fas-associated protein with death domain

GAD- Gal4 Activation Domain

GEF- Guanine nucleotide Exchange Factor

GFP- Green Fluorescent Protein

HA- Hemagglutinin

Hdj-1- Human DnaJ-1

HLA- Human Leukocyte Antigen

HRP- HorseRadish Peroxidase

Hsp70- Heat shock protein 70

IAP- Inhibitor of Apoptosis Protein

ICAD- inhibitor of Caspase-Activated Dnase

MHC- major histocompatibility complex

NLS- Nuclear Localization Sequence

NPC- Nuclear Pore Complex

pBS- pBlueScript/SK II$^+$

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

RHG motif- Reaper, Hid and Grim motif

SDS-PAGE- Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis

Smac- Second mitochondria-derived activator of caspase

TNF- Tumour Necrosis Factor

TNFR- Tumour Necrosis Factor Receptor

TRADD- TNFR-1 associated protein with death domain

TUNEL- Terminal dUTP Nick End Labelling

Ubq- Ubiquitin

X-gal- 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

CHAPTER ONE

Introduction

The human major histocompatibility complex (MHC) is a highly polymorphic region of DNA, which has been mapped to the short arm of chromosome 6. The MHC gene family has been divided into three major subclasses, encoded from centromere to telomere in the order of class II, class III and class I. Many of the genes within the class I and class II regions have been isolated and shown to be involved in the proper functioning of immunological processes. Only recently have we begun to appreciate the importance of those genes encoded within the MHC class III region. To date, several genes encoded within this region have been identified, some of which include the cytokines TNFα/β, the heat shock protein HSP70 and a series of nine genes known as HLA-B-associated transcripts (BATs). In 1990, Banerji *et al.* isolated two cDNA sequences that code for the proteins BAT2 and BAT3. The BAT2 and BAT3 nucleotide sequences are encoded on opposite strands of DNA and terminate just a few base pairs from each other. The proteins expressed from these genes possess a high percentage of charged amino acids and are rich in the amino acids proline and glycine (Banerji *et al.*, 1990). The BAT3 gene encodes for a protein of 1132 amino acids with a molecular mass of approximately 120 kDa. Within the amino terminal domain of BAT3 exists a stretch of amino acids that is 35% homologous to those of ubiquitin and a central cysteine/histidine rich region that is an imperfect copy of a canonical zinc finger motif (Banerji *et al.*, 1990). These zinc finger motifs are a common characteristic of many nucleic acid binding proteins. Recent investigations into the spatial and developmental expression of BAT3 in rodents, indicate that BAT3 is expressed in a wide array of

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tissues, with a predominant level of expression in rodent testis. This increase in testicular

expression is initiated 17 to 20 days after birth and progresses into adulthood (Wang and

Liew, 1994). Such results would suggest that BAT3 plays a potential role in

spermatogenesis, however such a notion requires further investigation.

Previous reports have shown that a short region within the amino-terminus of

BAT3 interacts with CAP (cyclase associated protein) (Hubberstey *et al.*, 1996), which as

the name implies is a protein linked with the enzyme adenylyl cyclase. Other studies

have also shown that CAP interacts with actin, a major constituent of the cytoskeleton

(Hubberstey *et al.*, 1996) (Freeman and Field, 2000) (Zelicof *et al.*, 1996). Thus it is

theorized that the protein CAP couples the events of signal transduction to those of actin

polymerization and cytoskeletal re-organization. The role of BAT3 in actin

polymerization and cytoskeletal re-organization has yet to be elucidated and is under

current investigation. Interestingly, the BAT3 protein does not show homology to any

other known family of proteins and thus its specific function remains unknown.

During the investigation of the potential interaction between BAT3 and CAP, a

Xenopus laevis protein Scythe was isolated (Thress *et al.*, 1998), which has provided

some insight into a possible role for the human BAT3 protein. The *Xenopus* Scythe

protein was recently isolated through its ability to associate with the protein Reaper; an

essential factor required for proper execution of apoptosis in *Drosophila* (Thress *et al.*,

1998). The Reaper interacting protein Scythe is 57% identical and 62% similar to the

human BAT3 protein (Figure 1). Like BAT3, the amino terminal domain of Scythe

contains a stretch of 80 amino acid residues that are 54% similar to ubiquitin. An

explanation for the existence of this ubiquitin-like domain has yet to be determined,

Figure 1: Primary amino acid sequence alignment between the human BAT3 protein and the *Xenopus laevis* homologue Scythe. Alignment of the sequences was carried out through the use of the CLUSTAL W multiple sequence alignment program. (∗) Conserved residues (:) Semi-conserved residues (.) Partially conserved residues.

```
BAT3    MEPNDSTSTAVEEPDSLEVLVKTLDSQTRTFIVGAQMNVKEFKEHIRASVSIPSEKQRLIYQGRVLQDDKKLQEYNVGGKVI
Scythe  MAAN----------EKMEVTVKTLDSQTRTFTVETEISVKDFKAHISSDVGISPEKQRLIYQGRVLQEDKKLKEYNVDGKVI
        *  .*           :.:** ********** * :::.**:** ** :.*.*.,**********;****:**** .****


BAT3    HLVERAPPQTHLPSGASSGTGSASATHGGGSPPGTRGPGASVHDRNANSYVMVGTFNLP------------------SDGS
Scythe  HLVERAPPQTQPSTGGPSTSSSTSPTSSNAAP----VPGAP--ERNGNSYVMVGTFNLPHVMSGLVRQQRPSVSTVNGNDGS
        **********  .:*..* :.*:*.* ...:*    ***. :**.************           .***


BAT3    AVDVHINMEQ-APIQSEPRVRLVMAQHMIRDIQTLLSRMETLPYLQCRGGPQPQHSQPPPQPPAVTPEPVALSSQTSEPVES
Scythe  TLDVHINLDQQLPVQSEPRVRLVLAQHILQDIQRILDRLEG------Q---------------------AVNEQAAEPMDT
        ::.*****::*  *:************:***;:.:*** :*.*:*       :                 *:..*::**:::


BAT3    EAPPREPMEAEEVEERAPAQNPELTPGPAPAGPTPAPETNAPNHPSPAEYVEVLQELQRLESRLQPFLQRYYEVLGAAATTD
Scythe  AES--E---GEASSRETLPQTTQNTDGQSNTTPT------S--HPSPSEYVEVLQSLSRVEERLAPFMQRYREILSSATSDA
        . *  .*   ..: .*..:* *   :  **         :  ****:*******.*.*:*.** **:**** *:*.:*::


BAT3    YNNNHEGREEDQRLINLVGESLRLLGNTFVALSDLRCNLACTPPRHLHVVRPMSHYTTPMVLQQAAIPIQINVGTTVTMTGN
Scythe  YENQEE-REQSQRIINLVGESLRLLGNALVAVSDLRCNLSSASPRHLHVVRPMSHYSGPMLLQQAAIPIQINVGTTVTATGN
        *:*:.* **:.**:************:**;*******:.:.************; **;******************* ***


BAT3    GTRP---PPTPNAEAPPPGPGQASSVAPSSTNVESS--AEGAPPPGPAPPPAT--SHPRVIRISHQSVEPVVMMHMNIQDSG
Scythe  GTHAGHMPSDGNAAQPP----STNTSEPQRPNTENQPPSNGERPASDAPPTSVPHPHPRVIRITHQTVEPVMMMHMNIQDSA
        **:.  *.  ** **    .:.:* *..*..* ::*  *.. ***.:.  .******;****;***:********.


BAT3    TQ-PGGVPSAPTGPLG---------PP-------------GHGQTLGQQVPGFPTAPTRVVIARPTPP--QARPSHPGGPP-
Scythe  SGGPTTIPPPTAGHGGSAHIHMPGLPPEFMQAISHQITQQAMAAASGQQIPGF-QAPPRFVFTRPAAPSFQFQPGTATTPPG
        :  *  :*...:* *        **        . . : ***:*** **.*.*::**:.* * :*. . **


BAT3    ---VSGTLQGAGLG--TNASLAQMVSGLVGQLLMQPVLVAQG---------TPGMAPPPAPATASASAGTTNTATTAGPAPG
Scythe  PGGATTTVPGATVGPAGNASLAQMISGLVGQLLMHPVVVAQGGSSTSSSTSSSTFTSTSSSASSSSSTDTTSTTTTSSTANP
        .: *: ** :*  *******:***********;**;****    :. ::...:.*::*:*:.**.**.*:**:..*


BAT3    GPAQPPPTPQPSMADLQFSQLLGNLLGPAGPGAGGPGVASPTITVAMPGVPAFLQGMTDFLQATQTAPPPPPPPPPPPPAPE
Scythe  TVSSVPSSQPPPGTDQHLSQLLGSLLGTASSGMSNITMGSPSITVTVPGMPAFLQGVTDILQATQTVPVSTAPTQSASQAPP
        :. *.:  *. :* ::*****.***.*...* .. :.**:***:;**:*****;**:;****.* ...*. ... **


BAT3    QQTMPPPGSPSGGAGSPGGLGLESLSPEFFTSVVQGVLSSLLGSLGARAGS-SESIAAFIQRLSGSSNIFEPGADGALGFFG
Scythe  PSSPPPP--P-AHSSPPPAAAPESLPPEFFTSVVQGVLSSMLGSLSAADQSGTESIAAFIQRLSGTHNIFQPDAEGPGGFFG
        .: ***  * . :..*  . ***.************:****.*   * ;************: **:*.*;*. ****


BAT3    ALLSLLCQNFSMVDVVMLLHGHFQPLQRLQPQLRSFFHQHYLGGQEPTPSNIRMATHTLITGLEEYVRESFSLVQVQPGVDI
Scythe  DLLTLICHNFSLVDMVMLLHGHSQPLQNLQPQLRSFFLQEYLHQVDPTPNNIQMASRNLTNGLEEYIRESFASVTVRDDVDI
        **:*:*:***:**:*******:****.*********;*.**.  :***.**:*::..* .*****;****: * *: .***


BAT3    IRTNLEFLQEQFNSIAAHVLHCTDSGFGARLLELCNQGLFECLALNLHCLGGQQMELAAVINGRIRRMSRGVNPSLVSWLTT
Scythe  TRTNIEFLQDQFNRITTHILHCADSTFGQRLLEMCNQSLFEWLALNLYCLRGDQNALTSVINERIRRLSLDVSPVLVSWVTS
        ***:*****;***.*:..;;***.** ** ****;***;*** ****;** *;** *::**.*****;*.*.* ****;*:


BAT3    MMGLRLQVVLEHMPVGPDAILRYVRRVGDPPQ-PLP---EEPMEVQGAERASPEPQRENASPAPGTTAEEAMSRGPPPAPEG
Scythe  VLSLRLQVLLGQMPVTEGEIQRHVRRVGDAPQVPEPSSQEQPMETMPVDCQNGAASPVLATHSGGVLFLPPQSSVPTICPDS
        ::,.*****:* ;*** . * *;*******.**  *   *;:***. .: . ..  *: : *.  . * *. .*:.


BAT3    GSRDEQDGASAETEPWAAAVPPEWVPIIQQDIQSQRKVKPQPPLSDAYLSGMPAKRRKTMQGEGPQLLLSEAVSRAAKAAGA
Scythe  DHPTQEDGG---SEQWAASVPPEWVPVIRQDMQNQRKIKQQPPLSDAYLSGMPAKRRKTMQGEGPHLSLSEAVSRAMKATGA
        .  ::;**. :*  ***;*****;**:;**:*:.**;* ***********************;*  .****** **;**


BAT3    RPLTSPESLSRDLEAPEVQESYRQQLRSDIQKRLQEDPNYSPQRFPNAQRAFADDP
Scythe  KPESSAECVRRELDNSEAQGRYREQLCQDIQKTLQDNESYSAQRFPNTQRAFRGDP
        :* :*.*.: *:*: .*.* **:** .**** **:: **.*****.**** **
```

Figure 1.

however it suggests that BAT3 and Scythe may play a role in the regulation of protein degradation. This high degree of homology between BAT3 and Scythe suggests that the human BAT3 protein likewise plays an important role in the regulation of mammalian apoptosis.

The Role of Apoptosis in Cell Death

Apoptosis is a genetically programmed process of cell suicide that culminates in the elimination of excess cells or those cells that have become genetically compromised; either through UV induced damage or viral infection. The clearing of excess cells is responsible for preserving the balance between cell proliferation and death, thus contributing to the maintenance of tissue homeostasis, proper immune function and fetal development. Aberrant apoptotic signal transduction leads to the occurrence of various forms of cancer, neurodegenerative disease and autoimmune diseases such as diabetes (Thompson, 1995).

The process of apoptosis is morphologically characterized by the occurrence of cell shrinkage, externalization of phosphatidylserine, chromatin condensation, membrane blebbing and DNA fragmentation. These features of apoptosis distinguish it from necrosis, where chromatin tends to form irregular clumps without any change in its distribution and cells rapidly swell until ultimately lysing (Cryns and Yuan, 1998). The transduction of apoptotic signals leads to the activation of a series of cysteine proteases referred to as caspases. These cysteine aspartate-directed proteases are synthesized as inactive zymogens that become activated through either autoproteolysis, cleavage in trans or simply through associations with other apoptotic factors. These proteases, once active,

are targeted toward structural proteins, where proteolytic degradation of these proteins leads to the loss of cell integrity. Activation of caspases is initiated through the transduction of apoptotic signals along one of two pathways. The first of these apoptotic pathways is triggered by the association of either the Fas/CD95 receptor or tumor necrosis factor receptor-1 (TNFR-1) with its corresponding ligand (e.g. TNF)(Figure 2). Upon binding of the ligand, these receptors form trimeric receptor complexes through interaction between homologous death domains (DD). The formation of this trimeric complex leads to the recruitment of other factors to the cytoplasmic face of the cell membrane. These factors include the adapter proteins FADD (Fas-associated death domain protein) and TRADD (TNFR-1 associated protein with death domain), which when bound to the death domains of either Fas/CD95 or TNFR-1, through their analogous death domains, leads to the recruitment of the cysteine protease caspase 8. This association is made possible through the interaction of homologous death effector domains (DED) of both the adapter molecules (TRADD and FADD) and caspase 8. Subsequent proteolytic cleavage of caspase 8, leading to the removal of DED, results in the translocation of caspase 8 to the cytosol, where it functions to activate the downstream protease caspase 3. Caspase 3, once active is free to initiate a cascade of caspase activation that leads to eventual cell destruction (Figure 2)(reviewed in Cryns and Yuan, 1998).

The apoptotic response may also be elicited through a second pathway that is stimulated by internal signaling cues, such as UV induced DNA damage, and is characterized by the release of cytochrome c from the inner membrane space of mitochondria (Figure 2). This cytosolic cytochrome c subsequently associates with the

Figure 2: Simplified schematic representation of mammalian cell death pathways. Apoptotic signals are carried along either the extrinsic pathway, which is initiated through binding of Fas/CD95 or TNF ligand to its corresponding receptor or through the intrinsic pathway initiated by release of cytochrome c from the mitochondria. Binding of Fas/TNF ligand leads to recruitment of caspase 8 to the receptor complex, through the assistance of the adapter proteins FADD or TRADD. The release of cytochrome c is believed to arise through the permeabilization of the mitochondrial membrane by pro-apoptotic factors such as Bax and Bim. The inhibitory proteins Bcl-2 and Bcl-X_L counteract the activities of these pro-apoptotic factors. Cytosolic cytochrome c associates with a downstream factor known as Apaf-1, which leads to recruitment of caspases. Both the extrinsic and intrinsic pathways ultimately lead to the activation of a cascade of caspases, which proteolytically cleave a wide range of substrates. **(DD)** Death Domain **(DED)** Death Effector Domain **(Apaf-1)** Apoptosis protease activating factor-1 **(TRADD/FADD)** TNFR1-associated protein with death domain/Fas-associated death domain protein **(IAP)** Inhibitor of Apoptosis Protein.
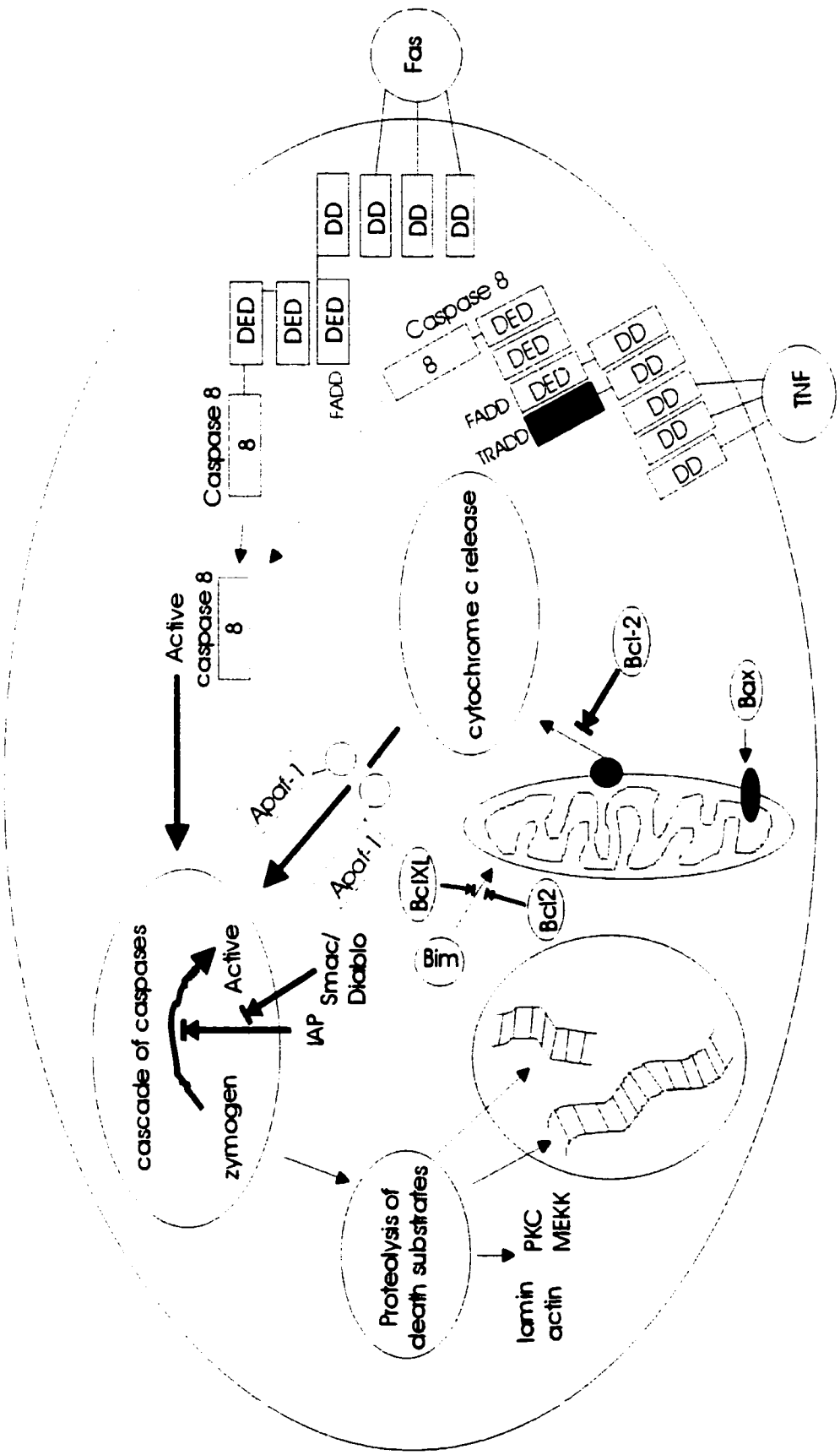
Figure 2

downstream apoptotic factor Apaf-1 (Apoptosis protease activating factor-1). Binding of cytochrome c to Apaf-1 leads to its oligomerization and association with caspase 9, facilitated through the interaction of analogous CARD (caspase-activation recruitment domain) domains. The complex consisting of cytochrome c, Apaf-1 and caspase 9 forms what has become known as the apoptosome (Bratton et al., 2001). Subsequent proteolytic cleavage of caspase 9 leads to its activation, resulting in downstream effects including the proteolytic processing of other related caspases, most notably caspase 3. Caspase 3 has been shown to function in a feedback loop, leading to further proteolytic processing of caspase 9. Once activated, this cascade of caspases targets a large number of structural proteins, leading to their destruction and subsequent death of the entire cell (Figure 2)(reviewed in Cryns and Yuan, 1998).

Recently, it was discovered that a particular class of proteins known as the inhibitor of apoptosis proteins (IAP's), could physically interact with caspases, leading to their attenuation (Figure 2). These IAP's were originally isolated as baculoviral proteins, capable of inhibiting apoptosis induced by viral infection. The IAP's are structurally related in that they possess a C-terminal RING finger domain, believed to be essential for protein-protein interaction. Likewise within the N-terminal domain, there exists anywhere from one to three baculoviral IAP repeat (BIR) domains that have been shown to be indispensable for blocking apoptotic signals. To date a number of IAP homologs have been identified in both insects and mammals. The human inhibitor of apoptosis proteins have been implicated in arresting the extrinsic pathway that leads through caspase 8 (Duckett et al., 1996). More recently it has been demonstrated that the X-linked IAP (XIAP/MIHA) is capable of binding to the processed form of caspase 9, but

not its inactive proenzyme form, suggesting that proteolytic processing of caspase 9 exposes regions capable of interacting with XIAP that are not available in the unprocessed protein (Srinivasula *et al.*, 2001)(Ekert *et al.*, 2001). Additionally, XIAP binding to the active form of caspase 3 and its association with the apoptosome complex, suggests that active caspase 3 is sequestered to the apoptosome, which prevents it from activating downstream targets (Bratton *et al.*, 2001). Counteracting the inhibitory effect of IAPs is a recently identified factor known as Smac/DIABLO, which is released from the mitochondria upon apoptotic induction (Du *et al.*, 2000) (Verhagen *et al.*, 2000). This newly identified apoptotic inducing protein has been shown to directly compete with caspase 9 for the same binding domain (BIR3) of XIAP (Srinivasula *et al.*, 2001). Thus Smac association with XIAP suppresses its ability to inhibit caspase 9 activation, leading to subsequent initiation of an apoptotic response. Thus the mitochondria elicit a 'two-factor response' to apoptotic induction, as cytosolic cytochrome *c* serves to assemble the apoptosome, while Smac/DIABLO relieves inhibition by XIAP.

Regulation of apoptosis at the level of cytochrome *c* release involves a family of proteins referred to as the Bcl-2 (B-cell lymphoma-2) family. Members of this family have been shown to possess either pro- or anti-apoptotic capabilities, which have been shown to function through antagonistic associations. It is believed that the concentration of these factors relative to one another, will determine the fate of the cell. The Bcl-2 family members are related based on the presence of homologous regions commonly referred to as the BH (Bcl-2 homology) domains. These domains have been shown to be essential for the interaction between family members. There exist four types of BH domains (BH$_1$-BH$_4$), which are critical for the association of Bcl-2 family members.

Some proteins contain all four BH domains (e.g. Bcl-2 and Bcl-X$_L$), while others possess simply one domain, namely the BH$_3$ domain (e.g. Bad and Bim) (Kelekar and Thompson, 1998). The BH$_3$ domains are particularly interesting since proteins that possess BH$_3$ sequences do not show any other sequence homology with other Bcl-2 family members. To date, all BH$_3$ only containing proteins have pro-apoptotic functions (e.g. Bad, Bid and Bim). Interestingly, BH$_3$ containing proteins can heterodimerize with anti-apoptotic Bcl-2 members, which presumably act to sequester anti-apoptotic factors. The anti-apoptotic members of the Bcl-2 family include the factors Bcl-2 and Bcl-X$_L$. Bcl-2 has been shown to render its effects by acting on mitochondria and preventing the translocation of cytochrome c to the cytosol and subsequently abrogating downstream caspase activation (Kluck *et al.*, 1997) (Yang *et al.*, 1997).

Counteracting the inhibition of cytochrome c release are the pro-apoptotic members Bax, Bak, Bid and the recently identified protein Bim (Bcl-2 interacting mediator)(Figure 2). Evidence suggests that Bim is regulated by anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-X$_L$, through interactions between their BH domains. Bcl-2 acts to suppress the death inducing activity of Bim, which has been shown to induce apoptosis when over-expressed in human cells (Strasser *et al.*, 2000). Original hypotheses suggested that the release of cytochrome c arises due to changes in mitochondrial membrane permeability, achieved through the actions of these pro-apoptotic Bcl-2 members. It has since been shown that the release of cytochrome c from the mitochondria precedes any changes in mitochondrial membrane potential, thus suggesting that alterations in membrane potential are a consequence of apoptotic

induction and not an event responsible for the initiation of a cell death response (Bossy-Wetzel *et al.*, 1998).

The Reaper Apoptotic Pathway and the Role of Scythe

Recently it was demonstrated that a specific region of DNA within the *Drosophila* genome is essential for the proper execution of apoptosis. This segment of DNA encompasses the genes *reaper*, *head involution defective* (*hid*) and *grim*, which when removed by deletion mutation results in the loss of apoptosis (White *et al.*, 1994) (Abrams *et al.*, 1993). This deficiency in apoptotic induction through the loss of Reaper, Hid and Grim has since been attributed to the inability to relieve the caspase inhibiting activity of IAP family members. While these potent apoptotic inducers do not contain defined structural domains, they do however share a highly conserved region within their N-terminal 14 amino acids, a region referred to as the RHG (Reaper, Hid, Grim) motif (Wing *et al.*, 2001). This motif has proven to be essential for the interaction with members of the inhibitor of apoptosis protein (IAP) family and is facilitated through binding to the IAP BIR domains (McCarthy and Dixit, 1998) (Vucic *et al.*, 1998) (Wang *et al.*, 1999). Although Reaper homologs have not yet been identified in mammals, recent investigations show that Reaper, Hid and Grim are capable of inducing apoptosis in human cells, suggesting that such factors have been conserved throughout the course of evolution (McCarthy and Dixit, 1998) (Claveria *et al.*, 1998) (Haining *et al.*, 1999). The functional similarity between the recently identified mammalian protein Smac/DIABLO and *Drosophila* Reaper, Hid and Grim, suggest that these factors are highly related. Further evidence of this relationship comes from the observation that this

group of proteins has sequence similarities in their N-terminal domains (Silke *et al.*, 2000). The similarities between these proteins provide an additional example of the level of conservation that has taken place through the course of evolution.

In 1998, Thress *et al.* isolated a novel member of the Reaper induced apoptotic pathway, referred to as Scythe. Evidence that Scythe is essential for the regulation of this pathway was demonstrated by immunodepletion of Scythe from *Xenopus* egg extracts. The removal of Scythe from these extracts led to the inhibition of Reaper-induced apoptosis. Since Reaper had been shown to be capable of inducing cytochrome c release, caspase activation and nuclear fragmentation in *Xenopus* egg extracts (Evans *et al.*, 1997), it was believed that the over-expression of the newly identified Scythe protein, would likewise initiate an apoptotic response. Surprisingly, this proved not to be the case, as the addition of recombinant bacterially expressed Scythe to isolated nuclei *in vitro* had no affect on nuclear morphology, suggesting that in order for Scythe to associate with downstream apoptotic effectors, it must first bind to Reaper. The binding of Reaper to Scythe would lead to a conformational change in Scythe, exposing previously unavailable domains and allowing it to associate with downstream apoptotic effectors. Consistent with this hypothesis, it was shown that over-expression of an amino-terminal truncated form of Scythe, expressing only the carboxyl-terminal 312 amino acid residues (ScytheC312), was sufficient to induce apoptosis in the absence of Reaper. The apoptotic activity of Scythe is specific to this region, as truncation to the terminal-terminal 235 amino acid residues, abolished the apoptotic inducing capabilities of Scythe. To investigate the role of Scythe in the release of cytochrome c, ScytheC312 was added to purified mitochondria in the presence of Reaper. Under these conditions, Scythe was

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unable to induce the release of cytochrome c from mitochondria, demonstrating that other cytosolic factors are required for cytochrome c release. Interestingly, the addition of ScytheC312 to egg extracts elicited an increased rate of cytochrome c release, which was in sharp contrast to the addition of full-length Scythe, which led to a decreased level of cytochrome c release. Further studies by Thress *et al.* (1999) showed that Scythe in fact carries anti-apoptotic capabilities, contrary to the previous notion that Scythe was a potent inducer of apoptosis. The results demonstrating that full-length Scythe suppressed Reaper induced cytochrome c release, was the first evidence suggesting that Scythe may posses' anti-apoptotic properties.

The immunodepletion of endogenous Scythe from egg extracts inhibited Reaper induced apoptosis, therefore it was hypothesized that Scythe binds a pro-apoptotic factor, which is likewise immunodepleted with Scythe. Upon the binding of Reaper to Scythe, it would lead to the release of the pro-apoptotic factor, allowing it to initiate cytochrome c release and the activation of downstream caspases. To investigate this hypothesis, Thress *et al.* (1999) immunoprecipitated full-length Scythe from egg extracts and subsequently incubated these precipitates with recombinant Reaper. If a pro-apoptotic factor was associated with Scythe and released upon Reaper binding, then the addition of recombinant Reaper would lead to the release of this factor into the supernatant. Upon the addition of a Reaper and Scythe depleted supernatant to an egg extract depleted of Scythe, resulted in caspase activation and release of cytochrome c, demonstrating that this unidentified factor is a direct link to cytochrome c release. This pro-apoptotic factor has yet to be identified, however it is suggested that it may be a member of the Bcl-2 family of proteins, as addition of Bcl-X_L to the released supernatant inhibited caspase

activity induced by the released factor. Furthermore, the C-terminus of Scythe contains a presumptive BH₃ domain, which may mediate these protein-protein interactions.

The Role of Hsp70 in the Regulation of Apoptosis

In recent studies by Mosser *et al.* (1997), the chaperone protein Hsp70 has been implicated in the regulation of apoptosis, as the over-expression of full-length wildtype Hsp70 protects cells from heat shock-induced cell death. Inhibition of apoptosis by Hsp70 is accomplished through the suppression of cytochrome *c* release, thereby inhibiting caspase activation (Mosser *et al.*, 2000). This ability to abrogate cell death is dependent on the chaperoning abilities of Hsp70, as chaperone-defective Hsp70 protein is incapable of preventing heat shock-induced cell death (Mosser *et al.*, 2000). Further investigations into the regulation of Hsp70 refolding, has led to the identification of a novel protein named BAG1 (Takayama *et al.*, 1997). This protein, like BAT3/Scythe possesses an N-terminal ubiquitin domain and a C-terminal domain that has been dubbed the "BAG domain" (Takayama *et al.*, 1999). Interestingly, BAG1 was originally identified as a Bcl-2 interacting protein and has been shown to have anti-apoptotic properties (Takayama *et al.*, 1995). This aspect of BAG1, in addition to the apparent presence of a BAG domain within the C-terminus of Scythe, suggested that Scythe likewise interacts with Hsp70 and functions to inhibit the protein refolding capabilities of Hsp70. Investigations into this potential theory were carried out by Thress *et al.* (2001) who have shown that Scythe binds to the ATPase domain of Hsp70 through its C-terminal BAG domain. The binding of Scythe to Hsp70 inhibits the ability of Hsp70 to renature structurally compromised proteins. Subsequent binding of Reaper to Scythe

relieves the physical interaction between Scythe and Hsp70, facilitating Hsp70 association with its co-chaperone protein Hdj-1 (Bukau and Horwich, 1998). The binding of these two factors leads to the re-establishment of the protein refolding process and likely results in the release of a pro-apoptotic factor that has the ability to liberate cytochrome c from the mitochondria (Figure 3). The discovery of Scythe's ability to bind Hsp70 is consistent with previous reports showing Scythe's ability to associate with an Hsp70-like protein called Stch (Kaye *et al.*, 2000). The Stch protein consists of the core ATPase domain of Hsp70 and lacks the C-terminal peptide-binding domain. Studies also show that the Stch protein binds a second ubiquitin containing protein known as Chap1, which is the human homolog of the yeast Dsk2 gene (Kaye *et al.*, 2000). Previous reports have demonstrated that Dsk2 is involved in regulating the G_2/M transition of the cell cycle in yeast (Biggins *et al.*, 1996). These findings provide evidence for the multifunctional capabilities of the heat inducible Hsp70 protein, as these results suggest that Hsp70 is involved in the regulation of both cell cycle and cell death events.

The Targeting and Import of Proteins into the Nucleus

The compartmentalization of the internal matrix of eukaryotic cells assists in the regulation of signal transduction pathways, as it allows for the spatial separation of associating factors. Likewise, the process of protein synthesis occurs within the cytoplasm, while many of the cell's proteins serve to function within various subcellular compartments. Thus the targeting and translocation of proteins from one subcellular compartment to another is fundamental for the co-ordination and propagation of signaling

Figure 3: Proposed mode of action of the Reaper/Scythe apoptotic pathway. In this model Scythe binds to the ATPase domain of Hsp70 and prevents the refolding of the unidentified pro-apoptotic factor X. Upon binding of Reaper, Scythe inhibition of Hsp70 protein refolding is alleviated, allowing for binding of the co-chaperone Hdj-1 and subsequent re-naturation and release of factor X. This unbound factor X is thus capable of inducing cytochrome c release and caspase activation. This figure has been adapted from Thress *et al.* (2001).
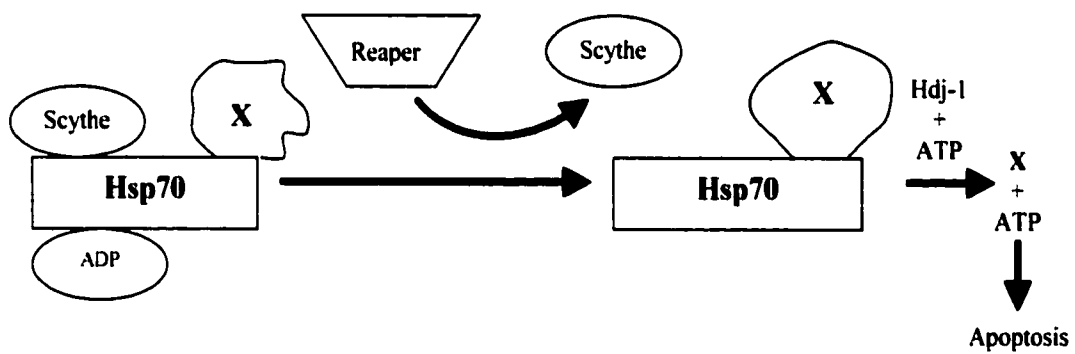
Figure 3.

responses. To facilitate this protein sorting process, many proteins possess specific amino acid sequences that direct them to their appropriate cellular locations.

Specifically, the import of proteins into the nucleus is facilitated by the existence of nuclear localization sequences (NLS) that are encoded within the primary amino acid sequence of target proteins. These nuclear localization sequences are commonly comprised of the charged amino acid residues arginine and lysine. Recognition and translocation of NLS carrying proteins is dependent on the activity of two shuttling carrier proteins, which in vertebrates are referred to as importin α and β. These two adapter proteins, while harbouring in the cytoplasm, form a heterodimer that is capable of binding to the NLS of target proteins. Once associated with the cargo protein, the trimeric complex of importin α/β and cargo are translocated across the nuclear envelope by passage through the nuclear pore complex (NPC)(reviewed in Nigg, 1997). This import of cargo proteins is dependent on the energy liberated from GTP hydrolysis by the enzyme Ran, which is a member of the Ras superfamily of small GTPases (Koepp and Silver, 1996). A GTPase-activating protein, called RanGAP1, stimulates the GTPase activity of Ran and the exchange of GDP by GTP is driven by the guanine nucleotide exchange factor (GEF) referred to as RCC1 (Klebe *et al.*, 1995). Once the nuclear-targeted cargo is released into the nuclear matrix, the importin α and β carrier proteins are shuttled back to the cytoplasm along pathways that are independent of the import process.

Thesis Objectives

The main objective of this thesis was to gain insight into the role of the human BAT3 protein and its potential as a regulator of apoptosis. To this end, experiments were performed that addressed three specific questions:

1. Where is BAT3 localized in the cell?
2. What are potential binding partners for BAT3?
3. What is the role of BAT3 in regulating cell survival?

We have found that the full-length BAT3 protein exhibits a predominant nuclear accumulation, with a small number of cells displaying cytoplasmic localization. Expression of the C-terminal domain of BAT3 displayed nuclear localization, while the amino-terminal domain localized to the cytoplasm. Based on these results we were able to deduce a presumptive NLS within the C-terminus of BAT3. The substitution of two amino acids within this presumptive NLS altered the localization pattern to a strict cytoplasmic accumulation (Chapter 2). The yeast two-hybrid system was used to isolate binding partners of BAT3. We have found that human Chap1 protein interacts with BAT3 through its C-terminal domain. Binding of Chap1 is specific to the amino-terminus of BAT3 as yeast two-hybrid results demonstrate that Chap1 does not interact with a C-terminal domain of BAT3. The interaction between these two proteins suggests that BAT3 plays a role regulating both cell cycle and cell death events (Chapter 3).

We sought to determine whether BAT3 was either a positive or negative regulator of apoptosis. We show that over-expression of a C-terminal 313 amino acid fragment of BAT3 leads to reduced cell viability and that the nuclear localization of this domain is

required to initiate this cell death response. However, the ability of BAT313C to kill cells is perhaps through apoptotic independent processes, as cells over-expressing BAT313C do not lead to the release of cytochrome c or stain positive for TUNEL, both indicators of apoptosis (Chapter 4). The significance of these results will be discussed in Chapter 5.

CHAPTER TWO

Subcellular Localization of BAT3 and the Isolation of a C-Terminal Nuclear Localization Sequence

Introduction

Eukaryotic organisms are distinguished from their prokaryotic counterparts through the existence of subcellular compartmentalization of their intracellular matrix. This division of the internal environment allows these diversified organisms to regulate their cellular processes at a level unobtainable in prokaryotic organisms. Cellular signals travel along a wide array of metabolic pathways that is dependent on the interaction of various factors with downstream counterparts. By separating and segregating these associating factors to different subcellular compartments, the eukaryotic cell inhibits the initiation of undesired cellular responses. Thus in order for signals to be carried along certain transduction pathways, it may be a requirement for some proteins to alter their subcellular localization and possibly translocate across particular intracellular membranes. The importance of such a phenomenon can be seen during several events of apoptosis. Perhaps the most well characterized example is the release of cytochrome c to the cytosol. This cytosolic cytochrome c associates with other downstream apoptotic effectors, which leads to the activation of caspases and subsequent execution of the cell. The sequestering of cytochrome c to the inner membrane of the mitochondria prevents the association between cytochrome c and these downstream effectors.

Many of the regulators of apoptosis are cytoplasmic (e.g. Apaf-1) or bound to the cell or mitochondrial membrane (e.g. Bcl-2). However, several translocate between the nucleus and cytoplasm. These include AIF (Apoptosis Inducing Factor), which travels

from the mitochondria to the nucleus upon apoptotic induction and at least five caspase members (caspases 1,2,3,6 and 9) enter the nucleus from the cytoplasm. The nuclear translocation of caspases initiates degradation of key substrates including nuclear lamins and ICAD (Inhibitor of Caspase-Activated DNase). Therefore, the precise subcellular localization of a protein is critical in executing its intended function.

In this study, we show that the human BAT3 protein localizes to the nucleus of transiently transfected mammalian cells and that this localization pattern is dependent on the NLS within the C-terminus of BAT3. Interestingly, the full-length BAT3 protein also displays some cytoplasmic localization in a small percentage of cells, suggesting that BAT3 may relocalize upon receiving a specific cell signal. The significance of this cytoplasmic localization remains unknown and is currently being investigated.

Materials and Methods

Cell Culture and Transfections

HeLa S3 and NIH 3T3 cell lines were maintained in DME medium supplemented with 10% fetal calf serum and antibiotics. Cultures were incubated at 37°C in a 5% CO_2 atmosphere. Transient transfection of cultured cells was carried out through the use of the Calcium Phosphate Transfection kit (Sigma), Superfect transfection media (QIAGEN) or Lipofectamine 2000 reagent (Life Technologies) as per manufacturers' instructions.

Plasmid Construction

All recombinant DNA procedures were performed using standard techniques (Samson, Fritsch and Maniatis, 1989). All restriction and modifying enzymes were purchased from MBI Fermentas, Promega or New England BioLabs. The coding sequence for the hemagglutinin-epitope tag (MYPYDVPDYASLGGP-MSTLD) was cloned into the Nhe I and Eco RI sites of the mammalian expression vector pCI, generating the plasmid pCI-HA (Hubberstey *et al.*, 1996). The BAT3 or deletion mutant cDNA sequence to be inserted into pCI-HA was generated through PCR using appropriate oligonucleotide primers (Appendix A). Resulting cDNA fragments were blunt end ligated into the Sma I site of pBlueScript/SK II[+] (pBS) and subsequently cut out using the restriction enzymes Xba I and Not I. Resulting cDNA fragments were cloned into the Xba I and Not I sites of pCI-HA, generating an in frame fusion between the HA-epitope tag and BAT3 or one of its mutants. Ligation of the cDNA inserts into the pCI-HA vector was facilitated through the engineering of Xba I and Not I restriction sites into the PCR primers. Sequencing of the HA epitope-tagged BAT3 plasmids was carried out by dideoxy chain termination (Sanger et al., 1977) using an ABI377 sequencing apparatus (York University, Canada) or a Visible Genetics "Long Read" system (University of Windsor). BAT3 containing the nuclear localization signal mutation (ΔNLS) was created through the use of an overlap extension mutagenic PCR strategy (Appendix B). Briefly, the 5' 710 nucleotides (nt) of the BAT313C cDNA sequence were amplified by PCR using the primers HABAT313-F and BATMUT-R, while the 3' 260 nt (nucleotides 679-939) of BAT313C were PCR amplified using the primers BATMUT-F and HABAT-R. The BATMUT-F and BATMUT-R primers that are incorporated into these resulting PCR fragments are

complementary to one another and contain the three mismatch nucleotides. These three mismatch nucleotides changed the wildtype amino acid sequence $_{1049}$KRRK$_{1052}$ into $_{1049}$KRSL$_{1052}$. Thus upon carrying out a second PCR with these products (the 710 nt and 260 nt fragments) as template, resulted in annealing between the two complementary regions of BATMUT-F and BATMUT-R. Using the primers HABAT313-F and HABAT-R, we were able to PCR amplify the entire BAT313CΔNLS sequence containing the desired mutation. The BATMUT-F and BATMUT-R primers introduce a Hind III site that is not present in the wildtype sequence, which facilitated screening for the introduced mutation. This product with Xba I and Not I cohesive ends was cloned into the corresponding sites of the plasmid pCI-HA.

To generate the full-length BAT3 carrying the NLS mutation, pCI-HABAT313CΔNLS was digested with the restriction enzymes Eco RI and Not I. This digest led to the removal of the 3' 705 nucleotides which contained the coding sequence for the mutated NLS (ΔNLS). This resulting DNA fragment was ligated into the corresponding Eco RI and Not I sites of full-length BAT3, which had been cloned into the Xba I and Not I sites of pBS. To facilitate the removal of the Eco RI/Not I fragment from full-length BAT3 (3' 705 nt of BAT3) in pBS, it was necessary to remove the Eco RI site from pBS. To delete the Eco RI site, pBS was digested with Eco RI and the resulting cohesive ends were made blunt through the use of the Klenow fragment of DNA polymerase. The pBS vector was then subsequently blunt end ligated, reconstituting the pBS plasmid with the Eco RI site deleted (pBSΔEco RI). This removal of the Eco RI site was necessary because an Eco RI/Not I digest of pBS-BAT3 would lead to the removal

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of the entire BAT3 sequence, thus rendering it impossible to insert the 3' 705 nt fragment into BAT3.

Western Blot Analysis

HeLaS3 cells were plated at a density of 5×10^3 cells/cm^2 on 100 mm culture dishes and transfected with the appropriate plasmid using Superfect transfection media, as per manufacturers instructions. Eighteen hours post transfection cells were scraped off and collected in RIPA buffer (10mM Tris pH 7.4, 150mM NaCl, 10mM KCl, 1mM EDTA, 0.5% Tween20) with 0.03 mM leupeptin, 0.37 mg/mL aprotinin and 0.02 mg/ml PMSF protease inhibitors. Following a brief sonication, cell debris was pelleted and supernatants transferred to fresh microfuge tubes. Protein assays were performed using the Bio-Rad Protein Bio-Assay Reagent. Twenty micrograms of each protein extract was resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose for Western blotting. Hemagglutinin-epitope tagged proteins were probed with an anti-HA monoclonal antibody 12CA5 (1:10 000), GFP-tagged proteins were probed with anti-GFP (1:1000) (Roche) and LexA-tagged proteins were probed with anti-LexA (1:10 000) (Clontech). Detection of proteins was carried out through the use of HRP-conjugated secondary antibody (1:1000) (Roche) and visualization was facilitated using a Lumi Light chemiluminescence detection kit (Roche).

Cells were seeded on acid washed glass coverslips in 35 mm culture dishes and afterward transfected with the appropriate DNA construct. Approximately eighteen to twenty hours post-transfection, cells were fixed in 3.7% formaldehyde and subsequently permeabilized in 0.5% Triton X-100. Detection of HA-epitope tagged proteins was carried out with the use of the anti-HA monoclonal antibody 12CA5 (1:50) and Alexa-488 conjugated goat anti-mouse secondary antibody (1:400) (Molecular Probes). Reduction of nonspecific binding of antibodies was facilitated with a final incubation in PBS+0.05% Tween20. Mounting of coverslips on glass slides was carried out with the use of the SlowFade mounting medium (Molecular Probes). Observing the subcellular localization of labeled proteins was achieved through the use of the MRC1024 laser scanning confocal microscope (BioRad). Post acquisition processing was carried out using Adobe PhotoDeluxe Home Edition 3.

Results

Localization of Human BAT3

In eukaryotic systems, the cell's internal matrix is subdivided into a series of highly ordered compartments. This division of the internal matrix allows for the separation of regulatory components and assists the cell in controlling the activation or inhibition of various metabolic pathways. Thus, determining the localization of a specific target protein can provide much insight into the possible role and method of regulation of that protein. We thus sought to determine the localization pattern of the human BAT3 protein, through the generation of a series of hemagglutinin (HA) epitope-tagged

proteins. PCR generated cDNA fragments of BAT3 were cloned into the expression vector pCI-HA, which contained the coding sequence for the hemagglutinin epitope-tag. Cloning of the specific BAT3 deletions into this expression vector created in-frame fusion proteins with the HA epitope-tag. Immunofluorescent detection and confocal imaging of transiently transfected HeLa cells revealed that the full-length BAT3 protein displayed a predominant nuclear localization (Figure 4 A, *i* and Figure 6 A, *i*). However, approximately 14% of cells expressing full-length BAT3, exhibit some degree of cytoplasmic localization (Figure 4 A, *ii*), while the remaining 86% displayed nuclear localization (Figure 4 A, *i*) (Figure 4 B). To further investigate this apparent redistribution of BAT3, a series of deletion mutants fused to the HA-epitope tag were generated (Figure 5 A). To verify that the HA epitope-tagged proteins were being expressed at their predicted molecular weights, total protein extracts were prepared from HeLa cells and subjected to Western blot analysis. All HA epitope-tagged proteins migrated at their predicted molecular size (Figure 5 C). The introduction of the two amino acid substitutions within the presumptive NLS (Figure 5 B) had no effect on the migration pattern of the HABAT313CΔNLS and HABAT3ΔNLS mutant proteins.

Previous studies investigating the functional role of the *Xenopus* BAT3 homolog Scythe have shown that over-expression of the carboxyl-terminal 312 amino acids leads to the initiation of caspase activation and nuclear fragmentation in *Xenopus* egg extracts (Thress *et al.*, 1998). A fusion protein between the HA epitope-tag and the C-terminal 313 amino acid residues of BAT3 was generated (HABAT313C) and was found to display a strict nuclear localization and exclusion from the nucleolar regions (Figure 6 A, *ii*). This localization pattern is in sharp contrast to that of the amino-terminal domain.

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Figure 4: Comparison of nuclear versus cytoplasmic localization of BAT3 (A) HeLa S3 cells were transiently transfected with DNA constructs expressing full-length BAT3 fused to the HA epitope-tag. Cells were fixed, permeabilized and stained with anti-HA and Alexa-488 conjugated secondary antibody. Visualization was achieved through confocal microscopy. Full-length BAT3 exhibits both nuclear (*i*) and cytoplasmic localization (*ii*). A population of cells expressing BAT3 was visualized at a lower magnification (200X) (*iii*). (B) Quantitative analysis of BAT3 localization in NIH 3T3 cells. The data (mean ± S.E.) are the percentage of transfected cells displaying either nuclear or cytoplasmic localization, as assessed through the observation of 5 fields of view.

A)

B)

Percentage of Cells

Nuclear   Cytoplasmic
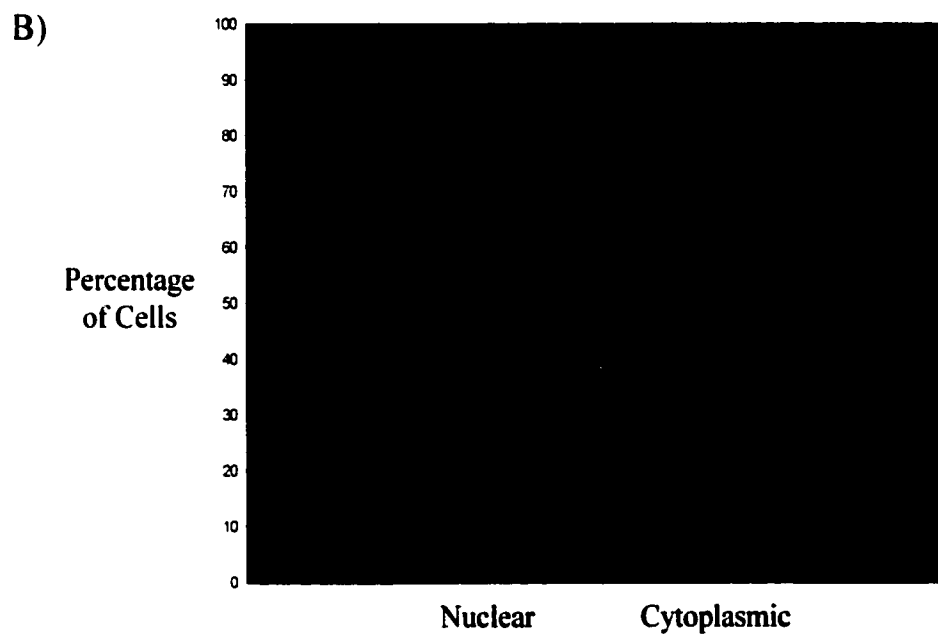
Figure 4.

Figure 5: **(A)** Schematic representation of hemagglutinin epitope-tagged BAT3 and deletion mutants. (Ubq) ubiquitin-like domain, (Pro) poly-proline region, (NLS) presumptive nuclear localization sequence, (BAG) BAG1 domain. **(B)** The proposed nuclear localization sequence and amino acid substitutions for the NLS mutant. **(C)** Western blot analysis of HA-epitope tagged BAT3 and deletion mutants.

A)

HABAT3

HABAT3ΔNLS

HABAT380N HABAT313C

HABAT656N HABAT313CΔNLS
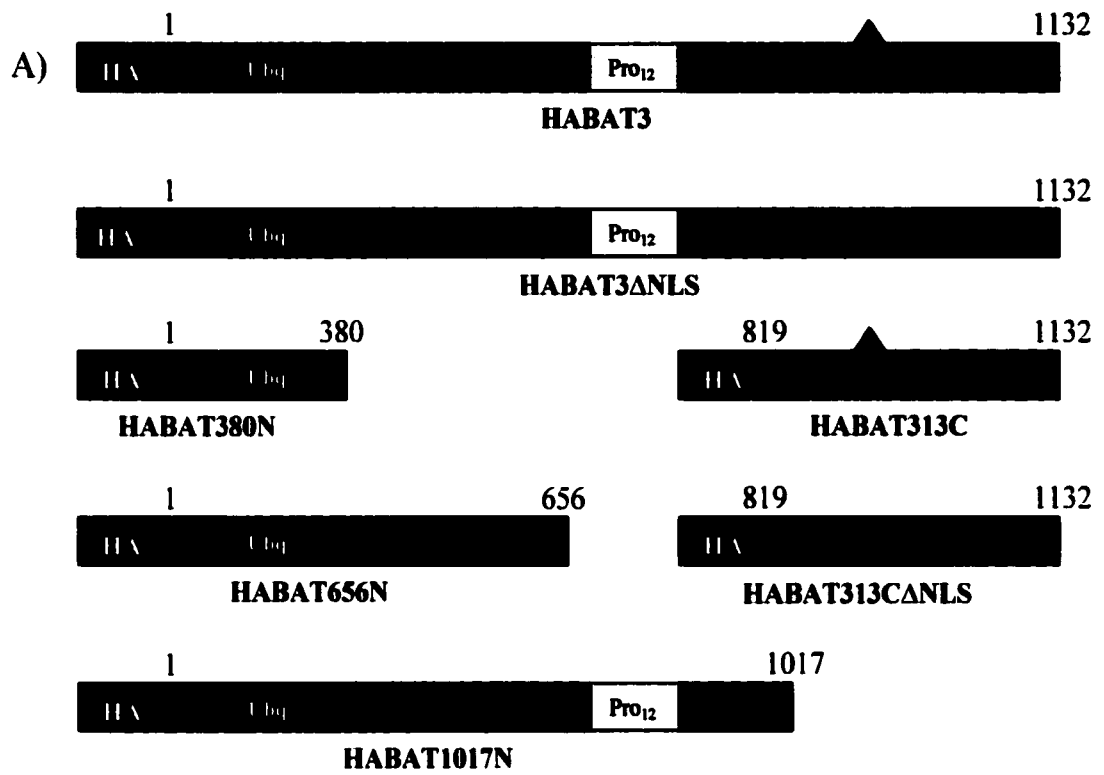
HABAT1017N

B)   **MPAKRRKTMQ** wildtype
     MPAKRSLTMQ mutant

C)

Figure 5.

When over-expressing either the amino-terminal 380 (HABAT380N), 656 (HABAT656N) or 1017 (HABAT1017N) amino acid residues, all cells revealed a strict and diffuse cytoplasmic localization (Figure 6 A, *iii-v*). Similar immunolocalization studies using NIH 3T3 fibroblast cells generated identical results (data not shown). The results obtained from these experiments suggested that the carboxyl-terminal domain of BAT3 carries a nuclear localization sequence (NLS), which targets BAT3 to the nucleus and potentially sequesters it within this subcellular compartment. Upon further examination of the BAT3 amino acid sequence, we were able to deduce a presumptive NLS (AKRRKT) within this region (amino acids 1048-1053). To investigate the possibility of BAT3 having a C-terminal NLS, we used a site-directed mutagenesis PCR strategy to introduce a two amino acid substitution into the presumptive NLS. The resulting BAT313CΔNLS and BAT3ΔNLS mutants carry an Arg_{1051} Lys_{1052} $\Rightarrow Ser_{1051}$ Leu_{1052} mutation (Figure 5 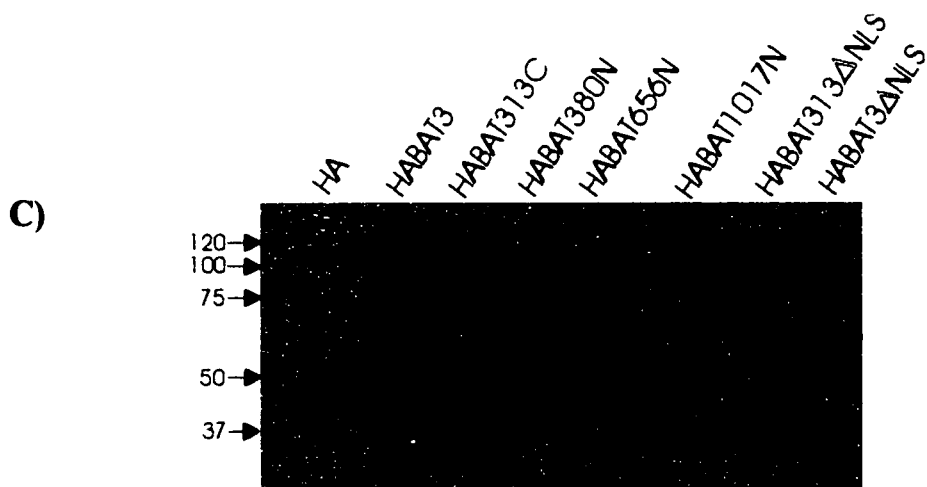B). The resulting ΔNLS ($_{1049}KRSL_{1052}$) cDNA fragments were fused in frame with the HA epitope-tag (see Materials and Methods) and immunofluorescent detection of these mutants was achieved using the primary antibody 12CA5. The localization pattern of BAT3ΔNLS displayed a strict cytoplasmic localization in contrast to the full-length BAT3 protein (contrast Figure 6 A, *i* and B, *i*), demonstrating that BAT3 contains a nuclear localization sequence in the C-terminal end of the protein. A similar result is observed in cells expressing BAT313CΔNLS which display a prevalent cytoplasmic localization, with some cells still exhibiting nuclear accumulation of the C-terminal domain of BAT3 (Figure 6 B, *ii* and *iii*). In cases where BAT313CΔNLS is concentrated in the cytoplasm, the localization pattern takes on a punctate appearance, with some cells showing the existence of large protein aggregates

Figure 6: General localization of BAT3 and the various mutant proteins. (A) HeLa cells were transiently transfected with DNA constructs expressing (*i*) full-length BAT3 (*ii*) BAT313C (*iii*) BAT380N (*iv*) BAT656N and (*v*) BAT1017N. Twenty-four hours post transfection cells were fixed, permeabilized and stained with the primary antibody 12CA5 (anti-HA). Detection was facilitated through the use of the Alexa 488 conjugated secondary antibody and visualization was achieved using confocal laser scanning microscopy. Full-length BAT3 and the C-terminal domain (BAT313C) display nuclear localization, while the amino terminal domain of BAT3 (BAT380N, 656N and 1017N) show cytoplasmic localization. (B) HeLa S3 cells expressing (*i*) full-length BAT3ΔNLS and (*ii* and *iii*) BAT313CΔNLS that contain a mutated nuclear localization sequence. (*Arrowhead*) protein aggregate formation in cells expressing BAT313CΔNLS.

A)
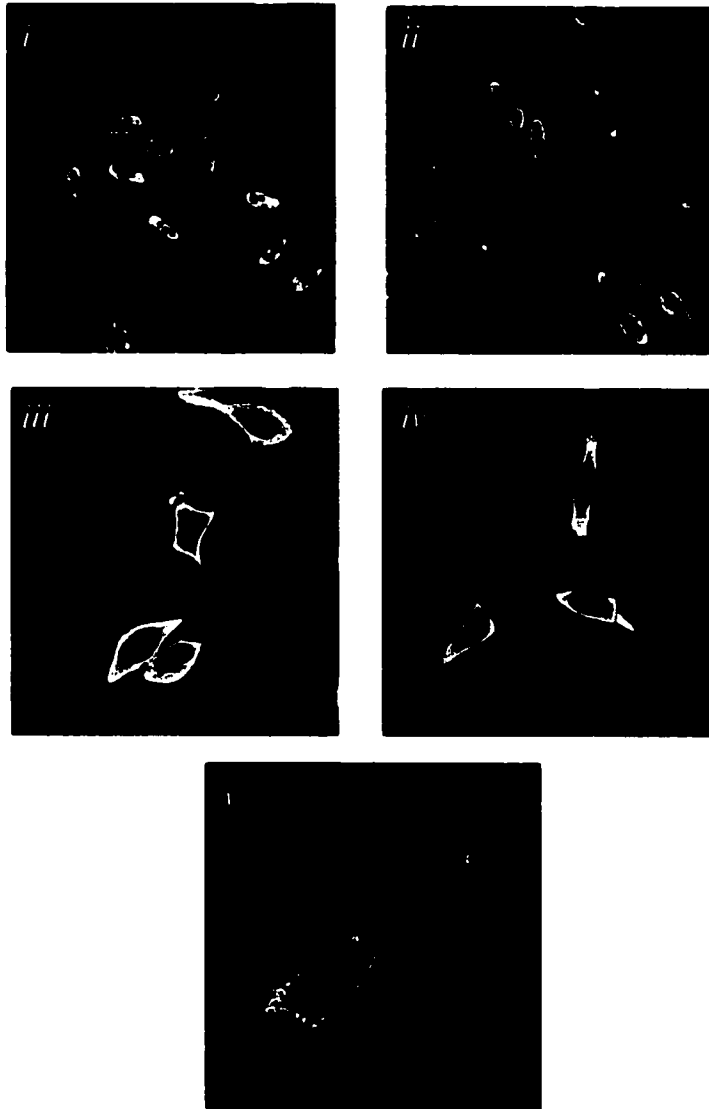
B)
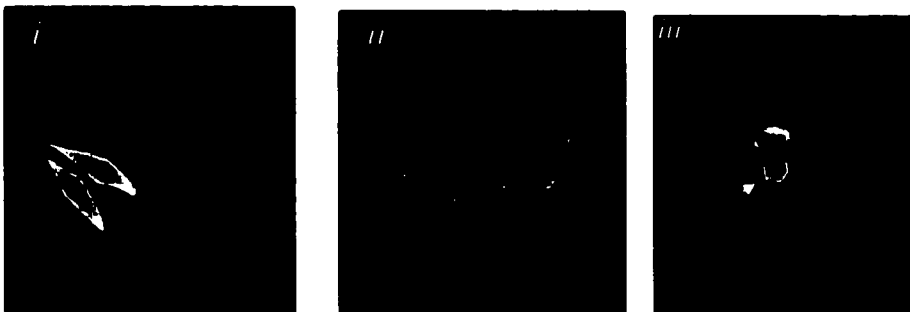
Figure 6.

positioned next to the nucleus (Figure 6 **B**, *iii*). Nuclear pore complexes are known to allow the passive diffusion of macromolecules of less than 40 kDa. Since BAT313CΔNLS is a protein of approximately 34 kDa, we suggest that the nuclear accumulation of this small protein results from its passive diffusion across the nuclear envelope. Once in the nucleus, BAT313CΔNLS may then interact with other nuclear localized proteins, which leads to its being trapped within the nucleus.

In an attempt to explain the appearance of both nuclear and cytoplasmic localization of full-length BAT3, we cell cycle synchronized HeLa cells that had been transiently transfected with the gene coding for a GFP-BAT3 protein. The synchronization of cells allowed us to assess the distribution of BAT3 at different stages of the cell cycle. We have found that the localization of BAT3 remains unaltered during the course of cell cycle progression (data not shown). Thus the significance of the apparent re-distribution of BAT3 remains unknown and is an area of current investigation.

Discussion

The targeting of proteins and other macromolecules to specific locations within the intracellular matrix of eukaryotic cells, allows for the regulation and co-ordination of signal transduction pathways at levels unachievable in prokaryotic systems. One of the most highly understood targeting mechanisms is the nucleocytoplasmic translocation of proteins through the nuclear pore complex. This transport of proteins into the nucleus is achieved through the recognition of a highly positive charged stretch of amino acids within the target protein, referred to as the nuclear localization sequence (NLS). Here we

provide evidence for the existence of an NLS (AKRRKT) within the carboxyl-terminal

domain of BAT3 (residues 1048-1053). This short stretch of amino acids is sufficient to

target BAT3 to the nucleus, resulting in its compartmentalization within this subcellular

region. Evidence in support of this region of BAT3 being critical for its function, lies in

the fact that a comparison of the BAT3 sequence with the *Xenopus* homolog Scythe,

reveals a stretch of 25 amino acids (between amino acids 1035-1060 including the NLS)

with 100% homology between the two proteins (see Figure 1). This is the longest region

of complete conservation between BAT3 and Scythe, suggesting that this region is an

extremely important structural domain that contributes to the proper functioning of the

proteins.

BAT3 Nuclear Localization and the Significance of Cytoplasmic Accumulation

The over-expression of full-length BAT3 protein in mammalian cells resulted in a

predominant nuclear localization pattern. However, this localization pattern was not

confined to the nucleus, as some cells showed cytoplasmic localization of BAT3. This

raised the possibility that BAT3 is a prevalent nuclear protein that may additionally

perform some as yet unknown function in the cytoplasm. The nuclear accumulation of

human BAT3 contradicts previous investigations into the subcellular localization of the

rodent BAT3 homolog (Ozaki *et al.*, 1999). Ozaki *et al.* (1999) showed that the rat

BAT3 protein, when fused to GFP, localized to the cytoplasm of transiently transfected

COS cells (Ozaki *et al.*, 1999). Upon further examination of this report, it was

determined that only the amino-terminal fifty-three residues of the rat BAT3 protein had

been fused to GFP. This finding is consistent with our results, as removal of the C-

terminus leads to a cytoplasmic localization of BAT3. However, our demonstrating that the full-length BAT3 protein concentrates predominantly in the nucleus is a more accurate reflection of the true localization of the BAT3 protein. We can only speculate about the physiological significance of the small number of cells displaying cytoplasmic localization, as our results demonstrated that the redistribution of BAT3 was not cell cycle dependent (data not shown). Thus BAT3 re-localization must be attributed to other cellular events and could potentially be a response to environmental stresses. We are currently investigating these possibilities.

Nuclear Localization of BAT3 and the Relationship With Reaper Binding

The results from our localization studies present evidence that raises some question about the true mode of action of the Scythe/BAT3 protein. Current understanding of the mode of action of the BAT3 homolog Scythe, proposes that Scythe regulates apoptosis through its interaction with the protein Reaper (Thress *et al.*, 1998). Previous reports using *Xenopus* egg extracts, show that Scythe sequesters an as yet unknown positive regulator of apoptosis that is subsequently released upon the binding of Reaper (Thress *et al.*, 1999). It is believed that Scythe associates with the chaperone protein Hsp70 and inhibits its ability to refold bound substrates. Upon the binding of Reaper to Scythe this inhibition of Hsp70 is alleviated, freeing Hsp70 and allowing it to fold an apoptotic inducing protein (Thress *et al.*, 2001). Such a hypothesis is difficult to comprehend, considering that earlier investigations into the subcellular localization of Reaper, report that Reaper is a cytoplasmic protein (McCarthy and Dixit, 1998). This poses the question of how Reaper is able to associate with Scythe/BAT3, knowing that

they are localized to differing cellular regions. During the course of apoptotic signal transduction, either Reaper or Scythe must translocate across the nuclear envelope. Since Reaper is a protein of 65 amino acids, it is plausible that this protein is able to move into the nucleus in an energy-independent manner, where it is able to interact with Scythe/BAT3 and lead to the release of an apoptotic inducer. The previous observation that the inhibitor of apoptosis protein (IAP) is able to associate with Reaper and lead to the development of a punctate perinuclear localization of Reaper (McCarthy and Dixit, 1998), suggests that cellular IAP proteins perhaps sequester Reaper in this region and prevent it from translocating across the nuclear envelope. Positioning the Reaper protein in this subcellular location would be advantageous in that it would allow for an immediate response to the receipt of apoptotic stimuli. Upon receipt of upstream signals, Reaper would dissociate from IAP, facilitating its translocation into the nucleus where it is able to associate with Scythe. In order for such a theory to be valid, the level of Reaper protein capable of binding and activating Scythe/BAT3 would have to be at a level undetectable in the localization studies of Reaper. However, this does not appear to be the case, as previous reports by Thress *et al.* (2001) show that Reaper must be present in a 1:4 (Scythe:Reaper) molar ratio, in order for it to reverse the inhibition of Hsp70 refolding.

The alternative scenario of Scythe/BAT3 translocating to the cytoplasm is an interesting one to consider. In order for Scythe/Bat3 to translocate to the cytoplasm, it must be accomplished through the inactivation of the C-terminal Scythe/BAT3 NLS that we have discovered. It is interesting to suggest that the re-localization of BAT3 to the cytoplasm is a consequence of a proteolytic processing event. Proteolytic cleavage of

BAT3 would lead to the liberation of an amino-terminal fragment that translocates to the cytoplasm, where it can direct its downstream effects. Although the predicted caspase cleavage site seen in the *Xenopus* BAT3 homolog Scythe (DDVD in Scythe) does not appear to be conserved (PGVD in BAT3), we have not ruled out the possible existence of other protease cleavage sites. The Reaper binding region has been localized to a region within the C-terminus (residues 819-897) of Scythe/BAT3. Thus if Scythe/BAT3 is proteolytically cleaved and liberates an amino-terminal fragment capable of binding Reaper, then such a cleavage site must be present downstream of this Reaper binding region. We do show evidence that a domain of BAT3 (BAT1017N) carrying this Reaper binding site, localizes to the cytoplasm and thus may potentially interact with a human Reaper-like protein. So, why does the expression of BAT1017N not lead to the induction of apoptosis if it interacts with Reaper in the cytoplasm? The fact that no apoptotic morphology in cells expressing BAT1017N generates more support for the theory that the apoptotic events induced by Scythe/BAT3 probably take place in the nucleus. It is also plausible to suggest that perhaps Scythe is not even a regulator of Reaper-induced apoptosis and the association of Scythe with Reaper is perhaps simply an artifact of using a cell free system such as egg extracts, as used in all of the published Scythe experiments (Thress *et al.*, 1998, 1999, 2000).

Protein Aggregate Formation When Expressing An Amino-Terminal Truncated Protein Lacking the Nuclear Localization Sequence

The observation of the presence of protein aggregates when expressing the C-terminal NLS mutant is interesting in that a number of neurodegenerative diseases, such as Huntington's, have been attributed to the development of dense protein aggregates

(Nasir *et al.*, 1996). It is believed that these protein aggregates lead to the induction of apoptosis and subsequent loss of neuronal cells. It will thus be interesting to see if such protein aggregating characteristics of BAT3, contribute to the development and progression of some form of neuropathology.

CHAPTER THREE

Identification of Chap1; a Protein That Binds to the Amino-terminus of BAT3

Introduction

The cDNA encoding the human BAT3 gene was identified several years ago (Banerji *et al.*, 1990), but has only recently received the attention of a number of researchers. The human BAT3 gene was found to encode for a large protein of 1132 amino acid residues and a molecular weight of 120 kDa. This large architectural framework of the BAT3 protein allows for the incorporation of a number of functional domains. It has long been known that the BAT3 protein possesses an amino-terminal domain highly homologous to ubiquitin. While ubiquitin has been shown to be involved in the proteolytic degradation of proteins, no such function has been attributed to the role of BAT3. Our efforts have defined a region that targets BAT3 to the nucleus and leads to its sequestration within this subcellular compartment. This nuclear localization sequence is encoded by a short stretch of positively charged amino acid residues within the C-terminus of BAT3. The work of Thress and others on the *Xenopus* BAT3 homolog Scythe have defined other domains within BAT3, one of which (the BAG domain) is involved in binding and regulating the activity of Hsp70 (Takayama *et al.*, 1997). The Hsp70 protein regulates the refolding of partially denatured proteins, which prevents them from developing into protein aggregates. Hsp70 is comprised of two functional domains, the C-terminal peptide-binding domain that recognizes exposed hydrophobic regions of structurally compromised proteins and an amino-terminal ATPase domain that

drives the refolding process (Bukau and Horwich, 1998). Thus Hsp70 prevents the accumulation of misfolded proteins and provides the cell with protection during times of stress (Bukau and Horwich, 1998). Interaction of BAT3 with Hsp70 occurs between the BAG domain of BAT3 and the amino-terminal ATPase of Hsp70. These findings are consistent with previous reports that demonstrated BAT3's ability to associate with the protein Stch (Kaye *et al.*, 2000). The Stch protein, isolated in 1994 by Otterson *et al.*, is a truncated version of Hsp70 that is 33% identical to the ATPase domain of Hsp70 and lacks the C-terminal peptide-binding domain. The precise function of Stch is unknown but is suggested to provide the cell with a broader range of protein processing capabilities (Otterson *et al.*, 1994).

The binding of BAT3 to the ATPase domain of Hsp70 leads to the inhibition of Hsp70 protein refolding capabilities. The alleviation of this inhibition is facilitated by interactions with a second BAT3 binding factor, the *Drosophila* protein Reaper. The Reaper binding region has been isolated within the C-terminus of BAT3, upstream of the BAG domain. Binding of Reaper or Reaper-like factors to this region of BAT3 leads to the release of BAT3 from Hsp70 and reactivates Hsp70 refolding activity (Thress *et al.*, 2001). Reactivation of Hsp70 refolding is believed to be involved in Reaper apoptotic induction. While BAT3 is thought to be regulated by *Drosophila* Reaper, no known mammalian Reaper homologs have been identified. These results demonstrate the significance of the functional domains within the C-terminus, however little has been identified or is known about other possible domains in other regions of the BAT3 protein.

In experiments reported here, we have used the yeast two-hybrid system to isolate other factors capable of interacting with the BAT3 protein. We have successfully

identified the human Chap1 protein as a potential interaction partner of BAT3. The interaction between these factors resulted from the binding of an amino-terminal domain of BAT3 with the C-terminus of Chap1. The binding of Chap1 was specific to the amino-terminus of BAT3, which demonstrates the direct association between these two proteins. These results present a possible second role for BAT3, namely the regulation of cell cycle events.

Materials and Methods

Molecular Cloning

The full-length or carboxyl terminal domain (BAT313C) cDNA fragment of the human BAT3 gene was PCR amplified from the plasmid pCI-MycBAT3 using the appropriate primers (Appendix A). Resulting PCR products were blunt end ligated into the Sma I site of pBlueScript/SK II$^+$ and subsequently subcloned into the Sal I site of the plasmid BTM116. Production of cDNA sequences encoding the amino-terminal domains of BAT3 (BAT380N and BAT656N) were generated through PCR amplification from the plasmid pCI-HABAT3 using appropriate primers (Appendix A). Cloning into pBlueScript/SK II$^+$ was not required for construction of these plasmids. Engineering of Xho I restriction sites into the primers used for PCR, facilitated the cloning procedure. Screening for proper orientation of the cDNA fragment was carried out through restriction digestion analysis. Sequencing of BAT3 clones was performed by dideoxy chain termination (Sanger et al., 1977) using an ABI377 sequencing apparatus (York University, Canada). The plasmid pGAD424-lamin C was a generous gift of Howard Worman (Columbia University, New York).

We screened a human brain library consisting of cDNA fragments 700-900 bp in length, cloned into the activation domain plasmid pGAD10 (Clontech). The bait plasmids used encoded either the amino-terminal 380 residues (LexBAT380N) or the carboxyl-terminal 313 residues (LexBAT313C) of BAT3, fused in frame with the LexA DNA binding domain (Appendix C). Approximately 1.7×10^{8} yeast transformants were screened for their ability to grow in the absence of histidine. For the screen using LexBAT380N as bait, the twenty-six His $^{+}$ transformants were subsequently analyzed for their ability to turn blue in the presence of the substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). The forty-one His^{+} transformants from the screen using LexBAT313C were subjected to the same β-galactosidase (β-gal) assay. From these β-gal assays, the transformants generating the strongest colorimetric reaction were selected for further analysis. To isolate the library plasmid, yeast transformants were plated on media containing the amino acid corresponding to the selectable marker gene of the bait plasmid. In this specific case, transformants were grown and replicated twice on media containing tryptophan. Complete loss of the bait plasmid was confirmed through conducting a β-gal assay on cured yeast. Transformants successfully cured of the bait plasmid were cultured overnight and purification of plasmids was achieved through membrane disruption with glass beads. Purified plasmids were recovered by electroporation into the *E. coli* strain DH10B. Sequencing of library plasmids was carried out using the dideoxy chain termination (Sanger et al., 1977) method and Visible

Genetics Long Read Tower sequencer and Gene Objects DNA analysis software (University of Windsor).

Results

Identification of Chap1 as a BAT3 Interacting Protein

We used the yeast two-hybrid system in an effort to isolate potential interacting partners of the human BAT3 protein (Appendix C). We generated DNA constructs that allowed for the expression of LexA DNA binding domain fusion proteins. These LexA fusion proteins were then used to screen for Gal4 activation domain fusion proteins, encoded by a human brain cDNA library. Using a fusion protein consisting of an amino-terminal domain of BAT3 (residues 1-380) fused to LexA, we isolated 8 positive clones capable of interacting with this region of BAT3 (Table 1). Seven of these clones encode overlapping regions consisting of a C-terminal domain of the human Chap1 protein, recently shown to be capable of binding to the ATPase domain of the HSP70-like Stch protein. We have also found that Chap1 interacts with the full-length BAT3 protein and an amino-terminal domain comprised of the first 656 amino acid residues of BAT3 (Figure 7 A). Chap1 interaction with BAT3 is specific to the amino-terminal domain, as yeast two-hybrid experiments show that Chap1 does not interact with the C-terminal domain of BAT3 (residues 819-1132)(Figure 7 A). The Chap1 protein contains an amino-terminal ubiquitin-like domain and a carboxyl-terminal ubiquitin-associated domain and shows homology to yeast Dsk2 and Xenopus DRP1 (Figure 7 B). The remaining clone encoded for the Herc2 protein, a protein believed to be involved in

Table 1: Analysis of clones isolated in the
yeast two-hybrid library screens using LexBAT380N
and LexBAT313C fusions as bait.

	Clone #	**Identity**
BAT380N	1	Chap1
	2	Chap1
	3	Chap1
	4	Chap1
	5	Herc2
	6	Chap1
	7	Chap1
	8	Chap1
BAT313C	1	Novel Protein
	2	NADH-dehydrogenase
	3	NADH-dehydrogenase
	4	No sequence obtained
	5	Novel Protein
	6	NADH-dehydrogenase
	7	NADH-dehydrogenase
	8	Herc2
	9	Herc2

Figure 7: Chap1 interacts specifically with the N-terminus of BAT3 and shows homology to yeast Dsk2 and *Xenopus* DRP1. (A) BAT3 interacts with Chap1. The yeast two-hybrid system was used to detect proteins that interact with either the amino- (residues 1-380) or carboxyl-terminus (residues 819-1132) of BAT3. Chap1 was tested for its ability to bind to various regions of BAT3 and was shown to interact with full-length BAT3 and either the amino-terminal 380 or 656 residues. Interactions were detected through the ability to induce β-galactosidase activity. (B) The domains of Chap1 along with Xenopus DRP1 and yeast Dsk2 showing the position of the ubiquitin-like domains (black) and ubiquitin-associated domains (spotted).
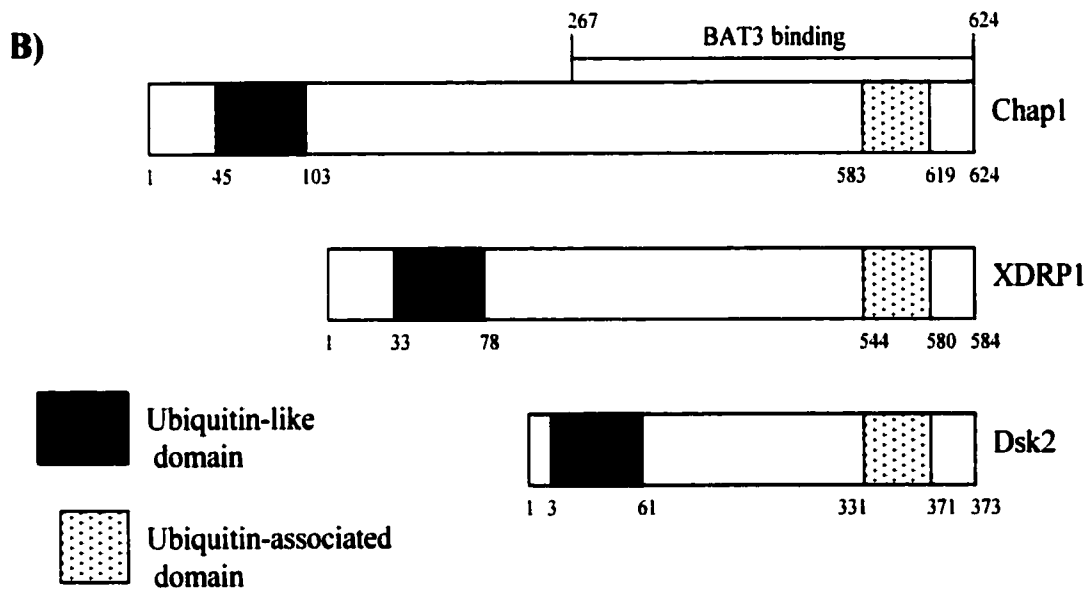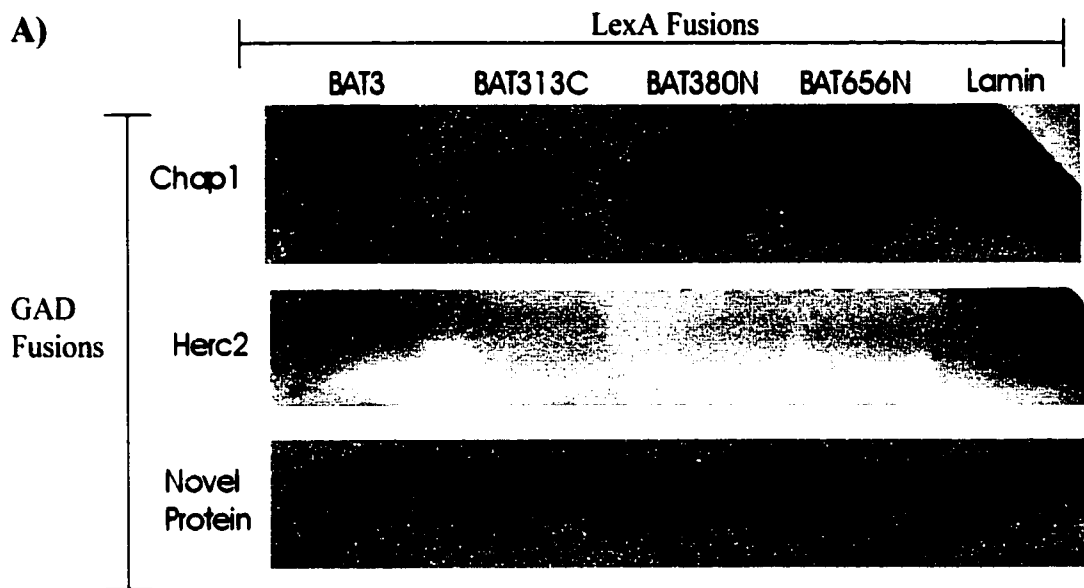
A)

B)

Figure 7.

protein trafficking and degradation pathways. However upon carrying out control experiments, whereby the bait plasmid was re-introduced into yeast cells carrying only the library plasmid, the interaction between these two proteins was lost (Figure 7 **B**).

In addition, we have screened the same human brain cDNA library for proteins that interact with the C-terminus of the human BAT3 protein (residues 819-1132). From this screen, we have isolated 9 positive clones that are capable of binding to this region of BAT3. Four of these clones encode overlapping regions of NADH-dehydrogenase subunit-2 and one of these clones cannot be resolved by dideoxy sequencing. Surprisingly, two of these clones encode overlapping regions of Herc2 and two clones encode overlapping sequences of a novel protein that remains to be characterized. Unfortunately, upon carrying out control experiments, no interaction between BAT3 and either of these proteins (Herc2 or the novel protein) were observed (Figure 7 **A**). Thus we have not attempted to further characterize these proteins.

Investigation Into the Potential Interaction of BAT3 With the Protein CAP

Previous reports by Hubberstey *et al.* (1996), have shown that BAT3 is capable of interacting with human CAP (cyclase-associated protein), a protein known to be capable of binding to actin and believed to be involved in remodeling of the cytoskeleton (Freeman and Field, 2000). This interaction has proven to be mediated through the binding of the amino-terminus of BAT3 (residues 246-360) with full-length CAP (Hubberstey *et al.*, 1996). Here, through the use of the yeast two-hybrid method, we have investigated the ability of the full-length BAT3 protein to interact with CAP. As can be seen in Figure 8 **A**, the full-length proteins do not interact in this system and thus we have not attempted to further characterize this interaction.

Figure 8:   Full-length BAT3 does not interact with CAP or nuclear Lamin C.

The yeast two-hybrid system was used to test the interaction of either **(A)** CAP or

(B) Lamin C with the various domains of BAT3. Detection was assessed on the ability to

induce β-galactosidase.

A)

LexA Fusions

| BAT3 | BAT313C | BAT380N | BAT656N |

CAP

VP16

LexA-VP16

B)

LexA Fusions

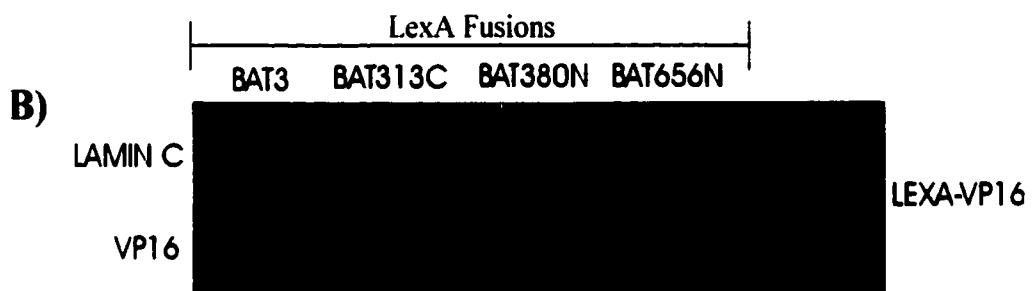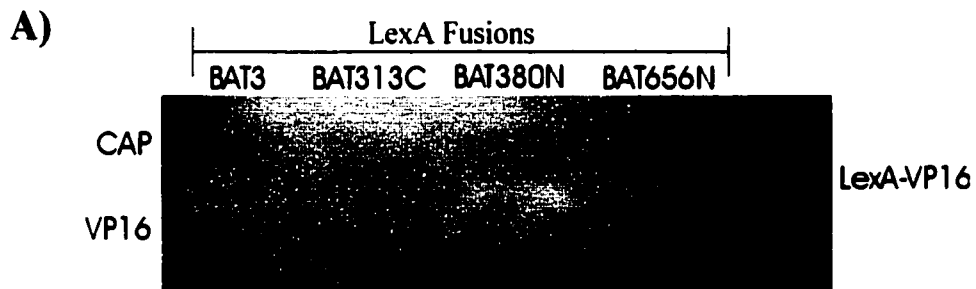| BAT3 | BAT313C | BAT380N | BAT656N |

LAMIN C

VP16

LEXA-VP16

Figure 8.

Binding Potential of BAT3 for Nuclear Lamin C

Previous observations by Hubberstey *et al.*, have also suggested that BAT3 may interact with nuclear lamin C, a major structural constituent of the nuclear envelope (unpublished results). In localization studies, the nuclear localized BAT3 protein was seen to form a ring-like arrangement around the nucleus, suggesting that BAT3 co-localizes in a similar location as nuclear lamin. To further investigate the potential association between BAT3 and lamin C, we performed a yeast two-hybrid test between full-length lamin C and BAT3. As can be seen in Figure 8 **B**, by the yeast two-hybrid system, full-length nuclear lamin C did not interact with the full-length BAT3 protein.

Discussion

In this report, we show evidence that BAT3 associates with a previously identified protein known as Chap1, a human homolog of the yeast Dsk2 protein kinase. This finding in combination with those demonstrating the ability of BAT3 to associate with the apoptotic regulator Reaper and Hsp70, suggests that BAT3 is a multifunctional protein that may regulate both cell cycle and cell death events. The full-length BAT3 protein does not interact with CAP, shedding doubt on the notion that BAT3 is involved in regulating actin polymerization. We also show that BAT3 does not associate with full-length nuclear lamin C, despite its apparent ability to localize to the same region as this protein.

BAT3 Association With Chap1 and Implications for Cell Cycle Regulation

Previous investigations had shown that Chap1 interacts with an Hsp70-like protein called Stch (Kaye et al., 2000). This Stch protein represents the core ATPase domain of Hsp70 and does not appear to have peptide binding capabilities seen with native Hsp70 members. While Chap1 contains both an amino-terminal ubiquitin-like domain and a C-terminal ubiquitin-associated domain, neither of these regions was required for Stch binding (Kaye et al., 2000). Previous reports show that the association between Chap1 and Stch is mediated by the interaction of centrally located Sti-1 repeats in Chap1 with the C-terminus of Stch (Kaye et al., 2000). These Sti-1 repeats have been shown to be required for binding to the chaperone proteins Hsp70 and Hsp90 (Lassle et al., 1997). Kaye et al. (2000) also demonstrated that the protein BAT3 had the ability to bind to Stch, in a manner similar to the binding characteristics of Chap1. While having demonstrated the interaction of Stch with both Chap1 and BAT3, Kaye et al. (2000) did not establish a direct interaction between BAT3 and Chap1. Previous reports on the role of Scythe/BAT3 in the regulation of Hsp70 function have demonstrated that Scythe/BAT3 binds to Hsp70 through its C-terminal BAG domain. Since Chap1 failed to interact with the C-terminus of BAT3 (BAT313C), in the yeast two-hybrid assay, we can conclude that the binding of Hsp70 did not bridge the interaction between Chap1 and BAT3. Although we have not determined the subcellular localization of Chap1, previous investigations report that Chap1 is localized predominantly in the nucleus (Davidson et al., 2000). This finding, in combination with our determination of the nuclear localization of BAT3, further supports the discovery of Chap1 binding to BAT3. Our results demonstrating that BAT3 interacts with Chap1, in combination with evidence

showing BAT3 association with Hsp70, suggests that BAT3 regulation involves the formation of protein complexes that include, in addition to BAT3, the proteins Hsp70 and Chap1.

The binding of BAT3 to Chap1 introduces the notion that perhaps BAT3 serves to regulate not just the events of apoptosis but perhaps couples cell death with cell cycle events. Progression through the cell cycle is characterized by cycles of cyclin degradation and synthesis. The destruction of these cyclins is accomplished through pathways that lead to the activation of the ubiquitin-proteasome. Work on the *Xenopus* Chap1 homolog DRP1 (Dsk2 related protein-1) protein has identified it as a cyclin A interacting protein (Funakoshi *et al.*, 1999). This binding of cyclin A is mediated through interactions with the amino-terminus of XDRP1. Although currently there is no evidence to suggest that either Chap1 or BAT3 is involved in regulation of degradation by the ubiquitin-proteasome, it would be interesting to see if human Chap1 protein likewise binds cyclin A in the same manner as XDRP1. Interestingly, the identification of a caspase cleavage site in cyclin A has lead to the discovery that early in embryonic development, cyclin A is degraded by caspase 3 (Stack and Newport, 1997). Thus perhaps BAT3 is involved in the regulation of cyclin A degradation through pathways dependent on the initiation of caspase activation.

Compounding evidence for the potential involvement of BAT3 in the regulation of the cell cycle, can be seen through studies carried out on the yeast Chap1 homolog Dsk2. The ability of Dsk2 to regulate cell cycle progression has been shown to be dependent on the activity of the Rad23 gene product. Thus *DskΔRad23Δ* yeast strains fail to enter mitosis due to defects in the duplication of the spindle pole body (Biggins *et*

al., 1996). Previous work on human Chap1 has shown that over-expression of Chap1 in *Dsk2ΔRad23Δ* yeast strains can rescue this cell cycle defect (Kaye *et al.*, 2000). Thus perhaps BAT3 regulates cyclin degradation, through an apoptotic process, that is dependent on the co-coordinated regulation of Chap1, Reaper and Hsp70. Proper functioning of this pathway may be essential for the proper development of the mitotic spindle, facilitating progression through mitosis.

Possible Role of BAT3 in the Regulation of Cytoskeletal Re-organization

Although CAP had previously been reported to be able to associate with the BAT3 protein through interaction with an amino-terminal domain (residues 246-360) of CAP, it was never reported whether or not the full-length CAP and BAT3 proteins had the ability to associate with one another (Hubberstey *et al.*, 1996). We thus tested this interaction by the yeast two-hybrid method and found that in this system the CAP and BAT3 proteins do not appear to interact with one another. These results unfortunately do not provide any further insight into the role of BAT3 and show that the interaction between BAT3 and CAP is not biologically significant. The fact still remains that the inability to detect an interaction between these two proteins could be due to limitations in the system used in this experiment.

Typically, when carrying out a yeast two-hybrid screen it is common practice to use a fusion between the LexA DNA binding domain and the nuclear protein lamin C as a negative control. However, when previously testing BAT3 interaction with a region of lamin C (residues 66-230), we in fact observed a result indicating that there existed a strong interaction between these two proteins (unpublished results). When we tested for

the potential association between the full-length BAT3 and lamin C proteins, the previously observed interaction was lost. Again, such a result suggests that the interaction seen between BAT3 and lamin C is not biologically significant, but may however be due to limitations in the system used. At this stage, we have not ruled out the possibility that BAT interacts with a region of lamin C that is not made available in the full-length protein, as localization studies demonstrate an apparent co-localization with the nuclear envelope. We are currently carrying out double labeling experiments to see if BAT3 truly co-localizes with nuclear lamin.

CHAPTER FOUR

The Potential Role of BAT3 as a Regulator of Apoptosis

Introduction

The process of apoptosis or programmed cell death has proven to be an essential process in embryonic development, maintenance of tissue homeostasis and proper functioning of the immune system. Morphologically, cells undergoing apoptosis display cell shrinkage, drastic membrane blebbing and nucleosomal fragmentation of DNA. Biochemically, apoptotic cells are characterized by the presence of cytosolic cytochrome c, externalization of phosphatidylserine on the outer leaflet of the plasma membrane and the proteolytic degradation of cellular proteins (Cryns and Yuan, 1998). Early experiments with the nematode *C. elegans* have been key to understanding how this process occurs. Initially, three genes in *C. elegans* were shown to have implications in the regulation of the cell death program. The genes Ced-3 and Ced-4 (*C. elegans* death) were proven to be positive regulators of apoptosis, whose function was attenuated by the product of the Ced-9 gene (Chinnaiyan *et al.*, 1997). Today, through the recognition of mammalian homologs of these nematode factors, we have begun to develop a much broader understanding of the regulation and significance of apoptosis.

The initiation of apoptosis is accomplished through one of two events, either the release of cytochrome c from the mitochondria or the binding of cytokines (Fas or TNF) to their corresponding receptor. The release of cytochrome c from the mitochondria leads to the formation of the apoptosome consisting of the ced-4 homolog Apaf-1 (Apoptosis

protease activating factor) and the proteolytic caspases 3 and 9. Formation of the apoptosome leads to the activation of these proteolytic enzymes, which subsequently targets a variety of protein substrates. Likewise, binding of cytokines to their receptor results in the recruitment of caspase 8, through the binding of homologous death effector domains (DED) to the adapter proteins TRADD and FADD. These adapter proteins are associated with the receptor through analogous death domains (DD). Recruitment of caspase 8 to the receptors leads to its autoproteolytic activation and initiation of the death response. Activation of apoptosis ultimately leads to the collapse of cellular structures and the formation of apoptotic bodies, which are subsequently engulfed by surrounding cells (reviewed in Cryns and Yuan, 1998).

Regulation of cytochrome c release is directed by the activity of a special family of proteins known as the Bcl-2 family, which are homologous to ced-9. This family of proteins is comprised of both anti- and pro-apoptotic members, which have been shown to regulate one another through concentration dependent effects. Only recently has it been discovered that apoptosis is further regulated at the level of caspase activation, through the activity of the inhibitor of apoptosis protein (IAP). This potent inhibitor of caspase activation has since been shown to be regulated by the newly identified factor Smac/DIABLO (Du et al., 2000) (Verhagen et al., 2000).

In this study, we investigated the possible role of the human BAT3 protein in the regulation of programmed cell death. Localization and TUNEL staining of cells expressing the full-length BAT3 protein, demonstrate that BAT3 does not have the ability to block cell death induced by staurosporine. However, a variety of cell death assays demonstrate that the over-expression of a C-terminal fragment of BAT3 (BAT313C)

leads to a reduced cell viability. Surprisingly, this reduction in viability does not appear to be a result of apoptosis, as assessed by measuring cytochrome c release and TUNEL staining. Additionally, the carboxyl-terminal mutant (BAT313CΔNLS), which displayed cytoplasmic localization, had a reduced effect on cell viability, suggesting that the C-terminus of BAT3 must be in the nucleus to initiate cell death. Currently we are employing other methods (e.g. Annexin V detection) to determine if apoptosis is occurring at an earlier stage in these cells.

Materials and Methods

Cell Death Assays

HeLa cells were seeded in 6 well plates at a confluency of 95% and subsequently transfected with either the control plasmid pCI-HA or one of the test plasmids HABAT3, HABAT313C or HABAT313CΔNLS using Lipofectamine 2000 Reagent. Forty-eight hours post-transfection, cells were stained with the nuclear specific dye Giemsa. Viable cells within a field of view were quantified from five separate fields. The percent viable cells were calculated based on the average number of cells in a field of view from a well of untreated cells.

Additionally, HeLaS3 cells were plated in 60 mm culture dishes at a density of 3.8×10^3 cells /cm2 and subsequently transfected with 2.5 µg of the reporter plasmid pCI-βgal and either the test plasmid pCI-HA, pCI-HABAT3 or pCI-HABAT313C using Superfect transfection media. Forty-eight hours post-transfection, cells were fixed in 2% formaldehyde/0.2% glutaraldehyde and stained for 16 hours with the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Cells were visualized and scored for

apoptotic morphology using phase contrast microscopy. Approximately 850 cells were quantified for each transfection (n=2) from five randomly selected fields and the mean of these was used to determine the percent cell death.

TUNEL Staining

Immunofluorescent detection of HA-tagged proteins was performed as described in Materials and Methods Chapter 2. To induce apoptosis, cells were treated with staurosporine (Sigma) at a concentration of 1 μM. TUNEL staining of cells was performed using the ApopTag detection kit as per manufacturers instructions (Intergen), with the following modifications. Transiently transfected cells on glass coverslips were fixed in 3.7% formaldehyde and permeabilized in 0.5% Triton X-100. Upon completion of incubation with anti-digoxigenin, cells were incubated in a 0.05% PBS+Tween20 solution for 10 minutes. All further procedures were performed exactly as outlined in the instructions supplied by the manufacturer.

Assessment of Cytochrome c Release

Cytochrome *c* release was measured in cells permeabilized with streptolysin O (Sigma). HeLa cells were transiently transfected with appropriate DNA constructs as described in Materials and Methods (Chapter 2). Approximately 10^6 cells were washed in PBS, centrifuged and subsequently resuspended in Streptolysin O buffer (20 mM HEPES-KOH [pH 7.5], 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10 μg of leupeptin and 2 μg of aprotinin/mL) containing 60 units of streptolysin O. After heating samples at 37°C for 30 min., permeabilized cells

were centrifuged at 15 000 rpm for 30 min. at 4°C. The supernatant containing cytosolic proteins was mixed with an appropriate amount of 5×SDS sample buffer. Cell pellets were resuspended in sample buffer and subjected to a mild sonication. Equal volumes of each sample were then resolved by 15% SDS-PAGE and transferred to nitrocellulose. Detection of cytochrome *c* was carried out using an anti-cytochrome *c* monoclonal antibody (1 μg/mL) (PharMingen).

Localization of BAT3 During Apoptosis

HeLa cells were seeded onto acid washed glass coverslips at a confluency of 50%. Cells were transfected with the GFP-BAT3 expressing construct with calcium phosphate, as per manufacturers instructions. Apoptosis was induced with staurosporine (1 μM) and localization of BAT3 detected by confocal imaging at the indicated times.

Results

Over-Expression of the C-terminal 313 Residues of BAT3 Leads to Cell Death

The apoptotic inducer Reaper has recently been shown to be capable of interacting with the *Xenopus* protein Scythe, a homolog (57% identity) of the human BAT3 protein. Previous reports have shown that the over-expression of a C-terminal fragment of Scythe (ScytheC312) can induce nuclear fragmentation and caspase activation in a cell free system (Thress *et al.*, 1998). Due to the high degree of homology between BAT3 and Scythe, we speculated that the human BAT3 protein was likewise a factor involved in the regulation of apoptosis. In agreement with results previously

shown for *Xenopus* Scythe, we have likewise found that the over-expression of a carboxyl-terminal domain of BAT3 (BAT313C) leads to an increase in cell death. HeLa cells that had been co-transfected with the various BAT3 DNA constructs, in conjunction with the reporter plasmid β-galactosidase, showed a reduced number of β–galactosidase expressing cells (Figure 9 **A**). In addition, cells transiently transfected with DNA constructs expressing the various BAT3 fusion proteins, revealed a markedly reduced number of viable cells when expressing BAT313C (Figure 9 **B**). Interestingly, cells expressing BAT313CΔNLS did show a reduced level of cell viability as compared to the full-length protein. However, those expressing the wildtype BAT313C exhibited a greater degree of cell death. Taken together, these results show that removal of the amino-terminal 819 amino acid residues of BAT3 can either activate cell death responses or inhibit cell survival processes and appears to be dependent on its localization to the nucleus.

TUNEL Analysis Demonstrates that BAT3 Generated Cell Death Does not Result in DNA Fragmentation

One of the hallmark characteristics of apoptosis is the cleavage of genomic DNA into fragments of varying lengths. We were interested in the possibility that over-expression of BAT313C in HeLa cells might lead to DNA fragmentation due to the induction of apoptosis. However, when we double labeled cells expressing HABAT313C with both anti-HA and TUNEL we were unable to detect any BAT313C expressing cells that were likewise positive for TUNEL (Figure 10 **A**, *ii*). Given this data, we reasoned that perhaps BAT313C must be localized to the cytoplasm in order for it to execute its

Figure 9: Quantification of cell death in HeLa S3 cells. **(A)** HeLa cells were co-transfected with the reporter plasmid pCI-βgal and either of the control plasmid pCI-HA, pCI-HABAT3 or pCI-HABAT313C. Following β-galactosidase staining, cells were visualized through phase contrast microscopy. The data (mean ± S.E.) represent the percent β-galactosidase expression per field of view (magnification: 200X) over two replicates. **(B)** Quantitative analysis of cell death induced by the over-expression of the human BAT3 protein in HeLaS3 cells. Cells were transiently transfected with DNA constructs expressing BAT3, BAT313C, BAT313ΔNLS or the vector control. Cells were allowed to recover for approximately 36 hours and subsequently stained with Giemsa. Represented are the average percent viable cells over a total of five fields of view.
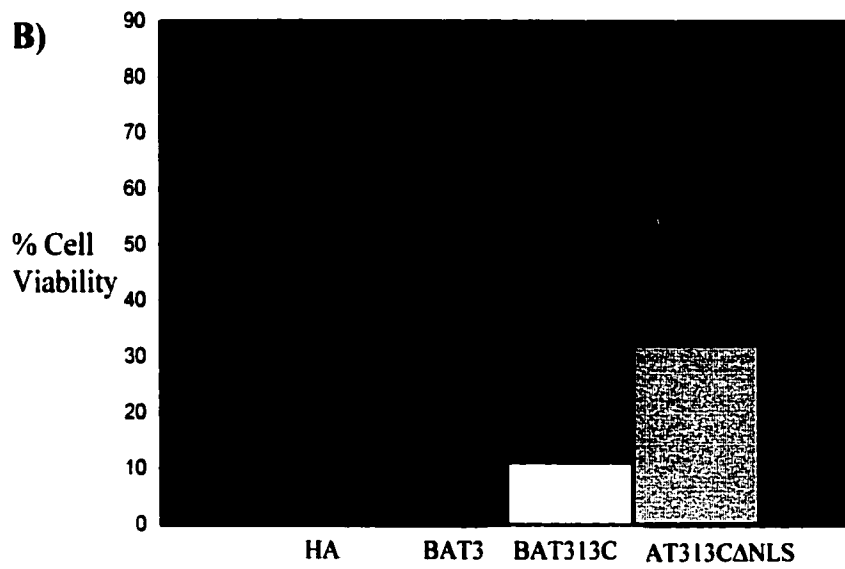
A)

% β-gal expression

None   BAT3   BAT313C

B)

% Cell Viability

HA   BAT3   BAT313C   AT313CΔNLS
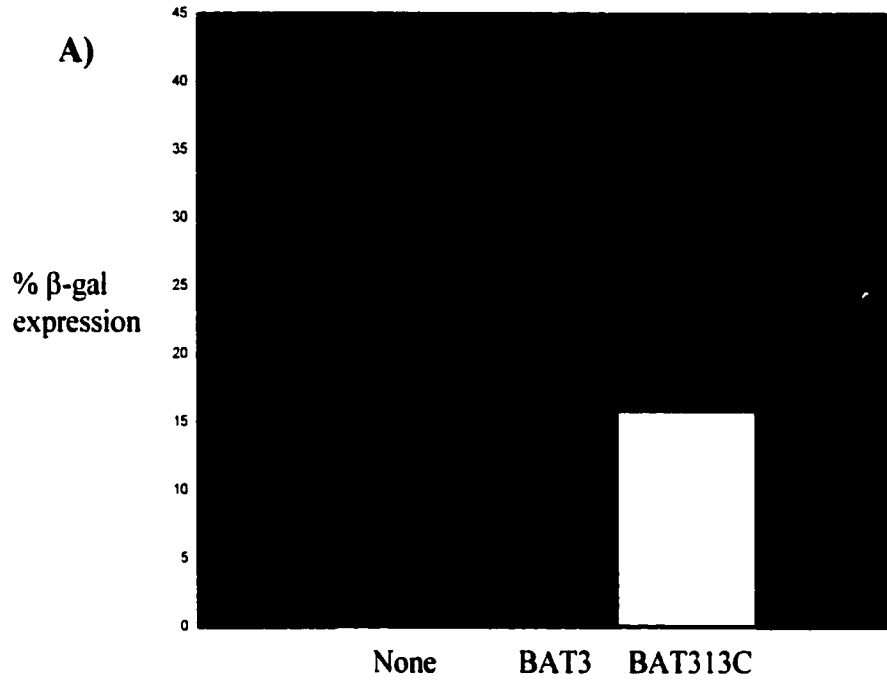
Figure 9.

Figure 10: BAT313C or BAT313CΔNLS induced cell death does not lead to DNA fragmentation and BAT3 expression does not inhibit staurosporine-induced apoptosis. **(A)** HeLa cells were stained for DNA fragmentation (*i*) and BAT313C and BAT313CΔNLS expressing cells were additionally labeled with anti-HA. Neither BAT313C nor BAT313CΔNLS expressing cells displayed DNA fragmentation as these cells were TUNEL negative (*ii, iii*). **(B)** Transiently transfected HeLa cells were treated with the apoptotic inducer staurosporine and subsequently assessed for DNA fragmentation based on TUNEL staining. Surprisingly, CAP2 over-expression prevented DNA fragmentation (*ii*), while BAT3 expression had no inhibitory effect (*iii*).

A)

TUNEL	anti-HA+TUNEL	anti-HA+TUNEL
i	*ii*	*iii*
HeLa + Staurosporine	HABAT313C	HABAT313CΔNLS

B)

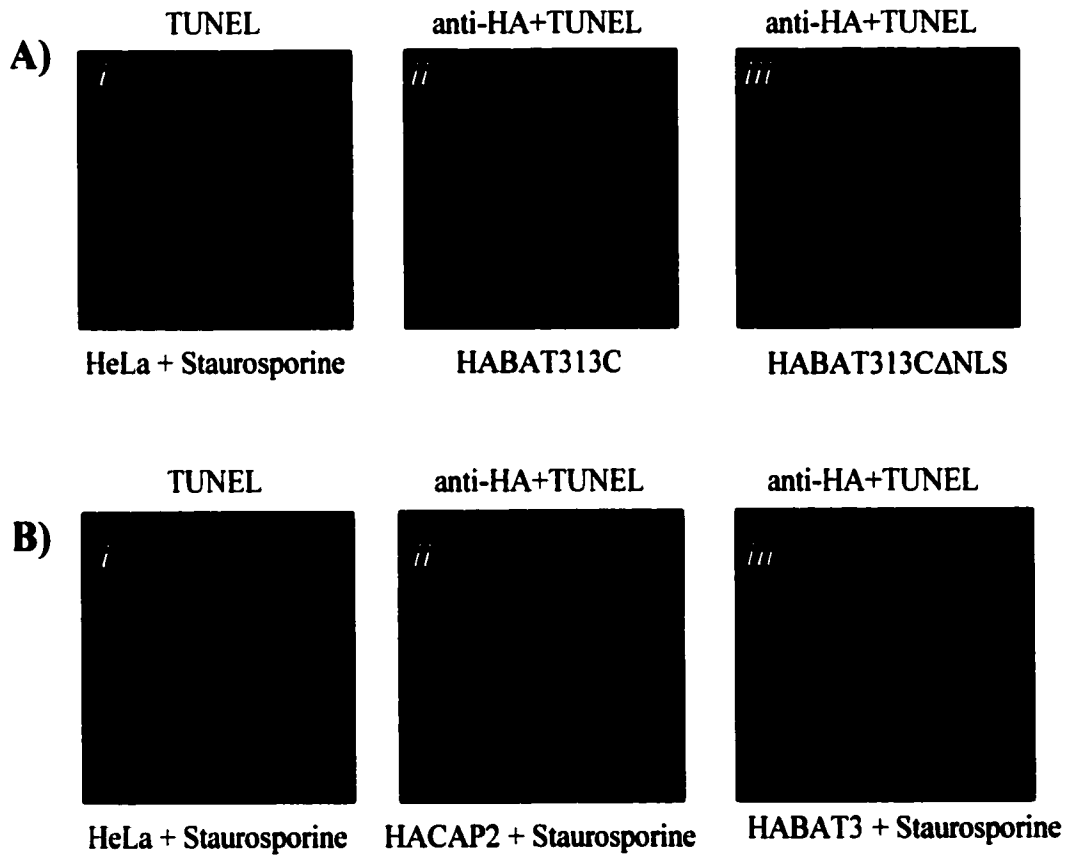| TUNEL	anti-HA+TUNEL	anti-HA+TUNEL
i	*ii*	*iii*
HeLa + Staurosporine	HACAP2 + Staurosporine	HABAT3 + Staurosporine

Figure 10.

cell death effects. However, when over-expressing BAT313C carrying the nuclear localization sequence mutation and subsequent staining with both anti-HA and TUNEL, we were unable to detect TUNEL positive BAT3 expressing cells (Figure 10 A, *iii*). This inability to detect TUNEL positive cells could not be attributed to inadequate TUNEL stain, as control cells treated with staurosporine were TUNEL positive (Figure 10 A, *i*). These data suggest that cell death induced by over-expression of either nuclear or cytoplasmic localized BAT313C, does not result in the DNA fragmentation typical of apoptosis.

BAT3 Does Not Inhibit Staurosporine Induced Apoptosis

Recent findings into the mode of action of the BAT3 homolog Scythe have suggested that Scythe/BAT3 in fact functions to prevent cell death through apoptosis. This ability to inhibit apoptotic signal transduction is thought to occur through the binding of some as yet unknown pro-apoptotic factor. We were thus interested in knowing if BAT3 had the ability to halt cell death induced by the apoptotic inducer staurosporine. Upon transfecting HeLa cells with the test DNA constructs, we triggered apoptosis by the application of staurosporine at a concentration of 1 μM. After five hours of apoptotic induction, HA tagged proteins were detected with anti-HA and cells were subsequently TUNEL stained. If either CAP2 or BAT3 were anti-apoptotic, then those cells expressing the test protein would be TUNEL negative, while all surrounding cells would stain positive for TUNEL. As a control for transfection efficiency, HACAP2 was transfected into cells and treated with staurosporine. Surprisingly, cells expressing HACAP2 were shown to possibly inhibit cells from undergoing apoptosis (Figure 10 **B**,

ii). Additionally surprising, in this assay, the BAT3 protein could not be detected while all cells exhibited TUNEL staining (Figure 10 **B**, *iii*). We speculate that this inability to detect HABAT3 is due to its being proteolytically degraded upon the induction of apoptosis, as subsequent experiments show that the level of BAT3 expression is reduced in cells undergoing staurosporine induced apoptosis. Upon carrying out the same experiment with cells expressing BAT313C, we likewise were unable to detect the HA-tagged protein (data not shown). Our inability to detect BAT3 or BAT313C may be due to proteolytic degradation initiated by apoptotic induction. Hence, these data suggest that the BAT3 protein is unable to prevent cell death induced by the apoptotic inducer staurosporine.

Cell Death Induced by BAT3 Does Not Involve Cytochrome *c* Release

The release of cytochrome *c* into the cytoplasm has become known as one of the hallmark characteristics of apoptotic induction. This cytoplasmic cytochrome *c* leads to the formation of the apoptosome and subsequent caspase activation. We hypothesized that if BAT3 was a regulator of apoptosis and that the over-expression of BAT313C resulted in cell death due to apoptosis, then cytoplasmic extracts from cells over-expressing this C-terminal domain would have a higher level of cytochrome *c* content. Surprisingly, upon examining cytochrome c content from cytoplasmic extracts of cells transfected with either HA alone, BAT3, BAT313C or BAT313CΔNLS, we did not observe any notable differences in those extracts prepared from BAT3 expressing cells, as compared to controls (Figure 11). These results suggest that the marked increase in

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Figure 11: Cytochrome c release in cells expressing various BAT3 proteins as measured by streptolysin O permeabilization. HeLa cells expressing the indicated BAT3 protein were permeabilized by incubation with streptolysin O and then centrifuged to separate the cytosolic proteins from the permeabilized cells. Equal volumes of each protein extract were loaded and analyzed by Western blotting with anti-cytochrome c antibody.
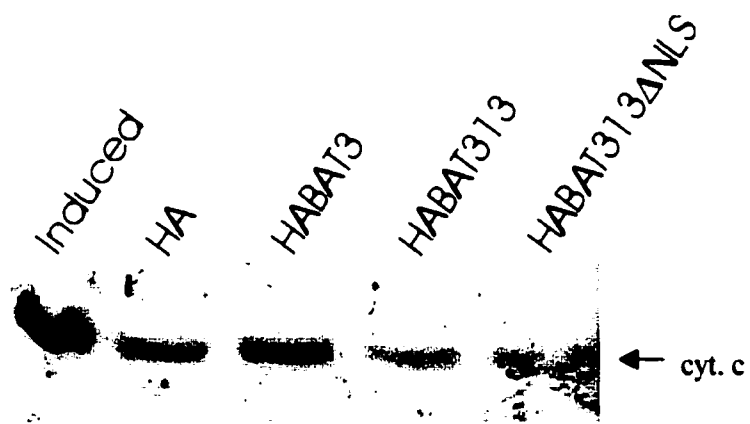
Figure 11.

cell death induced by the over-expression of BAT313C is not induced by the release of cytochrome *c* from the mitochondria.

BAT3 Localization is Altered Upon Apoptotic Induction

Since research on the role of Scythe suggests that it carries both anti- and pro-apoptotic capabilities, we hypothesized that upon inducing apoptosis, the integrity of cells expressing BAT3 would remain unaltered. We transfected HeLa cells with a DNA construct expressing a fusion between GFP and BAT3. We subsequently followed the localization of BAT3 after apoptotic induction with staurosporine. The localization of the GFP-tagged BAT3 was altered upon the addition of the apoptotic inducer, shifting from its typical nuclear localization to one of unknown classification (Figure 12). This data suggests that BAT3 does not have anti-apoptotic capability, as the nuclear localization of BAT3 was lost.

Discussion

Many factors shown to be involved in the regulation of apoptosis have proven to be conserved throughout the course of evolution. Evidence of this can be seen through the *Drosophila* protein Reaper's ability to induce apoptosis in mammalian systems, which suggests that mammalian Reaper-like factor(s) do exist (McCarthy, J.V. and Dixit, V.M., 1998). Given that the *Xenopus* apoptotic regulator Scythe displays a high degree of homology to BAT3, it is valid to suggest that BAT3 likewise carries the ability to regulate apoptosis. In this report, we show evidence that BAT3 is involved in regulating cell death processes. However, our findings propose that this reduction in cell viability is not achieved by events that lead to DNA fragmentation or cytochrome *c* release, events

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Figure 12: BAT3 does not protect cells from staurosporine induced cell death. HeLa S3 cells were transiently transfected with GFP-BAT3 and subsequently treated with 1 µM staurosporine. Localization of BAT3 at the indicated times was captured using confocal imaging. Upon apoptotic induction, the localization of GFP-BAT3 changes from a nuclear localization (t=0 min.) pattern to one that is undefined.
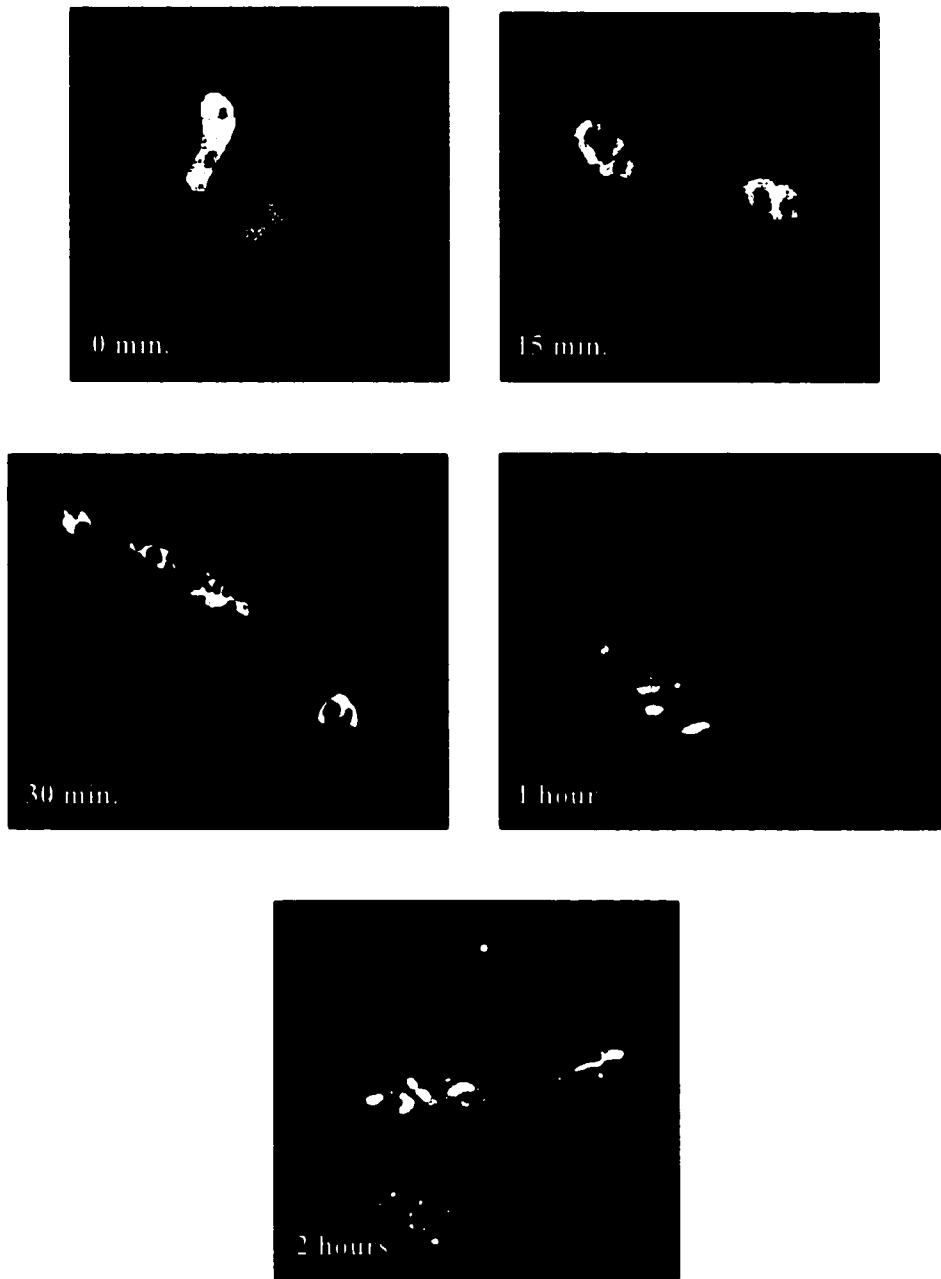
Figure 12.

characteristic of apoptosis. In addition, we show that BAT3 is not able to inhibit cell death induced by staurosporine.

Over-Expression of a C-terminal Domain of BAT3 Can Induce Cell Death That Is Not Dependent on Cytochrome c Release or DNA Fragmentation

We found that the over-expression of a C-terminal domain of BAT3 causes an observable reduction in cell viability, as cell numbers were drastically reduced as compared to controls. Those cells expressing the full-length BAT3 protein showed a markedly reduced ability to induce cell death. Interestingly, cells expressing the C-terminal 313 amino acids of BAT3, with mutations in the nuclear localization sequence, did not have the same effect on cell viability as the wildtype C-terminal protein. These results suggest that in order for BAT313C to carry out its cell killing activity, it must be localized to the nucleus. Previous work suggests that the cell death observed in BAT313C over-expressing cells arises due to the initiation of an apoptotic response (Thress *et al.*, 1998). However, our TUNEL staining and cytochrome *c* release experiments contradict these reports, as cells over-expressing BAT313C were both TUNEL negative and did not have elevated levels of cytosolic cytochrome *c*. These data suggest that the apoptotic pathway activated by the over-expression of BAT313C, does not require cytochrome c release or DNA fragmentation. Likewise, previous reports have shown that mutant Reaper unable to bind to the inhibitor of apoptosis protein (IAP), leads to the proteolytic processing of caspase 7, but does not lead to cell death (McCarthy and Dixit, 1998). Thus perhaps apoptosis regulated by BAT3 is dependent on some other factor(s) that is not present in our system. It is suggested that IAP can inhibit apoptosis downstream of Reaper and thus perhaps these IAP proteins are blocking apoptosis

downstream of BAT3 activity. An alternative explanation for this is that the cell death being observed is not due to apoptotic induction and is attributable to some other non-apoptotic mechanisms.

Over-Expression of Full-length BAT3 Does Not Inhibit Staurosporine Induced Apoptosis

Investigations attempting to elucidate the role of the *Xenopus* BAT3 homologue Scythe, have suggested that BAT3 has the ability to inhibit apoptosis through the regulation of Hsp70 (Thress et al., 2001). We tested this hypothesis by following the localization of BAT3 upon treating cells with the apoptotic inducer staurosporine. We found that the localization of BAT3 changed from its typically nuclear localization to one that suggests the formation of apoptotic bodies. Whether this BAT3 localization is associated with apoptotic body formation requires further experimentation. Based on these results, in combination with the inability to detect BAT3 in TUNEL experiments, suggest to us that BAT3 is degraded following the initiation of apoptosis. We will be attempting to determine if this inability to detect BAT3 is due to protein degradation through Western blot analysis.

Previously we have shown that BAT3 is potentially involved in the regulation of the cell cycle based on its association with Chap1. Chap1 binds to cyclin A, which has been shown to be degraded through apoptotic processes during the early stages of mitosis (Funakoshi et al., 1999). Thus perhaps over-expression of the C-terminal fragment of BAT3 can trigger the early events of apoptosis, but the pathway leading to cyclin A degradation is inhibited. Thus cells that appear to have died are in fact blocked at a particular stage of the cell cycle and are not capable of proliferating. Our cytochrome *c*

release experiments do not support the notion that the early events of apoptosis are initiated by BAT313C, since the release of cytochrome c is one of the primary events leading to cell death. Further investigations into cell cycle and apoptotic regulation by BAT3 and the implications for Chap1 binding will provide further insight into the links between these two cellular processes.

CHAPTER FIVE

General Discussion

The human BAT3 protein displays strong homology to a recently identified protein from *Xenopus* known as Scythe. This homologous protein was isolated through its ability to bind to the potent *Drosophila* apoptotic inducing protein Reaper (Thress *et al.*, 1998). Based on this association, it was postulated that Scythe was an additional factor involved in the regulation of Reaper induced apoptosis. Initial studies suggested that Scythe functioned as a positive regulator of apoptosis, as the addition of a C-terminal fragment of Scythe consisting of the last 312 amino acid residues, induced nuclear fragmentation and caspase activation in egg extracts (Thress *et al.*, 1998). Subsequent investigations into the role of Scythe determined that this protein was in fact an inhibitor of Reaper induced apoptosis, demonstrating the ability to sequester a factor(s) capable of initiating cytochrome *c* release and subsequent caspase activation (Thress *et al.*, 1999). Evidence now suggests that the chaperone protein Hsp70 is implicated in regulating this apoptotic pathway. This proposal stems from the recent isolation and identification of the BAG1 protein as a regulator of Hsp70 protein refolding (Takayama *et al.*, 1997). Binding of BAG1 to Hsp70 is mediated through the interaction of a small region within BAG1, known as the BAG domain, with the ATPase domain of Hsp70 (Takayama *et al.*, 1997). The identification of a BAG domain within the C-terminus of Scythe and the functional similarities (BAG1 inhibition of heat-induced apoptosis) between these proteins, suggested that Scythe likewise binds to Hsp70 and inhibits protein refolding. Recent investigations by Thress *et al.* (2001) have successfully identified Scythe as an Hsp70 binding protein that serves to block the protein refolding capabilities of Hsp70.

The reestablishment of Hsp70 refolding is facilitated by the binding of Reaper to Scythe, which leads to the dissociation of the Scythe-Hsp70 complex and refolding and release of a partially denatured pro-apoptotic factor that can induce cytochrome *c* release (Thress *et al.*, 2001).

In this study, we have explored the subcellular localization of the human BAT3/Scythe protein and a series of deletion mutants. Through these studies we have discovered an NLS in the C-terminus of the BAT3 protein, which serves to localize BAT3 to the nucleus. While in the nucleus, BAT3 is potentially sequestered through its association with the protein Chap1, as yeast-two hybrid experiments show that Chap1 binds to the amino-terminus of BAT3. These results put forward the notion that BAT3 is involved in regulating cell cycle events. While previous reports implicate BAT3 in the regulation of apoptosis, our cytochrome *c* release and TUNEL staining experiments do not support this hypothesis.

Subcellular Localization Studies Demonstrate That BAT3 Carries a Nuclear Localization Sequence Which Targets BAT3 to the Nucleus

Our studies show that full-length BAT3 localizes to the nucleus of transiently transfected HeLa cells. However, the localization of BAT3 was not restricted to the nucleus as some cells displayed cytoplasmic localization of BAT3. In an effort to determine if this apparent redistribution of BAT3 was cell cycle dependent, we synchronized cells in G_0 through serum starvation. We found that the localization of BAT3 did not change as cells progressed through the cell cycle. To date, the events leading to relocalization of BAT3 have not been identified, but are an area of current investigation. Previous reports have shown that BAT3 interacts with the Hsp70 ATPase

homolog Stch. We speculate that BAT3 localization will change in response to heat stress and thus we are currently investigating this possibility.

To further assess the cytoplasmic localization of BAT3, we generated a series of deletion mutants and observed their localization pattern in transiently transfected HeLa cells. We found that in those cells expressing the carboxyl-terminal 313 amino acid residues of BAT3 (BAT313C), they exhibited a strict nuclear localization. This localization pattern was distinct from those expressing the amino-terminal fragments of BAT3 (BAT380N, BAT656N and BAT1017), which displayed a strict cytoplasmic localization. These results suggested that BAT3 contained a nuclear localization sequence within its carboxyl-terminus. Further analysis of the BAT3 amino acid sequence identified a presumptive NLS spanning residues 1048 to 1053 (KRRK). Through the introduction of a two amino acid substitution within this region (KRRK⇒KRSL), we were able to show that this sequence is a bonafide nuclear localization sequence, as proteins carrying this mutation localized to the cytoplasm. The nuclear import of NLS carrying proteins is facilitated by the carrier proteins importin α and β. Thus it would be interesting to determine if binding of these carrier proteins to the NLS region facilitates the nuclear uptake of BAT3.

The demonstration that BAT3 is a nuclear protein does not correlate with previous reports suggesting that the BAT3 homolog Scythe associates with the apoptotic inducer Reaper. Previous reports have shown that Reaper displays a diffuse cytoplasmic localization and a punctate perinuclear localization when co-expressed with human IAP (McCarthy and Dixit, 1998). If BAT3 interacts with Reaper or Reaper-like factors to induce apoptosis, how is this achieved considering the two proteins are spatially

separated? One factor must alter its localization in response to apoptotic stimuli. One could argue that BAT3 is the candidate protein that changes localization, as we see both nuclear and cytoplasmic localization in cells expressing BAT3. However, when cells expressing full-length BAT3 fused to GFP were treated with the apoptotic inducer staurosporine, the localization of BAT3 did not shift to the cytoplasmic localization pattern seen when expressing the wildtype BAT3 protein in the absence of staurosporine. Perhaps the apoptotic pathway regulated by Reaper is not initiated in staurosporine-induced apoptosis. It would thus be interesting to see if BAT3 localization is altered upon treatment with varying apoptotic-inducing stimuli.

We have not ruled out the possibility that protease members not required for apoptosis proteolytically cleave BAT. If cytoplasmic localization is required for BAT3 interaction with Reaper-like factors, then we suggest that cleavage occurs in a region localized between the Reaper binding domain and the nuclear localization domain. The amino-terminal fragment alleviated from this cleavage event would be of high molecular weight and thus must translocate by active diffusion processes. Although we have not identified a nuclear export sequence, it is an area of future investigation.

The other alternative hypothesis suggests that Reaper must cross the nuclear envelope in order to facilitate interaction with BAT3/Scythe and induce cell death. This notion is attractive because Reaper is a protein of 65 amino acids. Due to this small size, nuclear uptake of Reaper could be achieved by passive diffusion through the nuclear pore complex. Co-localization studies show that Reaper develops a punctate perinuclear localization when expressed along with human IAP. Positioning of Reaper in this subcellular location would be a strategic advantage in transduction of apoptotic signals.

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Upon the receipt of an apoptotic stimulus, Reaper and IAP complexes dissociate allowing the nuclear uptake of Reaper. Once in the nucleus, Reaper can bind to BAT3/Scythe, alleviating Hsp70 refolding inhibition and subsequent apoptotic induction. Interestingly, it has been shown that Reaper has sequence homology within its amino-terminal domain to the recently identified protein Smac (Silke *et al.*, 2000). Smac is known to bind to IAP and prevent its ability to inhibit caspases (Du *et al.*, 2000). Thus the dissociation of Reaper from IAP would facilitate the inhibition of caspases through IAP binding. This suggests that Reaper serves to both induce apoptosis through binding to Scythe/BAT3 and yet at the same time inhibit the transduction of apoptotic signals through the release of IAP. Supporting this notion is the evidence that a deletion mutant of Reaper that is not able to bind to IAP leads to caspase 7 processing, but does not lead to cell death. Thus the apoptotic pathway regulated by Reaper must require the activity of other downstream factors in order to produce a full cell death response.

BAT3 Cell Death is not Attributable to Cytochrome c Release or DNA Fragmentation

Two hallmark characteristics of apoptosis are the release of cytochrome c and the fragmentation of DNA into nucleosomal fragments. Release of cytochrome c from the mitochondria results in the activation of downstream caspases, while DNA fragmenation is the result of proteolytic activation of the DNAse CAD. Our TUNEL staining results show that cell death induced by the over-expression of the C-terminal 313 amino acid residues of BAT3 does not lead to DNA fragmentation. Likewise, over-expression of the full-length BAT3 protein could not inhibit the induction of apoptosis and in fact appears

to be degraded during the apoptotic process. In addition, cytochrome c release was not

evident in cytoplasmic extracts from cells expressing either the full-length BAT3,

BAT313C or BAT313CΔNLS proteins. Together these results suggest that cell death

induced by BAT3 is not the result of initiation of apoptosis. However, we have not ruled

out the fact that perhaps BAT3 regulates a form of cell death that does not result in DNA

fragmentation or is initiated by cytochrome c release.

Interaction of BAT3 With Chap1; Implications for BAT3 Involvement in the Regulation of the Cell Cycle

Our yeast two-hybrid library screen has identified Chap1 as a putative BAT3

interacting protein. Binding of Chap1 is facilitated through the interaction of a C-

terminal domain of Chap1 with the amino-terminus of BAT3. The interaction of Chap1

is specific to this domain of BAT3, as we show that Chap1 is incapable of interacting

with the C-terminal domain of BAT3 (BAT313C). Previous reports have shown that this

C-terminal domain binds and regulates the activity of Hsp70 (Thress et al., 2001). Thus

the observation that BAT3 does not interact with Chap1 through its C-terminus

demonstrates that the interaction between these two proteins is not facilitated by Hsp70

interactions.

Chap1 is the human homolog of the yeast Dsk2 protein, which recently has been

shown to be related to the *Xenopus* protein XDRP1, which was isolated through a yeast

two-hybrid library screen using an amino-terminal domain of cyclin A as bait (Funakoshi

et al., 1999). Cyclin proteins are mediators of cell cycle progression and their regulated

synthesis and degradation is essential for proper co-ordination of growth, synthesis and

division. The interaction of the *Xenopus* Chap1 homolog with cyclin A, suggests that

BAT3 is involved in the regulation of cyclin degradation during the course of the cell cycle. Previous reports have shown that cyclin A degradation during certain stages of development are not due to ubiquitin-proteasome degradation, but is in fact a consequence of activation of the apoptotic program (Funakoshi *et al.*, 1999). This apoptotic degradation of cyclin A has been shown to occur during the interphase stage of division and is distinct from mitotic degradation of other cyclins. Cyclin A degradation has been shown to be mediated by the activation of the cysteine protease caspase 3 (Funakoshi *et al.*, 1999). It is thus interesting to suggest that BAT3 is involved in regulating cyclin degradation through the concomitant regulation of apoptosis.

BAT3 Protein Aggregate Formation and Implications in Neurodegeneration

The neurodegenerative disease Huntington's has been shown to arise due the expansion of a polyglutamine tract within the protein huntingtin. The length of the polyglutamine stretch has been correlated with timing and progression of the disease. Investigations into the role of mutant huntingtin in the neurodegeneration associated with Huntington's have revealed that the mutant protein is proteolytically cleaved, releasing an amino terminal fragment of huntingtin. This amino-terminal domain containing the polyglutamine expansion has been shown to translocate into the nucleus and form large protein aggregates (Hackam *et al.*, 1998). It is believed that these intranuclear protein aggregates contribute to the degeneration of cortical neurons. Recent evidence shows that members of the heat shock family of proteins can serve to prevent the formation of the protein aggregates (Muchowski *et al.*, 2000). Thus perhaps through regulation of Hsp70, BAT3 contributes to the regulation of protein aggregate formation by mutant

ataxin-1. We were intrigued by these findings as it appeared as though BAT3 functioned in a similar manner, proteolytic cleavage and translocation to the cytoplasm. Our results demonstrate that by introducing a mutation into the NLS of a C-terminal fragment of BAT3 leads to the development of a large protein aggregate localized in close proximity to the nucleus. Interestingly Davidson *et al.* (2000) recently discovered that the protein ataxin-1 interacts with the BAT3 binding protein Chap1. Expansion of a polyglutamine tract within ataxin-1 causes spinocerebellar ataxia type-1, a neurodegenerative disease that affects the brain stem, spinocerebellar tracts and the cerebellar Purkinje cells of afflicted patients. Additionally, ataxin-1 has been shown to be a predominantly nuclear protein. These data suggest that perhaps BAT3 is involved in regulating the events that contribute to the development of spinocerebellar ataxia type 1.

Summary

In conclusion, we have identified two additional functional domains within the BAT3 amino acid sequence, a nuclear localization sequence and a Chap1 binding region. In Figure 13, we can see the structural complexity of the BAT3 protein, as eight functional domains have been identified. We have shown that within the C-terminus there exists a nuclear localization sequence that is sufficient in targeting BAT3 to the nucleus. Under some as yet unknown condition BAT3 can be found concentrated in the cytoplasm. Additionally, we have shown that over-expression of a C-terminal fragment of BAT3 can induce cell death, which cannot be attributed to cytochrome c release or DNA fragmentation. This C-terminal fragment containing the nuclear localization sequence (NLS) localizes to the nucleus, suggesting that the function of BAT3 is carried

Figure 13: Schematic indicating the structural domains and protein binding regions of BAT3. Depicted are the following domains showing their relative position within the BAT3 amino acid sequence (**Ubq**) Ubiquitin-like, (**Pro$_{12}$**) polyproline region, (**?BH3?**) putative Bcl-2 Homology 3 domain, (*) nuclear localization sequence, (**BAG**) BAG domain.

Figure 13.

out in the nucleus. Furthermore, full-length BAT3 does not have anti-apoptotic activity since it was undetectable in staurosporine-induced cells, suggesting that it is degraded during the course of apoptosis. Lastly, we have identified the human Chap1 protein as a BAT3 interacting protein, which suggests that BAT3 is involved in regulating both cell cycle and cell death events.

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Table 1. Primers used for PCR amplification of BAT3 cDNA.

Primer	Sequence 5'-3'
HABAT-F	TTTCTAGAATGGAGCCTAATGATAGTACC
HABAT313-F	TTTCTAGACGGGAGAGTTTTTCCTTGGTGC
HABAT-R	TTGCGGCCGCCTAAGGATCATCAGCAAAGGCC
HABAT380-R	TTGCGGCCGCCTAGGCTGTCTGTGTTGCCTGCAAG
HABAT656-R	TTGCGGCCGCCTAATTGATCTGTATGGGAATGGCTG
BATMUT-F	GCCTGCCAAGAGAAGCTTGACGATGCAGGGT
BATMUT-R	ACCCTGCATCGTCAAGCTTCTCTTGGCAGGC
LEXBAT-F	TTCTCGAGGGATGGAGCCTAATGATAGTACC
LEXBAT-R	TTCTCGAGCTAAGGATCATCAGCAAAGGCC
LEXBAT313-F	TTCTCGAGTGCGGGAGAGTTTTTCCTTGG
LEXBAT380-R	TTCTCGAGCTAATTGATCTGTATGGGAATGGC
LEXBAT656-R	TTCTCGAGCTAGGCTGTCTGTGTTGCCTGCCTGCAAG
LEXEcoBAT-F	TTGAATTCATGGAGCCTAATGATAGTACC
VP16BAT-F	TTGCGGCCGCTAATGGAGCCTAATGATAGTACC

APPENDIX B

Schematic of overlap extension mutagenic PCR strategy used to create ΔNLS mutation.

PRIMERS

Xba I
HABAT313-F: 5'--TT<u>TCTAGA</u>CGGGAGAGTTTTTCCTTGGTGC--3'

Not I
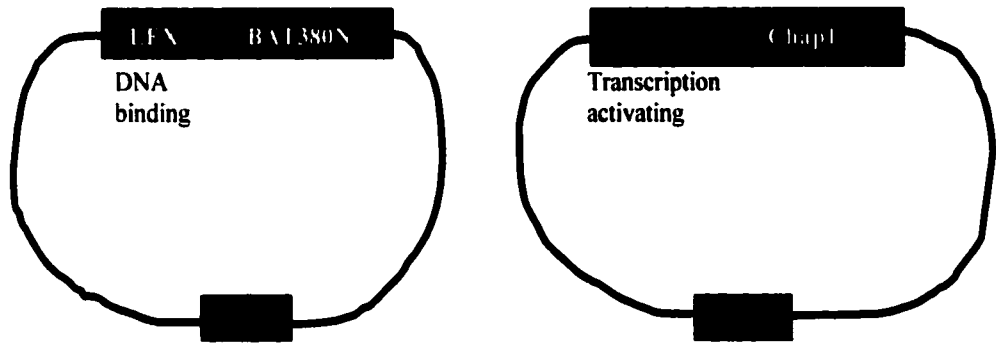HABAT-R: 5'--TT<u>GCGGCGCC</u>TAAGGATCATCAGCAAAGGCC--3'

BATMUT-F: 5'--GCCTGCC<u>AAGAGAAGCTTG</u>ACGATGCAGGGT--3'
                      K   **R**   **S**   **L**

BATMUT-R: 5'--ACCCTGCATCGTCAAGCTTCTCTTGGCAGGC--3'

PCR 1

5'--GCCTGCCAAGAGAAGCTTGACGATGCAGGGT--3'
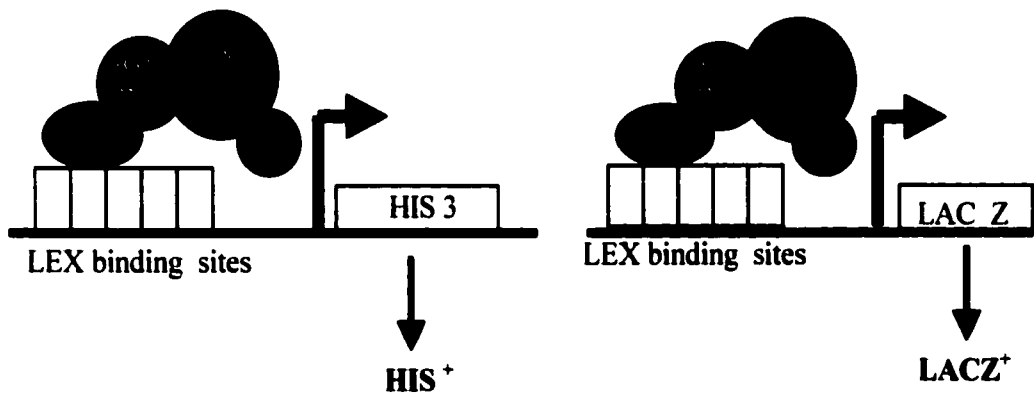3'--CGGACGGTTCTCTTCGAACTGCTACGTCCCA-5'

PCR 2

BAT313CΔNLS

95
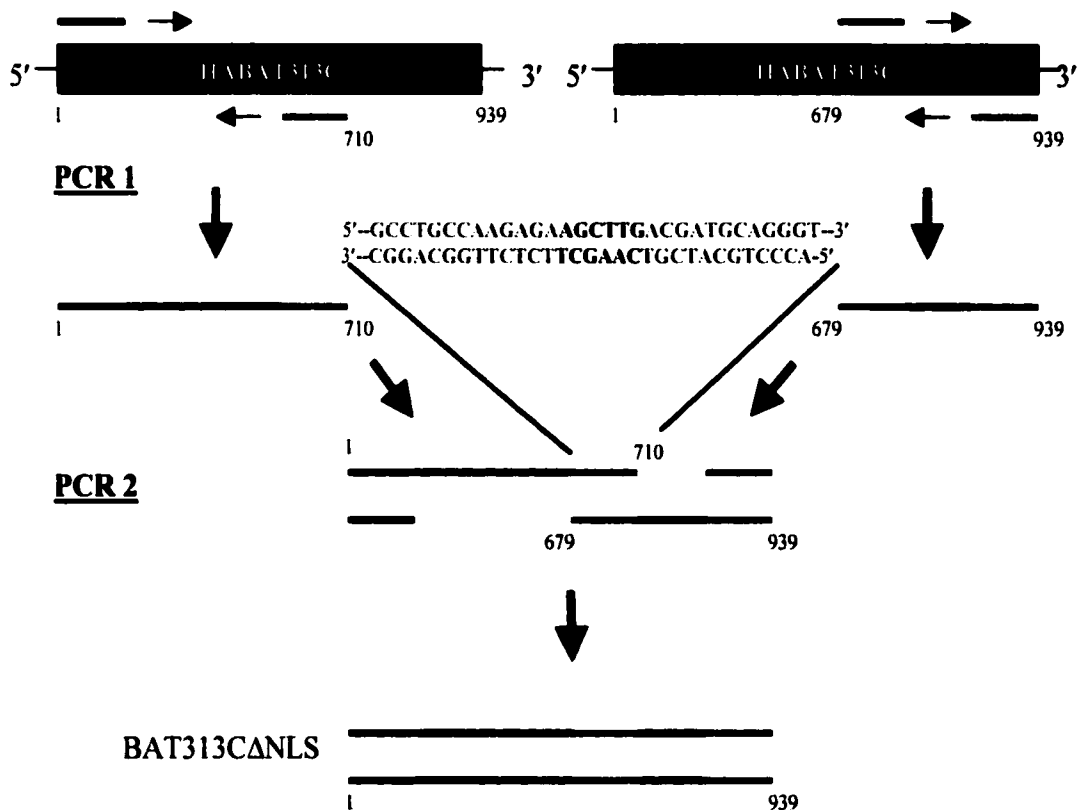
APPENDIX C

Schematic representation of the yeast two-hybrid system.

Transform plasmids into *S. cerevisiae* strain (trp⁻, leu⁻, his⁻)

If protein BAT380N and protein Chap1 interact:

Vita Auctoris

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Member of Departmental Fund Raising Committee, 1999-present

Member of Intramural Recreational Volleyball and Inner tube
Water polo championship teams, 2000.

**Poster
Presentations:**   <u>S. Manchen</u> and A. Hubberstey. Characterization and Subcellular
Localization of the Human BAT3 Protein, A Putative Apoptotic
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Contains a Nuclear Localization Sequence and Shows Both
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