MOLECULAR CLONING AND CHARACTERIZATION OF HUMAN BAG-1

XIAOLONG YANG

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BY

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This thesis is dedicated to my grandfather, Zicheng Yang, who taught me the philosophy of life and is fighting with cancer

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ABSTRACT

In the past ten years, a tremendous amount of work has demonstrated that enhanced cell proliferation caused by activation of oncogenes or inactivation of tumor suppressor genes is one of the major causes of human cancer. However, recently, mounting evidence strongly suggest that decreased cell death by apoptosis via inactivation of pro-apoptotic genes or activation of anti-apoptotic genes may also play an important role in carcinogenesis. Inactivation of the pro-apoptotic gene *p53* by mutations or DNA tumor viruses and overexpression of anti-apoptotic gene *bcl*-2 have been found in the majority of human cancers.

In an attempt to identify the transcription factors regulating JC virus expression, our lab isolated a protein called K1, which has an amino acids sequence identical to that of BAG-1. BAG-1 is a protein originally isolated from mouse embryonic cells as a Bcl-2 interacting protein. To understand the role of BAG-1 in human cancer, I isolated a 1.3 kb full length human BAG-1 cDNA by screening a human cervical cell cDNA library using mouse BAG-1 cDNA as a probe. The amino acid sequence of human BAG-1 cDNA is identical to a hormone receptor-binding protein, RAP46, and has 75% identity and 84% homology with that of mouse BAG-1. BAG-1 RNA was expressed in a variety of human tissues and at higher levels in the heart and pancreas. Characterization of BAG-1 protein *in vitro* and *in vivo* found that human BAG-1 protein is expressed as four isoforms, p50, p46, p33, and p29, by alternative translation initiation of the start codons through a leaky mechanism. Different BAG-1 isoforms have distinct subcellular localization.

Like mouse BAG-1, overexpression of human BAG-1 can inhibit apoptosis induced by various apoptotic stimuli such as UV irradiation, heat shock, staurosporine, cisplatin and doxorubicin. The C-terminal BAG-1 sequence is responsible for its anti-apoptotic activity. However, BAG-1 sensitizes 4-HPR-induced apoptosis by activation of caspase-3 through the BAG-1 central region. This result

suggests that BAG-1 can be a pro- or anti-apoptotic protein, depending on the apoptotic inducer.

Since Bcl-2 has been shown to be overexpressed in most human cancers, I examined whether the expression of Bcl-2-interacting protein, BAG-1, was also enhanced during tumorigenesis. In this study, enhanced expression of BAG-1 protein was detected in breast and cervical cancer cell lines and tissues. The increased BAG-1 protein in cancer cell lines was due to enhanced transcription rather than genomic rearrangement of the BAG-1 gene. Correlated with that of Bcl-2 and Bcl-X_L, the levels of BAG-1 proteins increased progressively after immortalization and transformation during multistage carcinogenesis of cervical cells. The increased BAG-1 protein was correlated with progressively increased resistance to apoptosis induced by staurosporine. Moreover, the level of BAG-1 protein correlated with that of other BAG-1-binding proteins such as Bcl-2 and Bcl-X_L only in human breast and cervical cell lines but not tissues.

To understand the molecular mechanism of overexpression of BAG-1 in human cancers, I cloned a 0.9 kb BAG-1 5'-flanking region from genomic DNA. Deletion analysis of this sequence localized the region of maximal BAG-1 promoter activity from nucleotide positions -353 to -54, upstream of the first start codon CTG. Sequence analysis of the BAG-1 promoter region showed the absence of a TATA box but identified a CCAAT box, several GC boxes, a CpG island and several transcription factor binding sites, which may be important in the regulation of BAG-1 transcription. Most importantly, functional characterization of the BAG-1 promoter *in vivo* demonstrated that gain-of-function p53 mutants derived from human tumors upregulated the transcription of BAG-1 RNA and the expression of a reporter gene from the BAG-1 promoter. These data indicated that overexpression of BAG-1 in some tumors may be due to upregulation of the human BAG-1 promoter by mutant p53.

In summary, human BAG-1 cDNA and promoter/enhancer were cloned and characterized in

this study. Further analysis of BAG-1 RNA and protein demonstrated that enhanced expression of BAG-1 may play an important role in carcinogenesis.

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LIST OF ABBREVIATIONS

AIF	apoptosis-inducing factor
Ad	adenovirus
Apaf-1	apoptotic proteinase activating factor-1
AR	androgen receptor
ARE	androgen response element
CAD	caspase-activated DNase
cdk	cyclin-dependent kinase
cDNA	complementary DNA
CML	chronic myeloid leukemia
CMV	cytomegalovirus
cpm	count per minute
CSC	cigarette smoke condensate
DAPI	4, 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EGF	epithelial growth factor
EGFR	EGF receptor
ER	estrogen receptor
FADD	Fas-associated death domain
FasL	Fas ligand
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FLIP	FLICE-inhibitory protein
GR	glucocorticoid receptor
hBAG-1	human BAG-1
HBV	hepatitis B virus
HEC	human ectocervical cells
HEN	human endocervical cells
HGF	hepatocyte growth factor
HIV	human immunodeficiency virus
HPV	human papillomavirus
HTLV-1	human T-cell leukemia virus type 1
IAP	inhibitor of apoptosis protein
ICAD	inhibitor of CAD
JCV	JC virus
kb	kilobase pair
kDa	kilodaltons
KGM	keratinocyte growth medium
LTR	long terminal repeat
mAb	monoclonal antibody
MAP	mitogen-activating protein

MAPK	MAP kinase
MAPKK	MAP kinase kinase
mBAG-1	Mouse BAG-1
MTN	multiple tissue Northern blot
NSL	nuclear localization signal
nt	nucleotide position number
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-drived growth factor
PDGFR	PDGF receptor
PDTC	pyrrolidine dithiocarbamate
pfu	phage forming unit
PI	propidium iodide
PMSF	phenylmethlsulfonyl fluoride
PR	progesterone receptor
RA	retinoic acid
RAR	retinoic acid receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcription
SDS	sodium dodecyl sulfate
Siah	SINA-homologous
Sina	Drosophila seven in absentia
SV40	simian virus 40
TGF	transforming growth factor
TGF-βRE	TGF-β response element
TNF-α	tumor necrosis factor-α
TR	thyroid receptor
TRADD	TNF-receptor I-associated death domain
U	units
UTR	untranslated region
UV	ultraviolet

SECTION I CHAPTER 1

GENERAL INTRODUCTION: APOPTOSIS AND CANCER

1.1 Definition of apoptosis

The phenomenon of apoptosis, also called programmed cell death, was defined by Kerr *et al.* in 1972. It is a process in which cells commit suicide under normal (embryogenesis, development, immune response, differentiation, aging, tissue atrophy, etc.) or stress [ultraviolet (UV) irradiation, growth factor deprivation, etc.] conditions. Apoptosis is an important form of cell death occurring in the human body. Distinct from the two other major types of cell death, senescence and necrosis, apoptosis has unique morphological and molecular characteristics, which are summarized in Table 1.1.

Apoptotic bodies are efficiently phagocytosed by neighbouring cells or macrophages and usually do not elicit an inflammatory immune response *in vivo* as occurs when cells undergo necrosis. Therefore, apoptosis is a better strategy to eliminate unnecessary, dying or harmful cells during normal development or under stress conditions without damaging adjacent tissues. Senescence also causes little tissue damage, but is less well regulated, possibly representing a housekeeping form of cell death, as occurs during normal epithelial differentiation (Simon and Gordon, 1995).

1.2 Role of cell proliferation versus apoptosis in carcinogenesis

It has been known for a long time that cell proliferation and cell death (apoptosis, senescence, and necrosis) are required to be balanced for tissue survival in a process called homeostasis. In the past ten years, a tremendous amount of work has been published on the role of dysregulated cell proliferation in the development of human cancers (for review, see Clurman and Roberts, 1998; Fearon, 1998; Park, 1998). It was found that cell proliferation can be enhanced due to activation of some oncogenes such as c-*myc* and H-*ras*, or inactivation of tumor suppressor genes such as *p53* and

Table 1.1 Changes occurring during apoptosis

A. Morphological changes

- Cell shrinkage
- Cell membrane blebbing
- Cytoplasm, nuclear and chromosome condensation
- Nuclear fragmentation
- Formation of apoptotic bodies

B. Biochemical and molecular changes

- Increased apoptosis inducing proteins to inhibiting proteins ratio
- Mitochondrial changes
  - Decrease of mitochondrial membrane potential
  - Release of death factors, such as cytochrome c and apoptosis-inducing factor (AIF)
  - Formation of reactive oxygen species (ROS)
- Loss of asymmetry in plasma membrane phospholiplids such as phosphatidyl serine
- Activation of caspases
- Cleavage of key cellular proteins
- DNA fragmentation into 50-300 kilobase pair (kb) large and/or internucleosomal DNA fragments
- Increase of plasma membrane permeability for vital dye

Rb. Increased proliferation without a compensatory increase in cell death can cause the accumulation of cells and, finally, lead to cancer through a multistage carcinogenesis mechanism. Recently, mounting evidence suggest that decreased cell death, especially by apoptosis, may also plays an important role in multistage carcinogenesis. Cell death can be repressed due to downregulation of cellular genes that induce it or upregulation of cellular genes that inhibit it. Decreased apoptosis without a similar change in cell proliferation can similarly cause cell accumulation and finally cancer (Fig.1.1). Although the molecular mechanism for dysregulation of cell proliferation in the development of human cancer has been extensively studied, the role of apoptosis in human cancer remains relatively unexplored.

1.3 Signal transduction pathways of apoptosis

Although apoptosis can be induced by a variety of apoptotic stimuli, its regulators, executioners, and substrates in the signal transduction pathway often overlap and are summarized in Fig.1.2. Apoptosis stimuli generally originate externally to the cells. Some such as the cytotoxic cytokines, tumor necrosis factor-α (TNF-α), Fas ligand (FasL), and TRAIL can bind to their receptors on the plasma membrane, causing dimerization of their receptors and thereby activation of an initiator caspase such as caspase-8 through interaction of the receptors with death adaptor proteins such as Fas-associated death domain (FADD) or TNF-receptor I-associated death domain (TRADD) (Nunez *et al.*, 1998; Baker and Reddy, 1998). On the other hand, cancer chemotherapeutic drugs, such as cisplatin, can reduce the mitochondrial membrane potential ($\Delta\psi$) and cause release of cytochrome c from mitochondria. This activates Apaf-1 (apoptotic proteinase activating factor-1), which activates initiator caspase caspase-9 (Li *et al.*, 1997; Zou *et al.*, 1997).

Fig.1.1 The effect of relative rates of cell proliferation and cell death on tumorigenesis (adapted from Thompson, 1995). Unchanged rates of cell proliferation and cell death are indicated by the same thickness of arrows. Increased rate of cell proliferation and decreased rate of cell death are indicated by thicker and thinner arrows, respectively.

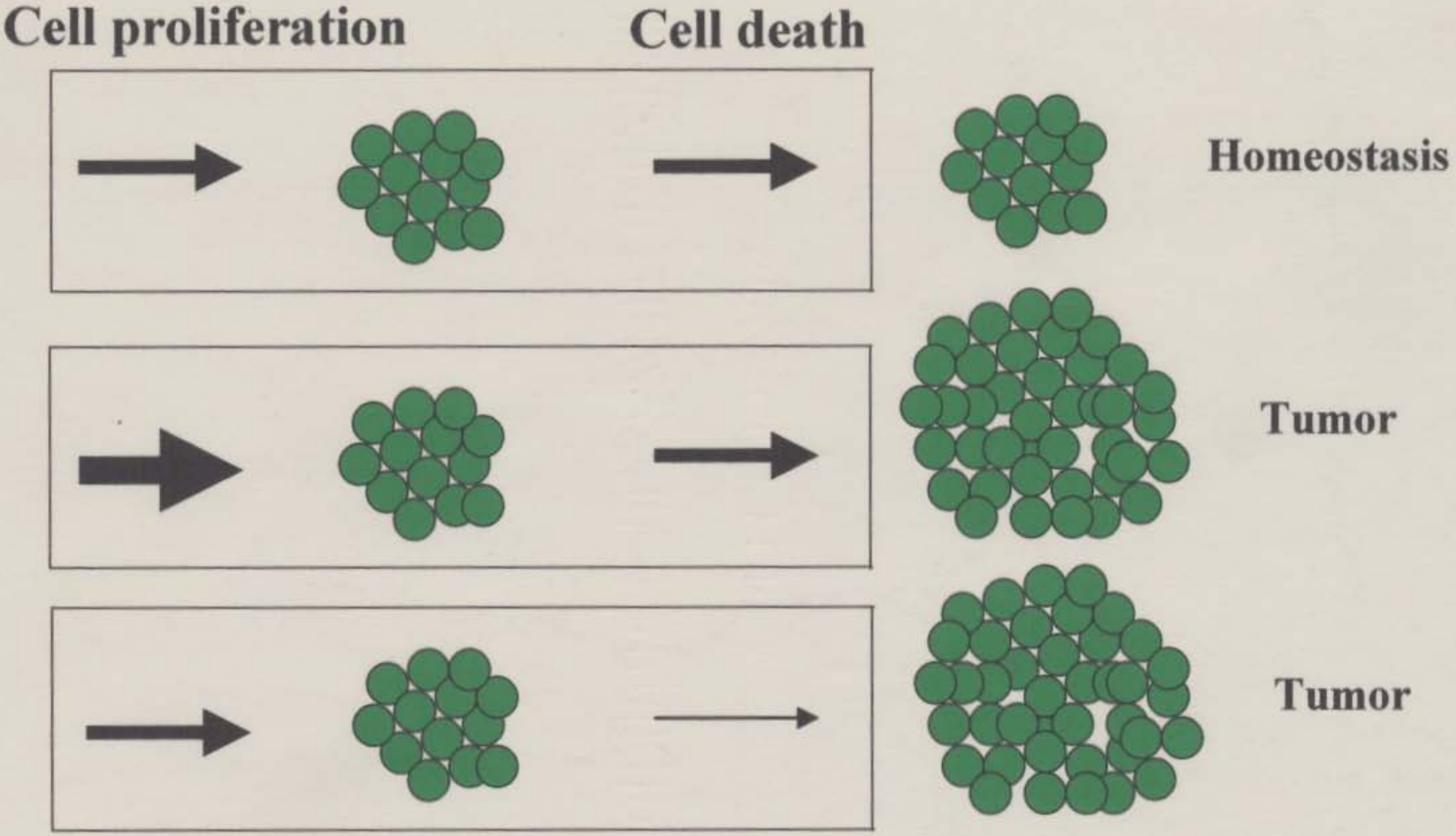
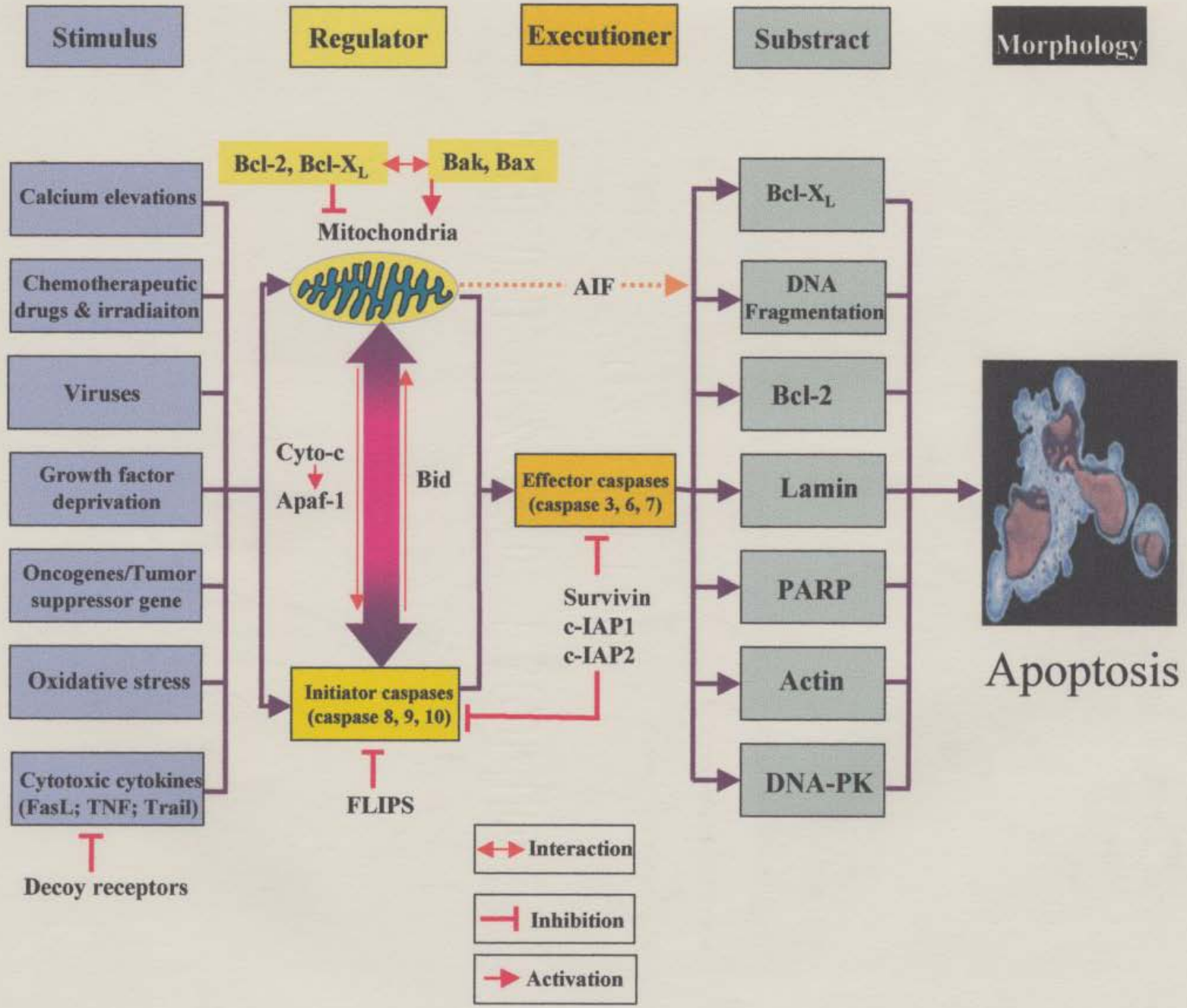

Cell proliferation
Cell death
Homeostasis
Tumor
Tumor

Fig.1.2 Signal transduction pathways of apoptosis (adapted from Reed, 1998b). Apoptosis stimuli activate downstream apoptosis executioners, which can subsequently cause apoptosis by cleaving DNA and key cellular protein substrates (See text for details).


Stimulus
Regulator
Executioner
Substract
Morphology
Bcl-2, Bcl-X$_L$
Bak, Bax
Mitochondria
Calcium elevations
Chemotherapeutic drugs & irradiaiton
Viruses
Growth factor deprivation
Oncogenes/Tumor suppressor gene
Oxidative stress
Cytotoxic cytokines (FasL; TNF; Trail)
Decoy receptors
AIF
Cyto-c
Apaf-1
Bid
Effector caspases (caspase 3, 6, 7)
Survivin
c-IAP1
c-IAP2
Initiator caspases (caspase 8, 9, 10)
FLIPS
Bcl-X$_L$
DNA Fragmentation
Bcl-2
Lamin
PARP
Actin
DNA-PK
Apoptosis
Interaction
Inhibition
Activation

The activated initiator caspase (e.g., caspase-8, -9 or -10) will subsequently activate an effector caspase (e.g., caspase-3) by proteolytic cleavage of their procaspases (Thornberry and Lazebnik, 1998). The decrease of mitochondrial membrane potential can also cause the release of effector caspases, such as caspase-3, and translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nuclear membrane and DNA fragmentation (Susin *et al.*, 1999). In a partially overlapping pathway, the pro-apoptotic Bcl-2 family member protein Bid is cleaved by Fas-activated caspase-8 and translocated from the cytosol to the mitochondrial membrane, and induces the release of cytochrome c, which in turn activates Apaf-1, which binds and activates the most upstream caspase, caspase-9 (Li *et al.*, 1998; Luo *et al.*, 1998).

Activated effector caspases can cleave important protein substrates such as PARP, lamin and actin, and anti-apoptotic proteins such as Bcl-2 and Bcl-X_L (Cryns and Yuan, 1998) or activate caspase-activated DNase (CAD) by cleaving/releasing inhibitor of CAD (ICAD) from CAD. The activated CAD can then be translocated to the nucleus and causes DNA fragmentation (Enari *et al.*, 1998; Sakahira *et al.*, 1998). Cleavage of proteins and DNA will finally cause apoptosis, which has the unique morphological characteristics described in Table 1.1 and Fig. 1.2.

1.4 Genes regulating apoptosis and their role in cancer

As described in Section 1.2, reduced cell death may cause the accumulation of cells and finally lead to human cancer. Therefore, reduced apoptosis may play an important role in carcinogenesis. The significance of apoptosis in the process of oncogenesis is evidenced by at least three important observations. The first is that most cancer chemotherapeutic drugs act to induce apoptosis (Martin and Green, 1995). The second is that the frequency of apoptotic cells in a tumor negatively correlates closely with outcome. That is, tumors with low apoptotic indices tend to be more aggressive than

those displaying higher incidence of apoptosis. Therefore, apoptosis is almost certainly an important factor controlling tumor growth (Arends *et al.*, 1994). The third observation is that a number of oncogenes and tumor suppressor genes have been found to regulate the process of apoptosis (Martin and Green, 1995; Bissonnette *et al.*, 1994; Hoffman and Liebermann, 1998; Kharbanda *et al.*, 1998; Amundson *et al.*, 1998). Therefore, studying the molecular mechanism of apoptosis will be useful for understanding and guiding the prevention of oncogenesis and design of cancer therapy.

Apoptosis can be promoted by proteins, such as Bak, Bax, Bik and Bid, or inhibited by proteins, such as Bcl-2 and Bcl-X_L, through, for example, modulation of mitochondrial membrane function (Green and Reed, 1998; Reed, 1998). Apoptosis can also be inhibited through inactivation of either initiator caspases by FLICE-inhibitory proteins (FLIPS) or effector caspases by survivin, c-IAP-1, and c-IAP-2 (Fig. 1.2; Thome *et al.*, 1997; Cryns and Yuan, 1998; LaCasse *et al.*, 1998). Since such proteins have essential roles in regulating apoptosis, their mechanisms of action and expression levels in human normal and cancer cell lines and tissues have been studied, as follows:

1.4.1 *bcl*-2 family

1.4.1.1 Pro-apoptotic genes

To date, about 12 human Bcl-2 family members (Bad, Bak, Bax, Bcl-X_S, Bid, Bik, Bim/Bod, Blk, Bok, Hrk/Dp5, Nip3 and Nix) have been identified that can enhance apoptosis (for review, see Chao and Korsmeyer, 1998; Zamzami *et al.*, 1998; Reed, 1998a). Of these, however, only Bak and Bax have been reported to be associated with carcinogenesis.

1) *bax*

Bax is a protein that can enhance apoptosis by binding to and inhibiting the anti-apoptotic function of Bcl-2 (Oltvai *et al.*, 1993). Bax expression is induced following a variety of DNA-

damaging treatments (Miyashita *et al.*, 1994a, b; Zhan *et al.*, 1994). It has recently been shown that the *bax* gene expression is induced by p53, which can bind to a p53-response element in the *bax* promoter and transactivate its transcription (Miyashita *et al.*, 1995). The following evidence suggest that Bax may be involved in human cancer: 1) reduced levels of *bax* RNA and protein have been reported in breast carcinomas and were correlated with poor responses to combination chemotherapy and shorter overall survival (Bargou *et al.*, 1995, 1996; Krajewski *et al.*, 1995); 2) mutations of *bax* have been found in a variety of human cancers including lymphomas, leukemias, and colorectal, endometrial, gastric, ovarian and stomach cancers (Rampino *et al.*, 1997; Brimmell *et al.*, 1998; Colella, 1998; Ouyang *et al.*, 1998); and 3) overexpression of Bax was found to suppress tumorigenesis *in vivo* (Yin *et al.*, 1997).

2) *bak*

bak is a pro-apoptotic gene that was originally identified by virtue of its homology to *bcl*-2 and the ability of its protein to interact and inhibit the anti-apoptotic activity of adenovirus (Ad) E1B 19K protein (Chittenden *et al.*, 1995; Farrow *et al.*, 1995; Kiefer *et al.*, 1995). Reduced expression of *bak* was found in breast, colon and skin cancers (Krajeska *et al.*, 1996; Tomkova *et al.*, 1997; Zapata *et al.*, 1998). In addition, overexpression of *bak* significantly inhibited *ras*-induced tumorigenicity (Rosen *et al.*, 1998), indicating that *bak* may act as a tumor suppressor gene in some cases.

1.4.1.2 Anti-apoptotic genes

To date, five human anti-apoptotic bcl-2 family members, *bcl*-2, *bcl*-X_L, *mcl*-1, *bcl*-w, and *bfl*-1, have been identified (Vaux *et al.*, 1988; Boise *et al.*, 1993; Choi *et al.*, 1995 Gibson *et al.*, 1996;

Lomo *et al.*, 1996). However, only *bcl*-2, *bcl*-X_L and *bfl*-1 have been shown to be involved in human cancers:

1) *bcl*-2

The *bcl*-2 gene was identified in 1984 while studying the t(14; 18) chromosome translocations that occur frequently in B cell leukemia and non-Hodgkin's follicular lymphoma (Yunis *et al.*, 1982; Tsujimoto *et al.*, 1984). The gene is located at chromosome 18q21, but the t(14; 18) translocation juxtaposes it with the immunoglobulin heavy chain (IgH) locus on chromosome 14. This bcl-2/IgH fusion gene expresses high levels of *bcl*-2 (Tsujimoto *et al.*, 1984; Tsujimoto *et al.*, 1985). Therefore, *bcl*-2 was originally regarded as an oncogene that contributed to the oncogenesis of B-cells. However, a later study demonstrated that *bcl*-2 functions differently from conventional oncogenes, since it neither promoted cell growth nor directly led to cellular transformation (Reed, 1994). Instead, overexpression of Bcl-2 contributed to oncogenesis by increasing cell survival through suppressing apoptosis, resulting in increased numbers of cells, which accumulated further oncogenic mutations (Fig. 1.1). For example, 15% of transgenic mice overexpressing Bcl-2 developed aggressive B-cell lymphomas. Approximately 50% of lymphomas expressed elevated levels of c-Myc, indicating that Bcl-2 overexpression leads to impaired apoptosis and thus increased mutations in genes, such as c-*myc*, controlling cellular proliferation (Vaux *et al.*, 1988). Later studies further demonstrated that overexpression of *bcl*-2, may be involved in the development of other human cancers. An enhanced level of Bcl-2 was found in a variety of human cancers, including neuroblastoma, and breast, cervical, colorectal, head and neck, lung, skin and prostate cancers (McDonnell *et al.*, 1992; Halder *et al.*, 1994; Ikegaki *et al.*, 1994; Liang *et al.*, 1995; Sinicrope *et al.*, 1995; Delehedde *et al.*, 1999; Pena *et al.*, 1999). Increased *bcl*-2 expression in human cancers

is due partly to the loss of its repression by p53 tumor suppressor after frequently occurring mutations of p53 (Harder *et al.*, 1994; Miyashita *et al.*, 1994a, b).

2) *bcl*-X_L

bcl-X_L is a *bcl*-2 family member that has anti-apoptotic activity. It is the long splice variant of the bcl-X gene (Boise *et al.*, 1993). Like Bcl-2, Bcl-X_L can inhibit apoptosis induced by many different apoptotic stimuli (Boise *et al.*, 1993; Chao and Korsmeyer, 1998; Reed, 1998a). In addition, Bcl-X_L can also inhibit some apoptosis pathways that transfection with *bcl*-2 does not affect (Gottschalk *et al.*, 1994). Overexpression of Bcl-X_L has been found in lymphomas, and bladder, colorectal, head and neck, pancreatic, and stomach cancers (Kondo *et al.*, 1996b; Krajewska *et al.*, 1996; Xerri *et al.*, 1996; Hirose *et al.*, 1997; Kirsh *et al.*, 1998; Miyamoto *et al.*, 1999; Pena *et al.*, 1999).

3) *bfl*-1

bfl-1 is a *bcl*-2 family member and the human homolog of mouse anti-apoptotic *A1* gene. Bfl-1 efficiently suppresses apoptosis induced by the p53 tumor suppressor protein and cooperates with the dominant Ad E1A oncoprotein in the transformation of primary epithelial cells *in vitro* (D'Sa-Eipper *et al.*, 1996). Unlike other Bcl-2 family proteins, expression of Bfl-1 can enhance cell proliferation (D'Sa-Eipper *et al.*, 1996). In addition, enhanced expression of *bfl*-1 was observed in lymphomas, and breast, colon, stomach and ovarian cancers (Choi *et al.*, 1995; Park *et al.*, 1997).

1.4.2 Death and decoy receptors

1.4.2.1 Death receptors

Death receptors are TNFR superfamily members, which are defined by their similar, cysteine-rich extracellular domains and homologous cytoplasmic death domain (Nagata, 1997). Death receptors can mediate apoptosis induced by death ligands (TNF-α, FasL, and TRAIL) by activating caspases through death domains-death adaptors (for review see Ashkenazi and Dixit, 1998; Baker and Reddy, 1998). Until now, about seven types of death receptors, Fas/CD95/Apo1, TNFR-I, TNFR-II, DR3/Apo3, DR4, DR5/Apo2/KILLER/TRAIL-R2, and DR6, have been identified (Ashkenazi and Dixit, 1998; Baker and Reddy, 1998).

Fas-mediated apoptosis plays an important role in lymphocyte homeostasis and killing of virus-infected or cancer cells (Varadhachary and Salgame, 1998). Therefore, inactivation of *fas* may cause enhanced lymphoproliferation and accumulation of cancer cells. Consistently, mutations of *fas* gene were found in leukemia and lymphoma (Beltinger *et al.*, 1998; Gronbaek *et al.*, 1998; Lamy *et al.*, 1998; Maeda *et al.*, 1999). Furthermore, reduced expression of *fas* was also reported in colon cancer (Butler *et al.*, 1998), indicating that downregulation of *fas* by either mutation or decreased expression may be responsible for the development of human cancers. Except in one case in which loss-of-function truncation mutation of a TRAIL death receptor gene, DR5/KILLER, was detected in a head and neck primary cancer (Pai *et al.*, 1998), no mutations of other death receptors have been found in human cancers.

1.4.2.2 Decoy receptors

Decoy receptors, such as DcR1, DcR2 and DcR3, are those which are structurally similar to death receptors, but lack an intracellular domain, are therefore incapable of transducing death receptor-like signals, and compete with death receptors for binding to the same ligands (Sheridan *et al.*, 1997; Pitti *et al.*, 1998; Ashkenazi and Dixit, 1998). Recently, it was found that DcR3 decoy

receptor for FasL was amplified in about half of 35 primary lung and colon tumors, indicating that tumors may block FasL-dependent immunocytotoxic attack by expressing a decoy receptor (Pitti *et al.*, 1998).

1.4.3 Inhibitors of apoptosis proteins (IAPs)

The IAP family members can suppress apoptosis by direct inhibition of caspase activity (Fig. 1.2; for review, see LaCasse *et al.*, 1998; Deveraux and Reed, 1999). They are characterized by an approximately 70 amino acids domain termed the baculoviral IAP repeat and a RING zinc finger. To date, six IAP members have been identified in humans: c-IAP1, c-IAP2, hILP, NAIP, XIAP, and survivin (Deveraux and Reed, 1999). Increasing reports indicate that IAPs may play a role in oncogenesis. The strongest evidence implicating IAP in cancer came from survivin studies. Survivin has been found to be expressed in a high proportion of the most common human cancers, such as breast, lung, colon and prostate cancers, but not in normal, terminally differentiated adult tissues (Ambrosini *et al.*, 1997; Adida *et al.*, 1998; Lu *et al.*, 1998; Kawasaki *et al.*, 1998).

1.5 Cellular oncogenes and their roles in apoptosis

Genes whose products have the ability to transform eukaryotic cells are called oncogenes. Most oncogenes are derived from normal genes, or proto-oncogenes, that encode proteins involved in essential cellular functions related to control of cell proliferation and differentiation, such as growth factors [e.g., epithelia growth factor (EGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), growth factor receptors [e.g., PDGF receptor (PDGFR) and EGF receptor (EGFR)], signal transducers (e.g., H-Ras), transcription factors (e.g., c-Myc, c-Fos and c-Jun) and cell cycle regulators (e.g., E2F). However, once activated by either overexpression or mutations,

oncogenes can increase cell proliferation, resulting in accumulation of cells and leading ultimately to cancer. In addition, oncogenes have been shown to be involved in tumorigenicity and metastasis (Vogelstein and Kinzler, 1993).

The conclusion that oncogenes are involved in apoptosis originated from studies of anti-apoptotic bcl-2 oncogene involved in B-cell follicular lymphoma as described in Section 1.3.1.2. Later studies demonstrated that many activated oncogenes, including c-*abl*, *AP-1* (*c-fos*/c-*jun*), c-*myc*, H-*ras*, and *mdm2*, can not only transform cells by increasing cell proliferation, but also have the ability to modulate apoptosis. Oncogenes can either enhance or inhibit apoptosis, depending on the specific gene, cell types or growth conditions, such as the level of growth factors (Colotta *et al.*, 1992; Schreiber *et al.*, 1995; Verheij *et al.*, 1996; Yuan *et al.*, 1997).

1.5.1 c-*abl*

The mammalian c-*abl* proto-oncogene was identified as the cellular homolog of the transforming v-*abl* oncogene of the Abelson murine leukemia virus (Goff *et al.*, 1980). It encodes a nonreceptor tyrosine kinase that is ubiquitously expressed in a variety of human tissues (Ben-Neriah *et al.*, 1986). c-Abl can not only phosphorylate proteins, but also transactivate some cellular genes by binding to an EP element found in the promoter/enhancer regions of certain cellular and viral genes (Dikstein *et al.*, 1992, 1996).

Previous reports indicated that the c-*abl* gene on chromosome 9 can be translocated to the *bcr* gene locus on chromosome 22, forming the Philadelphia chromosome (Kelliher *et al.*, 1990). This translocation produces a chimeric *bcr-abl* gene, the protein product of which exhibits an uncontrolled tyrosine kinase activity, which is thought to lead to uncontrolled cell growth and chronic myeloid leukemia (CML). Later, it was found that the resistance of a CML cell line (K562)

to several apoptotic stimuli, including cytotoxic drugs and serum withdrawal, can be overcome by downregulating expression of c-*abl* mRNA (McGahon *et al.*, 1994). Furthermore, transfection of K562 cells with v-*abl* restores their resistance to the same apoptotic stimuli, indicating that the c-*abl* gene has an anti-apoptotic function that may be responsible for the development of CML (McGahon *et al.*, 1994). However, several other studies demonstrated that c-Abl can provide pro-apoptotic function in cells deficient in p53 (Yuan *et al.*, 1997). In addition, c-Abl can bind to and enhance the pro-apoptotic activity of p73 (Kharbanda *et al.*, 1998). Therefore, depending on the cellular content, c-abl plays distinct roles in apoptosis.

1.5.2 c-*fos*/c-*jun*

AP1 is a transcription factor formed by dimerization of c-Fos and c-Jun proto-oncoproteins and can activate a variety of cellular genes associated with cell proliferation (Curran and Franza, 1988). *In vitro*, the expression of c-*fos* and c-*jun* mRNA and proteins is rapidly and transiently induced after stimulation of quiescent cells by growth factor (Sassone-Corsi and Verma, 1987). Therefore, c-*fos* and c-*jun* are immediate early genes responsible for proliferation in response to growth factor. *In vivo*, amplification of both genes has been found in osteosarcomas (Park,1998).

AP-1 was observed to increase apoptosis induced by withdrawal of cell survival factors. For example, c-*fos* and c-*jun* expression was rapidly induced after growth factor withdrawal from IL-6- and IL-2-dependent murine lymphoid cells. Blocking the expression of either c-*fos* or c-*jun* with antisense oligonucleotides increased lymphoid cell survival following growth factor deprivation (Colotta *et al.*, 1992). In addition, AP-1 can enhance apoptosis induced by stress (e.g., TNF-α, X-rays, UV light and heat shock) (Verheij *et al.*, 1996). However, in some cases, AP-1 can act as an anti-apoptotic protein. For example, it was shown that loss of AP-1 function can increase apoptosis

induced by short-wavelength UV light in c-*fos* null mouse 3T3 fibroblasts (Schreiber *et al.*, 1995). Therefore, AP-1 appears to function as a positive and negative regulator of apoptosis, depending on the cell types or growth conditions.

1.5.3 c-*myc*

c-*myc* is a proto-oncogene that has been shown to play a pivotal role in cell proliferation and prevention of differentiation in response to mitogenic stimuli (Evan and Littlewood, 1993; Ryan and Birnie, 1997). Overexpression of c-*myc* can promote cell proliferation by activating one or more cellular genes associated with the cell cycle such as cyclin A, cyclin E, and cdc25A (Dang *et al.*, 1999). Therefore, dysregulation of c-*myc* may cause uncontrolled cell growth and finally cancer. Increased expression or amplification of c-*myc* has been consistently found in a variety of human cancers including lymphomas, and breast, cervical, colon, esophageal, lung and ovarian cancers (Park, 1998).

Recent studies indicate that deregulated expression of c-*myc* not only promotes proliferation, but also can either induce or sensitize cells to apoptosis (Evan *et al.*, 1992; Shi *et al.*, 1992). c-Myc promotes cell proliferation in the presence of growth factors, whereas it promotes apoptosis in their absence (Evan *et al.*, 1992; Shi *et al.*, 1992). Therefore, c-Myc expression affords a proliferative advantage and also makes cells particularly susceptible to apoptosis under growth factor limitation conditions.

In cooperation with c-Myc, mutation of other oncogenes or overexpression of anti-apoptotic genes can cause either escape from growth factor dependency or inhibition of apoptosis, leading to tumor growth. For example, Bcl-2 can inhibit c-Myc-induced apoptosis in some cell systems (Packham and Cleveland, 1995; Thompson, 1998), while having no effect on the proliferative

function of c-Myc (Fanidi *et al.*, 1992; Packham and Cleveland, 1995). *In vitro* co-transfection studies showed that c-*myc* and *bcl*-2 can cooperate to achieve immortalization of several cell types (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992). Furthermore, *in vivo* studies demonstrated that c-*myc* and *bcl*-2 double transgenic mice develop tumors much more rapidly than c-*myc* transgenic mice (Strasser *et al.*, 1990). This interaction between c-*myc* and *bcl*-2 provides an explanation for the cooperation of c-*myc* and *bcl*-2 in cell transformation (Vaux *et al.*, 1988; Bissonette *et al.*, 1992). Thus, c-*myc* and *bcl*-2 synergize during multistage tumorigenesis.

1.5.4 *mdm2*

Mdm2 is a proto-oncoprotein originally cloned by virtue of its interacting with p53 (Oliner *et al.*, 1992). While its mRNA expression can be induced by p53, Mdm2 can bind to and inhibit the function of wild-type p53 protein (Momand *et al.*, 1992). Overexpression of Mdm-2 can transform murine fibroblasts (Momand and Zambetti, 1997). Furthermore, amplification or overexpression of the *mdm*-2 gene has been detected in various human tumors, including leukemias, neuroblastomas, soft tissue tumors, and breast, cervical, lung and ovarian cancers (Momand and Zambetti, 1997).

Recently, it was shown that Mdm-2 is involved in apoptosis by abrogation of p53 pro-apoptotic activity. Overexpression of *mdm*-2 can inhibit apoptosis induced by a variety of apoptotic stimuli (Kondo *et al.*, 1995; Chen *et al.*, 1996; Haupt *et al.*, 1996). These results suggest that inhibition of cell death by Mdm-2 through inactivation of p53 may play an important role in oncogenesis.

1.5.5 *ras*

The c-*ras* proto-oncogene product is a GTPase that mediates signal transduction from growth hormones to cellular proteins associated with proliferation. Upon mutation at amino acid positions

12 or 61, c-Ras can be activated and continuously activate its target proteins. This leads to uncontrolled cell proliferation and cancer. Consequently, mutation of *ras* is responsible for its oncogenic activity. In fact, about 30% of human cancers were found to contain activation mutations in a *ras* (N-, Ki and H-*ras*) gene (Park, 1998).

ras has been found to be involved in the regulation of apoptosis. Overexpression of *ras* gene can not only increase mitotic indices but additionally cause apoptosis (Wyllie *et al.*, 1987). Nevertheless, mutation-activated *ras* can inhibit apoptosis (Fenton *et al.*, 1998). Tumor cells expressing activated Ras exhibit greatly enhanced mitotic indices while retaining lower apoptotic indices than parental cells (Wyllie *et al.*, 1987). Consequently, activated *ras* may be involved in oncogenesis, not only by enhancing proliferation, but also by inhibiting apoptosis. The mechanism for the inhibition of apoptosis by oncogenic *ras* is still unclear. However, it was found that oncogenic *ras* can inhibit *fas*-induced apoptosis by inhibition of Fas (CD95) expression (Fenton *et al.*, 1998).

1.6 Viral oncogenes and their role in apoptosis

Apoptosis is implicated in the elimination of viral-infected cells whose survival might be harmful to the organism. Apoptosis can be triggered by cytotoxic T lymphocytes or tumor necrosis factor, which induce endonucleases that can target replicating viral nucleic acids and prevent virus production at early stages of viral infection. To proliferate in host cells, many viruses have acquired genes encoding proteins that effectively suppress or delay apoptosis long enough for the production of sufficient numbers of progeny. On the other hand, a growing list of viruses are now known to induce apoptosis actively at late stages of infection (Table 1.2).

In general, viruses regulate apoptosis by the following mechanisms:

1) Inactivation/degradation of p53

The best characterized viruses that inhibit apoptosis by inactivating p53 are tumor viruses, such as Ad, hepatitis B virus (HBV), human papillomavirus (HPV), and simian virus 40 (SV40). DNA tumor virus oncoproteins, such as Ad E1B (19 and 55 kDa), HBV pX, HPV E6 and SV40 large T antigen, can bind p53 and inactivate p53-dependent apoptosis (White *et al.*, 1991; Rao *et al.*, 1992; Shen *et al.*, 1994; Symonds *et al.*, 1994; Wang *et al.*, 1995; Tsang *et al.*, 1995; Thomas *et al.*, 1996). However, inhibition of p53-induced apoptosis by these viruses involves distinct events. For example, E6 protein of high risk HPVs can abolish p53-induced apoptosis through ubiquitin-mediated p53 degradation (Thomas *et al.*, 1996). However, SV40 large T antigen prevents p53-induced apoptosis by interacting with the p53 DNA binding domain and blocking p53-mediated transactivation activity (Bargonetti *et al.*, 1992; Symond *et al.*, 1994; also see section 1.7.1).

2) Inactivation of RB

Some viral oncoproteins, such as Ad E1A and high-risk HPVs E7, can induce apoptosis by binding and blocking the anti-apoptotic activity of RB (see section 1.7.3). In addition, these oncoproteins can also induce p53 accumulation and p53-dependent apoptosis (Debbas and White, 1993; Jones *et al.*, 1997).

3) Transcripitional regulation of apoptosis-associated genes

Some retrovirus oncoproteins, such as human immunodeficiency virus (HIV) Tat and human T-cell leukemia virus type 1 (HTLV-1) Tax can induce apoptosis by downregulation of anti-apoptotic Bcl-2 (Yamada *et al.*, 1994; Sastry *et al.*, 1996; for detailed function of Bcl-2, see Section 1.4.1.2). In addition, HIV-1 Tat can activate FasL, causing Fas-dependent apoptosis (Mitra *et al.*, 1996). On the other hand, Epstein Barr virus (EBV) LMP1 inhibits apoptosis through induction of anti-apoptotic Bcl-2 (Henderson *et al.*, 1991).

Table 1.2 Viral gene products regulating apoptosis

Viruses	Product	Effect on apoptosis	Mechanism	References
Ad	E1A	+	Inactivation of RB	Debbas and White, 1993
	E1B19kDa	-	Inactivation of p53	White *et al.*, 1991; Shen *et al.*, 1994
	E1B55kDa	-	Inactivation of p53	Rao *et al.*, 1992
Baculovirus	p35	-	Caspase inhibitor	Clem *et al.*, 1991
	IAP	-	Caspase inhibitor	Crook *et al.*, 1993
Cowpox virus	CrmA	-	Caspase inhibitor	Gagliardini *et al.*, 1994
EBV	LMP1	-	Induction of Bcl-2	Henderson *et al.*, 1991
	BHRF1	-	Bcl-2 homology	Henderson *et al.*, 1993
HSV	$\gamma_1$34.5	-	Restore cellular protein synthesis	Chou and Roizman, 1992
HBV	pX	-	Inactivation of p53	Wang *et al.*, 1995
HIV-1	Tat	+	Downregulation of Bcl-2, upregulation of Bax, FasL, activation of caspase	Sastry et al., 1996 Mitra *et al.* (1996) Bartz and Emerman (1999)
HPV	E6	-	Deregulation of p53	Tsang *et al.*, 1995; Thomas *et al.*, 1996
	E7	+	Inactivation of RB	Jones *et al.*, 1997
HTLV-1	Tax	+	Downregulation of Bcl-2	Yamada *et al.*, 1994
CMV	IE1 and IE2	-	Inactivation of p53	Zhu *et al.*, 1995
SV40	Large T antigen	-	Inactivation of p53	Symonds *et al.*, 1994

4) Regulation of caspase activity

Baculovirus p35 and IAP and Cowpox virus CrmA can inhibit apoptosis by inhibiting caspase activiation (Clem *et al.*, 1991; Crook *et al.*, 1993; Gagliardini *et al.*, 1994). While wild-type p35 can inhibit host cell apoptosis during infection, *p35* mutant viruses result in extensive induction of apoptosis and a reduction in infectivity both *in vitro* and *in vivo* (Clem *et al.*, 1991; Clem and Miller, 1993). Baculovirus IAP was the first IAP found to inhibit caspases. Several mammalian and Drosophila homologs have been isolated and have similar function (for detail, see Section 1.4.3). Cowpox virus CrmA is another caspase inhibitor. It can inhibit apoptosis caused by cytotoxic T cells (Tewari *et al.*, 1995) and FasL (Tewari and Dixit, 1995). Moreover, HIV-1 has been shown to induce apoptosis by activating caspase-8 (Bartz and Emerman, 1999)

1.7 Tumor suppressor genes and their roles in apoptosis

Tumor suppressor genes are genes whose mutation, deletion or inactivation is associated with the development of malignancy. Tumor suppressor genes have at least one of the following characteristics: 1) loss-of-function mutations accompanied by loss of heterozygosity in tumors; 2) mutations in inherited syndromes that predispose to cancer; 3) somatic mutations in spontaneous tumors; 4) inhibition of transformed cell growth *in vitro*; and 5) knockout mutation transgenic mice showing a predisposition to cancer that mirrors a human cancer syndrome.

1.7.1 *p53* and *p73*

p53 is a nuclear DNA-binding phosphoprotein that is a transcriptional activator of specific target genes, and can exert transcriptional repression, probably by interaction with transcription factors or

general transcriptional machinery proteins such as TATA-binding protein (for review, see Levine, 1997). *p53* has been regarded as a tumor suppressor gene for the following reasons: 1) *p53* mutations are found in 50-55% of human cancers (Hollstein *et al.*, 1994); 2) transfection of *p53* into carcinoma cells represses tumor growth and arrests cells in the G1 phase (Levine, 1991); and 3) p53 knockout mice exhibit a predisposition to various cancers (Donehower *et al.*, 1992). The p53 protein is normally present at very low levels, but is up-regulated rapidly by DNA damage, viral infection or other factors, causing a G1- or G2-specific cell-cycle arrest, allowing subsequent DNA repair (for reviews, see Levine, 1991; Zambetti and Levine, 1993; Levine, 1997). Therefore, loss of p53 cell cycle checkpoint and subsequent loss of genomic stability in human cells has been regarded as the most important cause of human cancer.

Recent experiments suggested that p53 can suppress tumor growth and progression by induction of apoptosis (Symonds *et al.*, 1994; Gottlieb and Oren, 1996). Apoptosis induced by agents such as irradiation, oncogene activation and cytokine deprivation is p53-dependent (for review, see Amundson *et al.*, 1998). Ectopic expression of wild-type *p53* can induce apoptosis in various cell lines. On the other hand, inactivation of *p53* by either mutations or DNA tumor virus oncoproteins (see Section 1.6.1) can cause resistance to apoptosis induced by different apoptosis stimuli (Yonish-Rouach, 1991, 1993; Ryan *et al.*, 1993; Lowe, 1995; White, 1996; Levine, 1997; Amundson *et al.*, 1998; Bates and Vousden, 1999).

Several studies indicate that sequence-specific transactivation is a required function for p53-mediated apoptosis in some experimental systems (Attardi *et al.*, 1996; Sabbatini *et al.*, 1995). An increasing number of p53-responsive genes are associated with apoptotic pathways (Table 1.3).

bax is a pro-apoptotic gene that is up-regulated by *p53* in many systems undergoing *p53*-dependent apoptosis (Selvakumaran *et al.*, 1994; Miyashita *et al.*, 1994; Zhan *et al.*, 1994). A *p53*-

Table 1.3 Apoptosis-regulating genes transactivated or repressed by wild-type p53

Gene	*p53* effect	Reference
Pro-apoptotic		
bax	transactivation	Miyashita *et al.* (1995)
c-*fos*	transactivation	Elkeles *et al.* (1999)
DR5	transactivation	Wu *et al.* (1997)
fas	transactivation	Owen-Schaub *et al.*(1995)
IGF-BP3	transactivation	Buckbinder *et al.* (1995)
PAG608	transactivation	Israeli *et al.* (1997)
Anti-apoptotic		
bcl-2	repression	Miyashita *et al.* (1995)
IGF-IR	repression	Prisco *et al.* (1997)
MAP4	repression	Murphy *et al.* (1996)

response element has been identified in the bax promoter that mediates *p53*-induced transactivation (Miyashita *et al.*, 1995).

c-*fos* is a proto-oncogene that can induce apoptosis in a number of conditions (Section 1.5.2). Using the suppression subtractive hybridization differential screening technique, c-*fos* was identified as a target for transcriptional activation by p53 in cells undergoing p53-mediated apoptosis (Elkeles *et al.*, 1999). Overexpression of wild-type p53 can induce c-*fos* mRNA and protein both *in vitro* and *in vivo*. Interestingly, the basal c-*fos* promoter is not regulated by p53. Rather, p53 binds a distinct region within the first c-*fos* intron and transactivates c-*fos* promoter (Elkeles *et al.*, 1999). Therefore, activation of c-*fos* by p53 may be partially responsible for *p53*-induced apoptosis.

DR5/KILLER is a member of the TNFR family that is also induced by p53 (Wu *et al.*, 1997) or p53-independent genotoxic stress (Sheikh *et al.*, 1998). Interaction of DR5 with its ligand, TRAIL, can activate the cytoplasmic death domain of DR5, which in turn induces apoptosis through activation of the caspase cascade (Ashkenazi and Dixit, 1998).

fas is another mediator of apoptosis that can be upregulated by p53 in several cell types (Owen-Schaub *et al.*, 1995). Binding of FasL to Fas can induce apoptosis by caspase recruitment and activation (for review, see Ashkenazi and Dixit, 1998). Therefore, p53 can induce apoptosis by enhancing signal transduction through the FasL/Fas and caspase pathway.

bcl-2 (Miyashita *et al.*, 1994, 1995), *IGF-IR* (Prisco *et al.*, 1997), and *MAP*4 (Murphy *et al.*, 1996) are three anti-apoptotic proteins whose expression has been shown to be inhibited by p53, suggesting another mechanism by which p53 promotes apoptosis. This explains why, while transactivation of some apoptosis-inducing genes can lead to apoptosis, *p53* mutants lacking the wild-type sequence-specific transactivation function have also been shown to induce apoptosis in a cell type-specific manner (Haupt *et al.*, 1995b; Haupt and Oren, 1996).

p73 was originally identified as a candidate tumor suppressor gene in neuroblastomas. It encodes a protein with considerable homology with p53 tumor suppressor and transactivates genes containing p53-responsive elements, such as *WAF*1 (Kaghad *et al.*, 1997). Nevertheless, *p73* is different from *p53* in several aspects. For example, *p73* is not induced by DNA damage and its protein is not targeted for inactivation by viral oncoproteins, such as SV40 T antigen, adenovirus E1B and HPV E6 (Kaghad, 1997; Marin *et al.*, 1998). In addition, unlike *p53*, *p73* mutations are rare in human cancers (Mai *et al.*, 1998; Sunahara *et al.*, 1998; Han *et al.*, 1999; Tsao *et al.*, 1999). However, like p53, overexpression of *p73* in *p53*-null human cancer cells or in baby hamster kidney cells induced apoptosis (Jost *et al.*, 1997). The molecular mechanism for the pro-apoptotic function of *p73* remains to be elucidated.

1.7.2 *WAF*1/*Cip*1/*Sdi*1/*p21*

*WAF*1 is a gene that is directly transactivated by wild-type *p53* (El-Deiry *et al.*, 1993). It encodes a 21 kDa protein that functions as a cyclin-dependent kinase (cdk) inhibitor, and has been shown to block predominantly the G1-S phase cell cycle transition (Sherr and Roberts, 1995). Like *p53*, overexpression of *WAF*1 can suppress tumor growth (El-Deiry *et al.*, 1993), demonstrating that it is a tumor suppressor gene.

Although *WAF*1 plays an important role in *p53*-induced G1 arrest, its role in *p53*-induced apoptosis remains controversial. *WAF*1 has been shown to either promote (El-Deiry *et al.*, 1994; Kondo *et al.*, 1996a) or inhibit (Gorospe *et al.*, 1997) p53-induced apoptosis, depending on the apoptotic stimuli or cell lines used. For example, in one study, it was shown that *WAF*1 was activated during p53-dependent cisplatin-induced apoptosis of U87-MG glioma cells, and overexpression of *WAF*1 caused apoptosis (Kondo *et al.*, 1996a). However, in another study,

overexpression of *WAF*1 protected human melanoma cells against *p53*-mediated apoptosis (Gorospe *et al.*, 1997).

1.7.3 *RB*

RB is a gene that is inactivated by deletion or mutation in many different human cancer cell lines, including retinoblastoma, small-cell lung cancer, bladder cancer and osteosarcoma lines (for reviews, see Lee, 1991; Hamel *et al.*, 1993). Transfecting wild-type *RB* cDNA into tumor cell lines lacking functional RB can reverse the transformed phenotype (Huang *et al.*, 1988; Xu *et al.*, 1991; Antelman *et al.*, 1995), indicating that RB can act as a tumor suppressor. RB has the ability to suppress cell proliferation after its cell cycle-dependent phosphorylation. In G0, RB is hypophosphorylated and can bind to transcription factors such as E2F and inhibit transcriptional activation of cellular genes, such as c-*myc* and *fos*, by E2F. In proliferating cells, RB was phosphorylated in late G1 by cdk. Phosphorylation of RB releases E2F, which can induce entry into S phase by induction of cellular genes associated with DNA replication or cell proliferation, such as c-myc, cdc2, DNA polymerase-α, thymidine kinase, and dihydrofolate reductase.

Apart from the role of RB in tumorigenesis and cell proliferation, several studies demonstrated an additional function for RB: regulating apoptosis. RB deficient mice have been shown to exhibit extensive apoptosis in their lens and nervous system (Clarke *et al.*, 1992; Morgenbesser *et al.*, 1994; Macleod *et al.*, 1996). On the other hand, overexpression of RB can inhibit apoptosis induced by ceramide, IFN-γ, irradiation, and p53 (Haupt *et al.*, 1995; McConkey *et al.*, 1996; Ishii *et al.*, 1997). Furthermore, RB is cleaved and inactivated by caspase-3 during apoptosis induced by TNF-α and Fas, suggesting that RB degradation contributes to the activation of apoptosis. RB may inhibit apoptosis by inhibiting the pro-apoptotic function of p53 through interaction with Mdm2 (Hsieh *et*

al., 1999).

1.7.4 *p16* and *p19ARF*

Several experimental results indicate that *p16* is a tumor suppressor and involved in cancer development: 1) *p16* deletions or mutations have been found in a variety of human cancers; in addition, inactivation of p16 expression by hypermethylation of its promoter has also been found in colon, non-small cell lung and head and neck cancers, and high-grade glioma; 2) *p16* germline mutations have been found in melanoma-prone kindreds; 3) overexpression of p16 inhibits tumor growth; and 4) *p16*-null mice develop cancer and are susceptible to DNA-damage reagents (for review, see Kamb, 1998). Overexpression of p16 can induce apoptosis in various cell systems (Urashima *et al.*, 1997; Frizelle *et al.*, 1998; Schreiber *et al.*, 1999). It is postulated that p16 induces apoptosis by inhibiting the function of anti-apoptotic RB (Frizelle *et al.*, 1998).

p19ARF is a tumor suppressor gene transcribed by alternative splicing from genomic sequence shared with the *p16* transcript but encoding a different open reading frame (Haber, 1997). Like p16, p19ARF exerts its tumor suppressor function by inducing G1 arrest. However, unlike p16, p19ARF cannot inhibit cdk activity. Instead, it blocks inhibition of p53 by Mdm2 (Pomerantz *et al.*, 1998). Consistent with the link between p19ARF and p53, p19ARF can induce apoptosis through a p53-dependent pathway (Radfar *et al.*, 1998).

1.8 Functions of BAG-1

BAG-1 is regarded to be a multifunctional protein that regulates several cellular processes, including apoptosis, signal transduction, hormone response, growth arrest, and protein folding (Fig. 1.3).

1.8.1 Anti-apoptosis

BAG-1 was a novel protein first cloned from mouse cells by screening proteins interacting with the anti-apoptotic protein, Bcl-2. (Takayama *et al.*, 1995). Cotransfection with mouse BAG-1 (mBAG-1) was shown to increase Bcl-2-mediated protection from apoptosis triggered by several stimuli, including staurosporine, anti-Fas antibody and cytolytic T cells (Takayama *et al.*, 1995). In addition, overexpression of BAG-1 alone in mouse 3T3 fibroblasts and lymphocyte cells can prolong cell survival following apoptotic stimulus treatment or cytokine withdrawal (Takayama *et al.*, 1995; Clevenger *et al.*, 1997). Recently, mouse BAG-1 was shown to bind the plasma membrane-associated receptors for hepatocyte growth factor (HGF) and PDGF and enhance their inhibition of apoptosis (Bardelli *et al.*, 1996).

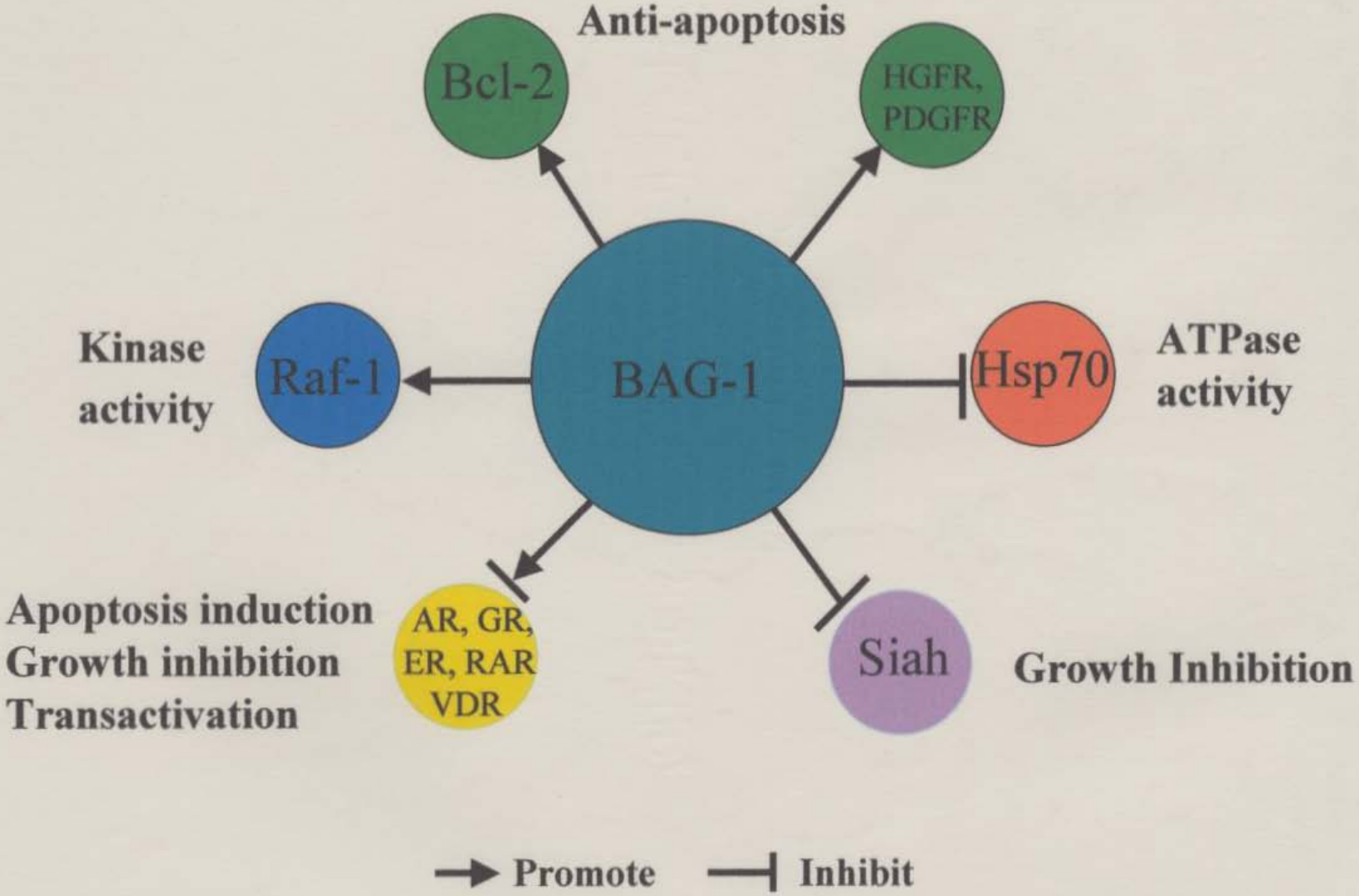
1.8.2 Signal transduction pathways

BAG-1 was reported to interact with serine/threonine-specific protein kinase, Raf-1, *in vitro* and *in vivo* and increase its kinase activity through a Ras-independent mechanism (Wang *et al.*, 1996). Raf-1 is a mitogen-activated protein (MAP) kinase kinase (MAPKK) that plays a central role in the Ras signal transduction pathway in response to receptor tyrosine kinases, such as EGF receptor (Park, 1998). Therefore, BAG-1 may play a role in signal transduction by phosphorylating and activating Raf-1.

1.8.3 Regulation of hormone receptor function

Recently, human BAG-1 was shown to be identical to a protein, RAP46, which binds to many members of the nuclear steroid hormone family receptors including estrogen receptor (ER),

Fig. 1.3. Interaction and modulation of cellular proteins by BAG-1 (see text for details).


Anti-apoptosis
Bcl-2
HGFR,
PDGFR
Kinase
activity
Raf-1
BAG-1
Hsp70
ATPase
activity
Apoptosis induction
Growth inhibition
Transactivation
AR, GR,
ER, RAR
VDR
Siah
Growth Inhibition
Promote
Inhibit

glucocorticoid receptor (GR), progesterone receptor (PR), androgen receptor (AR), thyroid receptor (TR), and retinoic acid receptor (RAR) (Zeiner and Gehring, 1995; Liu *et al.*, 1998; Yang *et al.*, 1998). Binding of BAG-1 protein to AR can enhance the ability of AR to transactivate reporter gene plasmids containing an androgen response element (ARE) (Froesch *et al.*, 1998). On the other hand, binding of BAG-1 to GR and RAR can inhibit their ability to transactivate other genes and induce apoptosis (Liu *et al.*, 1998; Kullmann *et al.*, 1998). Thus, elevated BAG-1 level in cancer cells (see Section 4) potentially contributes to their resistance to androgen, glucocorticoid and retinoid response by inhibiting the function of their receptors.

1.8.4 Inhibition of growth arrest

BAG-1 also has been shown to bind to p53-downstream human homologs of Drosophila seven in absentia (sina) proteins that are called SINA-homologous (Siah) proteins. In cotransfection experiments, wild-type BAG-1 but not a C-terminal deletion mutant of BAG-1, can abolish Siah-induced growth arrest in 293 and GM701 cells (Matsuzawa *et al.*, 1998). In addition, BAG-1 can prevent growth arrest following UV-irradiation-induced genotoxic injury without interfering with accumulation of p53 protein or activation of WAF1, suggesting that BAG-1 can suppress cell-cycle arrest by inhibiting p53-downstream Siah function (Matsuzawa *et al.*, 1998).

1.8.5 Chaperone cofactor

BAG-1 was found to bind the ATPase domain of Hsp70/Hsc70 both *in vitro* and *in vivo* through the BAG-1 C-terminal domain (Takayama *et al.*, 1997; Zeiner and Gehring, 1995; Hohfeld and Jentsch, 1997). Furthermore, BAG-1 can bind other proteins such as c-Jun through Hsp70 (Zeiner and Gehring, 1995), indicating that BAG-1 may act as an Hsp70 chaperone cofactor to regulate the

function of other proteins. Binding of BAG-1 to Hsp70/Hsc70 can either inhibit Hsp/Hsc70-mediated *in vitro* refolding of an unfolded protein substrate (Takayama *et al.*, 1997; Zeiner and Gehring, 1995) or cooperate with Hsp40 to stimulate the steady-state ATP hydrolysis activity of Hsc70. These results indicate that the observed anti-apoptotic function of BAG-1 may be exerted through modulation of Hsc70 chaperone activity.

1.9 Purpose of this study

To isolate the factors regulating the expression of JC virus (JCV), a protein called K1, which can bind to the NF1-binding site in the JCV enhancer and transactivate its promoter, was isolated from mouse P19 embryonal carcinoma cell cDNA library (Kumar *et al.*, unpublished data). A homology search in Genbank found that the amino acid sequence of K1 was identical to that of mouse BAG-1, an anti-apoptotic protein (Takayama *et al.*, 1995). Since dystegulation of Bcl-2 was found to be involved in carcinogenesis (Tsujimoto and Croce., 1986; Hollstein *et al.*, 1991; McDonnell *et al.*, 1992; Halder*et al.*, 1994; Ikegaki *et al.*, 1994; Reed, 1994; Liang *et al.*, 1995; Sinicrope *et al.*, 1995), my hypothesis was that BAG-1, a Bcl-2-interacting protein, also plays a role in human cancer.

Because human BAG-1 was not cloned when I started my PhD program several years ago, in order to study the role of BAG-1 in human cancer, the first purpose of my study was to clone and analyze the human homolog of mouse BAG-1 cDNA. Second, since mouse BAG-1 was originally identified as an anti-apoptotic protein (Takayama *et al.*, 1995), I tried to examine whether human BAG-1 plays similar role in apoptosis. Third, I had previously developed an *in vitro* cell model of multistep cervical oncogenesis (Yang *et al.*, 1996). To examine whether BAG-1 and other apoptosis-regulating proteins are involved in the different stages of cervical carcinogenesis, I assayed expression of pro- and anti-apoptotic proteins and its correlation with the sensitivity of different cervical cells

to apoptosis. Fourth, it had been found that enhanced expression of anti-apoptotic genes, such as *bcl-2*, is involved in human cancers (Tsujimoto and Croce., 1986; Hollstein *et al.*, 1991; McDonnell *et al.*, 1992; Halder *et al.*, 1994; Ikegaki *et al.*, 1994; Reed, 1994; Liang *et al.*, 1995; Sinicrope *et al.*, 1995). To study the possible role of BAG-1 in human cancer, BAG-1 RNA and protein levels were examined in cell lines and tissues of two common cancers in women, breast and cervical cancer. Fifth, many apoptosis-regulating genes are transcriptionally transactivated or repressed by key molecules for regulating apoptosis and oncogenesis, such as p53 (Selvakumaran *et al.*, 1994; Miyashita *et al.*, 1994; Zhan *et al.*, 1994; Miyashita *et al.*, 1995). Therefore, the last purpose of my study was to clone the BAG-1 promoter and test whether it can be regulated by other transcription factors.

SECTION 2

CLONING AND ANALYSIS OF HUMAN BAG-1

CHAPTER 2

HUMAN BAG-1 cDNA CLONING, SEQUENCE ANALYSIS AND RNA EXPRESSION

2.1 INTRODUCTION

At the beginning of my studies, a mouse BAG-1 cDNA was cloned in our lab. Preliminary data indicated that BAG-1 plays an important role in the transcription of JC virus, a human DNA tumor virus (Kumar, unpublished data). To study the role of human BAG-1 in apoptosis and carcinogenesis, I isolated a human BAG-1 cDNA clone by screening a human cervical cell cDNA library using mouse BAG-1 cDNA as a probe. The sequence of BAG-1 cDNA was analyzed. Tissue-specific expression of BAG-1 RNA was also examined.

2.2 MATERIALS AND METHODS

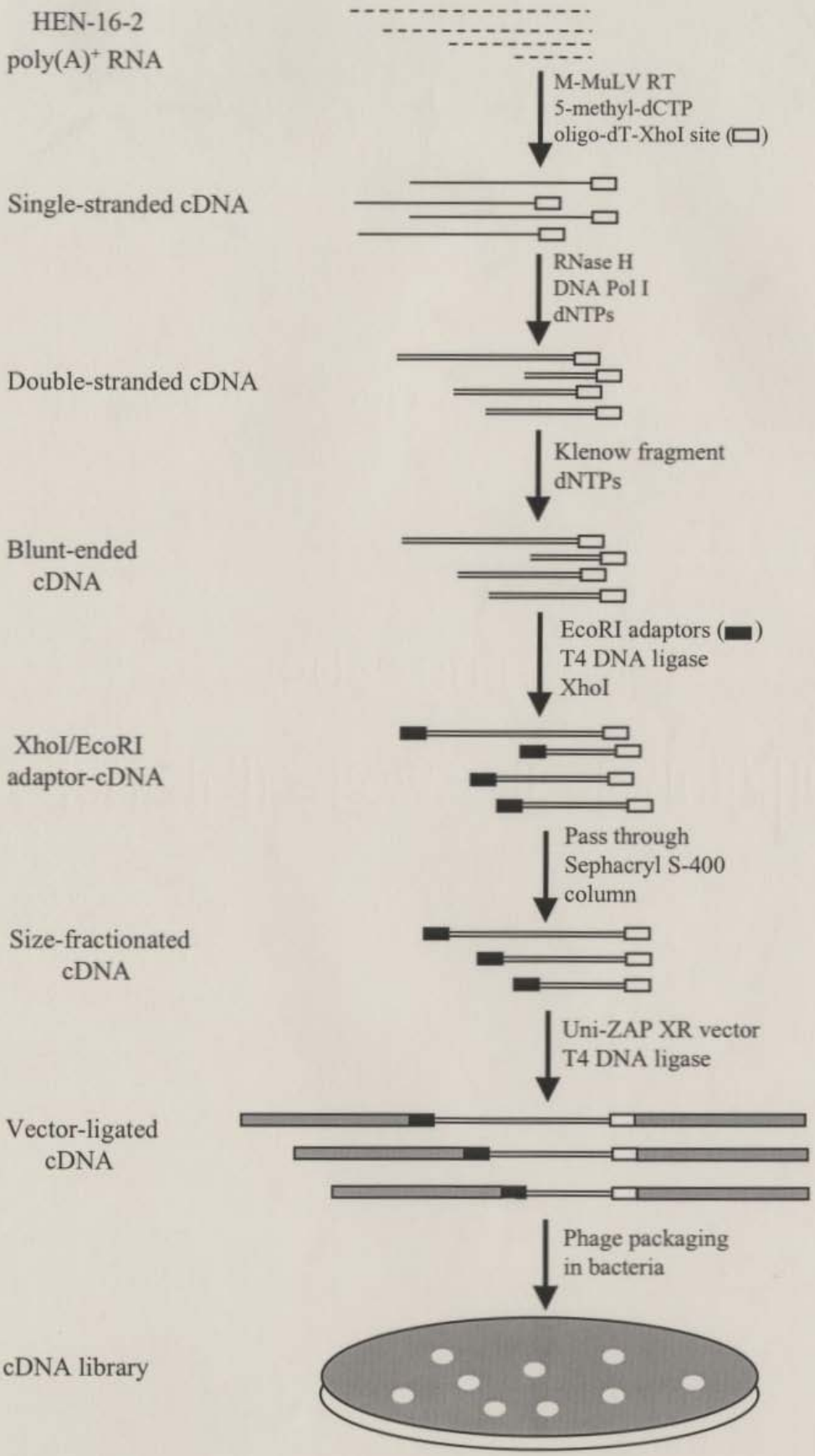
2.2.1 Construction of human cervical cell cDNA library

The λ phage ZAP-cDNA synthesis kit (Stratagene) was used for constructing an HEN-16-2 HPV16-immortalized human cervical cell cDNA library. The procedure is illustrated in Fig. 2.1 and described as follows:

1) Extraction of poly $(A)^+$ mRNA

Total RNA was extracted from HEN-16-2 cultured to 70% confluence using the CsCl gradient centrifugation method (Sambrook et al., 1989). Poly$(A)^+$ mRNA was extracted by passing total RNA

Fig. 2.1 Flow chart of cDNA library construction. See the text for description.


HEN-16-2
poly(A)+ RNA
M-MuLV RT
5-methyl-dCTP
oligo-dT-XhoI site
Single-stranded cDNA
RNase H
DNA Pol I
dNTPs
Double-stranded cDNA
Klenow fragment
dNTPs
Blunt-ended
cDNA
EcoRI adaptors
T4 DNA ligase
XhoI
XhoI/EcoRI
adaptor-cDNA
Pass through
Sephacryl S-400
column
Size-fractionated
cDNA
Uni-ZAP XR vector
T4 DNA ligase
Vector-ligated
cDNA
Phage packaging
in bacteria
cDNA library

twice through oligo(dT)-cellulose columns, washing the columns with high salt buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 M NaCl) and low salt buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl), and eluting poly(A)$^+$ mRNA from the columns with 65 ^{0}C elution buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) included in the mRNA purification kit (Pharmacia). The purity and integrity of the poly(A)$^+$ mRNA was examined by electrophoresis on agarose gels.

2) First strand cDNA synthesis

About 5 μg of poly (A)$^+$ RNA were incubated at 37 °C for 1 h in a premixed solution containing 1× first strand buffer, 10 mM DTT, 0.6 mM first strand methyl-nucleotide mixture, 56 ng/μl of linker-primer, 0.8 U/μl of RNase Block, and 1 U M-MuLV reverse transcriptase. As a control, 5 μCi α-[^{32}P]-dATP (Amersham) were mixed with 1/10 volume of the above reaction solution and incubated in the same conditions. The nonradioactive first stand reaction mixture was placed on ice after incubation until it was used for second strand synthesis. The radiolabelled reaction mixture was used for gel electrophoretic analysis of first strand synthesis products on a 1% alkaline agarose gel.

3) Second strand cDNA synthesis

The nonradioactive first strand reaction mixure was incubated in 1× second strand buffer, 4 mM DTT, 0.15 mM second strand nucleotide mixture, 20 μCi α-[^{32}P]-dATP, 4.5 U RNase H, and 100 U DNA polymerase at 16 °C for 2.5 h. The DNA was phenol/chloroform-extracted, precipitated and resuspended in sterile water. Then, 3 μl were analyzed for second strand synthesis by gel electrophoresis on a 1% alkaline agarose gel.

4) Preparation of cDNAs and their ligation into vector

To blunt-end the cDNA termini, the second strand reaction mixture was incubated in 1× Buffer#3 (supplied in the Stratagene kit), 125 μM dNTP mix and 2.5 U Klenow fragment of DNA polymerase I at 37 °C for 30 min. The blunt-ended cDNA was ligated with XhoI/EcoRI adaptor by incubation in ligation buffer (1× Buffer #3, 0.28 μg/μl XhoI/EcoRI adaptor, 1 mM γ-ATP, and 4 U T4 DNA ligase) at 8 °C overnight. The ligase was heat-inactivated and the EcoRI ends on the cDNA were phosphorylated by incubating in kinase buffer (1× Buffer#3, 2 mM γ-ATP and 10 U T4 polynucleotide kinase) at 37 °C for 30 min. XhoI digestion was then performed by incubation of the kinase reaction mix with 120 U XhoI in 1× XhoI buffer.

For size fractionation of cDNA, it was first loaded on a Sephacryl S-400 spin column and the first fraction was collected by centrifuging the column in a tube at 400× g for 2 min. The second and third fraction were collected by loading 60 μl of 1× STE buffer on the column and centrifuging, as for fraction 1. The size of cDNA in each fraction was examined by electrophoresis of 1/10 of the fractions on a 5% nondenaturing acrylamide gel. The cDNA was quantified by comparing the fluorescence intensity of 1 μl of each fraction with those of DNA standards on an ethidium bromide-containing plate irradiated by short wavelength UV light.

To ligate the cDNA into Uni-ZAP XR λ phage vector arms, approximately 100 ng of cDNA were incubated overnight at 12 °C in ligation mixture (1× Buffer #3, 1 mM γ-ATP, 1 μg/μl Uni-ZAP XR vector, and 2 U T4 DNA ligase).

5) Packaging recombinant cDNA into phage

GIGAPACK II packaging extract (Stratagene) was used to package recombinant cDNA-vector arms. In brief, 1 μl of ligated cDNA-vector arms mixed with Freeze-Thaw extract (Stratagene) was placed on ice, 15 μl of Sonic extract (Stratagene) was added, the mixture was incubated at room

temperature for 2 h. 500 µl of SM buffer and 20 µl of chloroform were then added into the tube and the supernatant containing the packaged cDNA was collected.

6) Plating and titrating the recombinant cDNA library

One µl of the final packaged reaction product was first mixed with 200 µl of XL1-Blue MRF' cells (OD_{600}=0.5). After incubation at 37 °C for 15 min, 3 ml of top agar, 15 µl of 0.5M IPTG and 50 µl of X-gal (250 mg/ml) were added and the mixture was plated immediately onto the NZY plates, which were further incubated at 37 °C for 6-8 h. Blue plaques were considered to be background, while white plaques were considered as recombinant plaques. The number of white plaques was used to calculate the titer of cDNA library.

7) Amplification of the cDNA library

Aliquots of packaged mixture or library suspension containing approximately 50,000 phage forming unit (pfu) were mixed with 600 µl of host bacteria at OD_{600} = 0.5 in Falcon 2059 tubes (Fisher). After incubation of the tubes for 15 min at 37 °C, 6.5 ml of melted top agar (cooled to approximately 48 °C before use) was added into the tubes. The mixture was immediately spread evenly onto 150-mm plates containing bottom agar. The plates were then incubated at 37 °C for 6-8 h to achieve plaques in the 1-2 mm range, overlaid with 10 ml SM buffer (100 mM NaCl, 20 mM $MgSO_4$, 50 mM Tris-HCl, pH 8.0, and 0.01 % gelatin), and incubated at 4 °C overnight. The phage-containing SM buffer was removed from the plates, collected, titrated, as above, and stored at 0.3% chloroform at 4 °C.

2.2.2 Screening the cDNA library

To screen the cDNA library, mixtures of about 5×10^4 pfu phages, 600 µl of XL1-blue cells (OD_{600} = 0.5) and 6.5 ml of top agarose were overlaid on 150-mm NZY plates. The plates were incubated at 37 °C for 8 h and chilled for 2 h at 4 °C. The plaques were then partially transferred for 2 min to nitrocellulose membranes, denatured in 1.5 M NaCl and 0.5 M NaOH for 2 min, neutralized in 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) for 5 min, and rinsed in 0.2 M Tris-HCl, pH 7.5, and 2× SSC for 30 sec. The membranes were then baked at 80 °C for 2 h, prehybridized at 60 °C in QuickHyb hybridization buffer (Amersham) for 0.5 hr, and hybridized QuickHyb buffer containing ^{32}P-labelled mouse BAG-1 cDNA probes (2×10^6 cpm/ml hybridization buffer) at 60°C for 2 h. The membranes were washed twice with 2× SSC and 0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min and twice with 0.1× SSC and 0.1% SDS at 60 °C for 10 min. Then, the membranes were sealed in clean plastic bags and exposed to Kodak XAR film overnight at -80 °C. Putative positive clones were identified on the films and aligned with the plates. One cm diameter disks of bacteria and phage lysates surrounding the positive clones were scraped off the plates with tooth picks and transferred to tubes containing 1 ml SM buffer and 20 µl of chloroform. To titrate phages, 5 µl of vortexed mixtures were spread on 100 mm NZY plates. The procedure used for the first screening was repeated until all plated plaques were aligned with positive signals for each clone.

2.2.3 Recovery of recombinant cDNA phagemids

The cDNA inserts in the final positive clones were then excised from the Uni-ZAP XR phage vector using the ExAssist/SOLR system (Stratagene). In brief, single plaques from agar plates were transferred to sterile microfuge tubes containing 500 µl of SM buffer and 20 µl of chloroform. Tubes were incubated at 4 °C overnight, and mixtures of 100 µl phages, 200 µl of XL1-Blue bacteria

(OD_{600} = 1.0) and 1 µl of ExAssist filamentous helper phage were incubated at 37 °C for 15 min. Three ml of 2× YT media were then added and the mixtures were incubated in 50 ml conical tubes at 37 °C for 2.5 h with shaking. The tubes were heated at 70 °C for 20 min and centrifuged for 15 min at 4,000× g. One µl of supernatants containing the packaged phagemids were then incubated with 200 µl of SOLR bacteria at 37 °C for 15 min and plated on LB-ampicillin (50 µg/ml) plates. The plates were incubated at 37 °C overnight and the colonies containing the pBluescript double-stranded cDNA phagemid were scraped from the plates with tooth picks and grown in LB-ampicilin (50 µg/ml) medium at 37 °C overnight. The phagemids were purified from the bacteria using a plasmid purification kit (Qiagen).

2.2.4 Testing cDNA clones

To test positive cDNA clones, dot and Northern blot analysis were performed. For dot blot analysis, approximately 100 ng of phagemid DNA were applied to nitrocellulose membrane. The membranes were baked at 80 °C for 1 hr, prehybridized, and hybridized with ^{32}P-labelled mouse BAG-1 probe (2×10^6 cpm/ml hybridization buffer) using the same conditions as for cDNA library screening. For Northern blot analysis, approximately 20 µg total RNA were size-fractionated in denaturing 1% agarose gels and transferred to Hybond nitrocellulose membrane (Amersham). The blots were hybridized in QuickHyb hybridization buffer containing ^{32}P-labelled putative human BAG-1 cDNA probes (2×10^6 cpm/ml hybridization buffer) at 65 °C for 1 h. The blots were then washed twice with 2× SSC and 0.1% SDS at room temperature for 15 min and twice with 0.1× SSC and 0.1% SDS at 60 °C for 10 min. Then, the membranes were exposed to Kodak XAR film overnight. For the expression of β-actin internal control RNA, membranes were subsequently stripped, reprobed with ^{32}P-labelled β-actin cDNA (5×10^5 cpm/ml hybridization buffer), washed

and exposed to film, as described above.

2.2.5 Sequencing and sequence analysis of cDNA clones

The positive cDNAs confirmed by dot and Northern blot analysis were sequenced from both 5'- and 3'-ends using a Sequenase Version 2.0 kit (USB). Sequence homology searches were performed with the BLAST program (NCBI) through internet (http://www.ncbi.nih.gov). An analysis for α-helical structure was performed using a protein secondary structure analysis program provided by Baylor College of Medicine (http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predic.html).

2.3 RESULTS

2.3.1 cDNA library construction

The first and second strand cDNA was synthesized and ranged from 0.5 kb to 8 kb (Fig. 2.2), which includes bands with the sizes of almost all the mRNA species expressed in eukaryotic cells. While the primary cDNA had a total titer of 4×10^6 pfu, the amplified cDNA library had a titer of 5×10^{10} pfu/ml. The insert sizes of 20 plaques examined by PCR in the cDNA library ranged from 0.5 kb to 3.5 kb.

2.3.2 Screening and testing BAG-1 cDNA clones

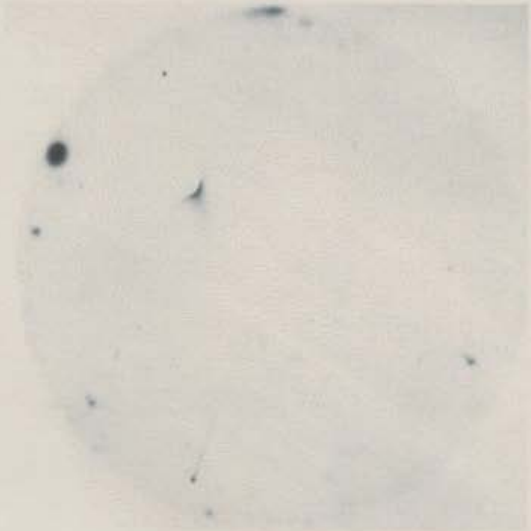
After primary screening of 5×10^5 pfu, 22 positive phage clones were identified, recovered and subjected to further screening. After three rounds of screening, seven positive clones were identified and isolated. An example of a screening result is shown in Fig. 2.3. In general, after the third screening, all plaques were separated from each other and hybridized strongly with the mouse BAG-

Fig. 2.2 Alkaline agarose gel analysis of first and second strand cDNA synthesis. Five µl of reaction mixture from radiolabelled cDNA synthesis were size-fractionated on 1% alkaline agarose gels, which were subsequently vacuum dried at 80 °C, before exposure to X-ray film at -80 °C overnight. Molecular weights in kb are shown on the left.

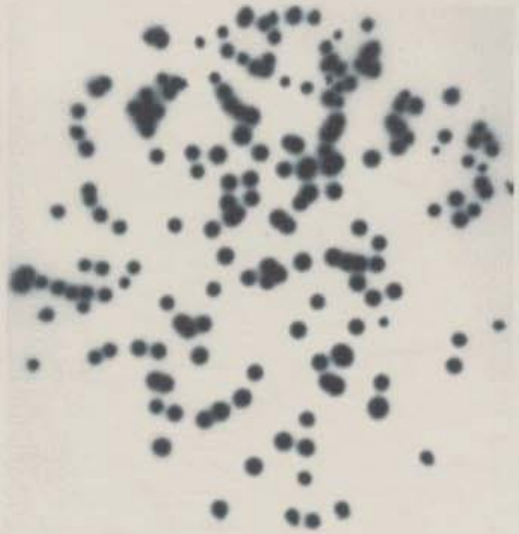
kb
5.0
4.0
3.0
2.0
1.0
0.5
Marker
First strand
Second strand

Fig. 2.3 cDNA library screening. 5×10^4 pfu phages were plated, incubated at 37 °C for 6-8 h, and transferred to nitrocellulose membrane. The membranes were hybridized with ^{32}P-labelled mouse BAG-1 cDNA probe in QuickHyb hybridization buffer at 60 °C for 2 h and washed before exposure to X-ray film at -80 °C overnight. The putative positive clones and their surrounding plaques were taken from the plates and subjected to similar second and third screening.

1st screening



2nd screening



3rd screening

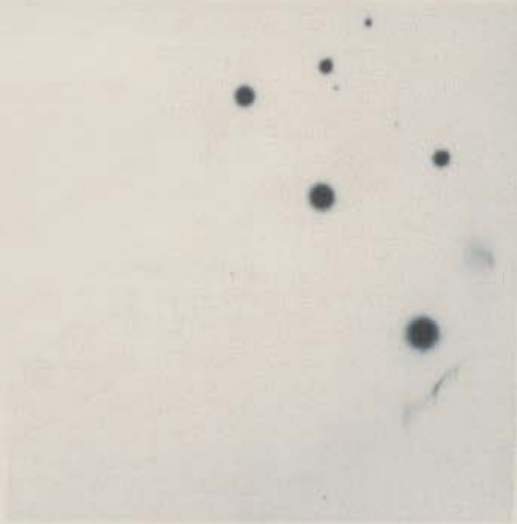
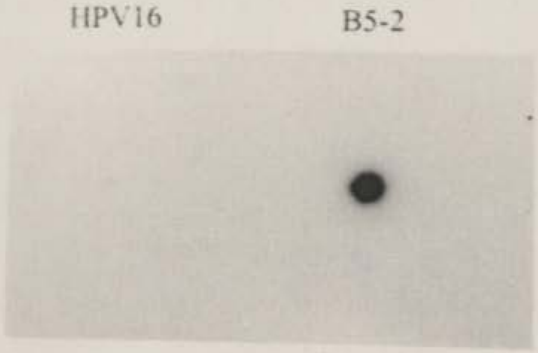
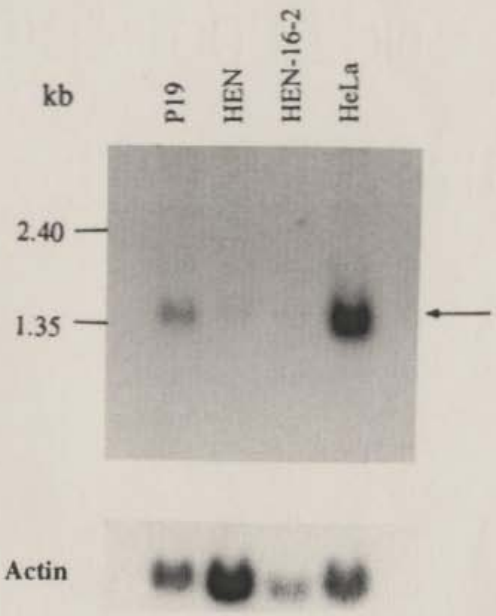


Fig. 2.4 Dot blot analysis of B5-2 cDNA and Northern blot analysis of BAG-1 RNA. **A.** Dot blot analysis of homology of B5-2 to mouse BAG-1 cDNA. 100 ng of HPV16 and B5-2 plasmid DNA were spotted on nitrocellulose membrane, which was subsequently dried, hybridized with ^{32}P-labelled mouse BAG-1 cDNA probe and washed before exposure to X-ray film at -80 °C overnight. **B.** Northern blot analysis of BAG-1 RNA. 20 μg of total RNA from mouse (P19), and human (HEN, HEN-16-2 and HeLa) cells were size-fractionated on a 1% formaldehyde agarose gel, transferred to Hybond nylon membrane, and hybridized with ^{32}P-labelled B5-2 cDNA probe in QuickHyb hybridization buffer at 60 °C overnight. The membrane was exposed to X-ray film at -80 °C overnight. Actin was used as internal control. Size marker are shown on the left and BAG-1 RNA is indicated on the right.

A



B



1 cDNA probe.

Each positive clone was then tested for homology with mouse BAG-1. Fig. 2.4 shows results of dot and Northern blot analysis for the longest (1.3 kb) cDNA clone, B5-2. Mouse BAG-1 cDNA probes hybridized strongly with B5-2 cDNA, but not with the negative control HPV16 DNA (Panel A). When B5-2 cDNA was used as a probe, a single 1.35 kb transcript was detected in the RNA from all four cell types, mouse P19 embryonic carcinoma cells, HEN human endocervical primary cells, HEN-16-2 HPV16-immortalized human endocervical cells and HeLa human cervical carcinoma cells (Panel B). The same results were obtained when mouse radiolabelled BAG-1 cDNA was used to probe the same blot after it was stripped, indicating that the B5-2 cDNA cloned is the human BAG-1 cDNA in full-length.

2.3.3 Sequences and functional domains of BAG-1 cDNA

Sequencing of B5-2 cDNA showed that it has a sequence of 1,312 bp containing an open reading frame (from the first ATG start to nucleotide position 1098 TGA stop codons) encoding a protein of 274 amino acids (Fig. 2.5). Human BAG-1 mRNA was also found to be translated from an upstream CTG start codon, resulting in a full-length 345 amino acid BAG-1 called p50 (see Chapter 3 for details). In addition, a polyadenylation signal, AATGAA, was also identified 44 nucleotides upstream of a poly(A) tail (Fig. 2.5). A search for sequences homologous to B5-2 human BAG-1 cDNA in the GenBank and EMBL databases revealed 100% identity to RAP46, a gene encoding a hormone receptor-binding protein that was isolated from a human liver cDNA library (Zeiner and Gehring, 1995). Comparison of amino acid sequences of human and mouse BAG-1 revealed 75% identity and 84% similarity. While the sequences of C-terminal amino acids were nearly identical in human and mouse BAG-1 cDNA, those of N-terminal were found to be less conserved (Fig. 2.6).

Fig. 2.5 Nucleotide and amino acid sequences of human BAG-1. The positions of the 1.3 kb cDNA nucleotide sequence and the deduced 345 amino acid sequence are shown on the left. ATG (M) or CTG (L) translation start codons and termination codon (*) are in boldface. The polyadylation site signal sequence (AATGAA) is boldfaced and underlined.

```
   1                                                                  TCG
   4 GGCGGGGTTGTGAGACGCCGCGCTCAGCTTCCATCGCTGGGCGGTCAACAAGTGCGGGC
  63 CTG GCT CAG CGC GGG GGG GCG CGG AGA CCG CGA GGC GAC CGG GAG
   1  L   A   Q   R   G   G   A   R   R   P   R   G   D   R   E
 108 CGG CTG GGT TCC CGG CTG CGC GCC CTT CGG CCA GGC CGG GAG CCG
  16   R   L   G   S   R   L   R   A   L   R   P   G   R   E   P
 153 CGC CAG TCG GAG CCC CCG GCC CAG CGT GGT CCG CCT CCC TCT CGG
  31   R   Q   S   E   P   P   A   Q   R   G   P   P   P   S   R
 198 CGT CCA CCT GCC CGG AGT ACT GCC AGC GGG CAT GAC CGA CCC ACC
  46   R   P   P   A   R   S   T   A   S   G   H   D   R   P   T
 243 AGG GGC GCC GCC GCC GGC GCT CGC AGG CCG CGG ATG AAG AAG AAA
  61   R   G   A   A   A   G   A   R   R   P   R   M   K   K   K
 288 ACC CGG CGC CGC TCG ACC CGG AGC GAG GAG TTG ACC CGG AGC GAG
  76   T   R   R   R   S   T   R   S   E   E   L   T   R   S   E
 333 GAG TTG ACC CTG AGT GAG GAA GCG ACC TGG AGT GAA GAG GCG ACC
  91   E   L   T   L   S   E   E   A   T   W   S   E   E   A   T
 378 CAG AGT GAG GAG GCG ACC CAG GGC GAA GAG ATG AAT CGG AGC CAG
 106   Q   S   E   E   A   T   Q   G   E   E   M   N   R   S   Q
 423 GAG GTG ACC CGG GAC GAG GAG TCG ACC CGG AGC GAG GAG GTG ACC
 121   E   V   T   R   D   E   E   S   T   R   S   E   E   V   T
 468 AGG GAG GAA ATG GCG GCA GCT GGG CTC ACC GTG ACT GTC ACC CAC
 136   R   E   E   M   A   A   A   G   L   T   V   T   V   T   H
 513 AGC AAT GAG AAG CAC GAC CTT CAT GTT ACC TCC CAG CAG GGC AGC
 151   S   N   E   K   H   D   L   H   V   T   S   Q   Q   G   S
 558 AGT GAA CCA GTT GTC CAA GAC CTG GCC CAG GTT GTT GAA GAG GTC
 166   S   E   P   V   V   Q   D   L   A   Q   V   V   E   E   V
 603 ATA GGG GTT CCA CAG TCT TTT CAG AAA CTC ATA TTT AAG GGA AAA
 181   I   G   V   P   Q   S   F   Q   K   L   I   F   K   G   K
 648 TCT CTG AAG GAA ATG GAA ACA CCG TTG TCA GCA CTT GGA ATA CAA
 196   S   L   K   E   M   E   T   P   L   S   A   L   G   I   Q
 693 GAT GGT TGC CGG GTC ATG TTA ATT GGG AAA AAG AAC AGT CCA CAG
 211   D   G   C   R   V   M   L   I   G   K   K   N   S   P   Q
 738 GAA GAG GTT GAA CTA AAG AAG TTG AAA CAT TTG GAG AAG TCT GTG
 226   E   E   V   E   L   K   K   L   K   H   L   E   K   S   V
 783 GAG AAG ATA GCT GAC CAG CTG GAA GAG TTG AAT AAA GAG CTT ACT
 241   E   K   I   A   D   Q   L   E   E   L   N   K   E   L   T
 828 GGA ATC CAG CAG GGT TTT CTG CCC AAG GAT TTG CAA GCT GAA GCT
 256   G   I   Q   Q   G   F   L   P   K   D   L   Q   A   E   A
 873 CTC TGC AAA CTT GAT AGG AGA GTA AAA GCC ACA ATA GAG CAG TTT
 271   L   C   K   L   D   R   R   V   K   A   T   I   E   Q   F
 918 ATG AAG ATC TTG GAG GAG ATT GAC ACA CTG ATC CTG CCA GAA AAT
 286   M   K   I   L   E   E   I   D   T   L   I   L   P   E   N
 963 TTC AAA GAC AGT AGA TTG AAA AGG AAA GGC TTG GTA AAA AAG GTT
 301   F   K   D   S   R   L   K   R   K   G   L   V   K   K   V
1008 CAG GCA TTC CTA GCC GAG TGT GAC ACA GTG GAG CAG AAC ATC TGC
 316   Q   A   F   L   A   E   C   D   T   V   E   Q   N   I   C
1053 CAG GAG ACT GAG CGG CTG CAG TCT ACA AAC TTT GCC CTG GCC GAG
 331   Q   E   T   E   R   L   Q   S   T   N   F   A   L   A   E
1098 TGAGGTGTAGCAGAAAAAGGCTGTGCTGCCCTGAAGAATGGCGCCACAGCTCTGCCGTC
      *
1157 TCTGGAGCGGAATTTACCTGTTTCTTCAGGGCTGCTGGGGGCAACTGGCCATTTGCCAA
1216 TTTTCCTACTCTCACACTGGTTCTCAATGAAAAATAGTGTCTTTGTGATTTTGAGTAAA
1275 GCTCCTATCTGTTTTCTAAAAAAAAAAAAAAAAAAAA
```

Fig. 2.6 Comparison of amino acid sequences of human (h) and mouse (m) BAG-1. Identities are indicated by vertical lines and similarities by two dots. The amino acids M or L encoding the potential in-frame translation initiation start codons are in boldface. The potential NLS is boxed. Numbers on the left indicate amino acid positions.

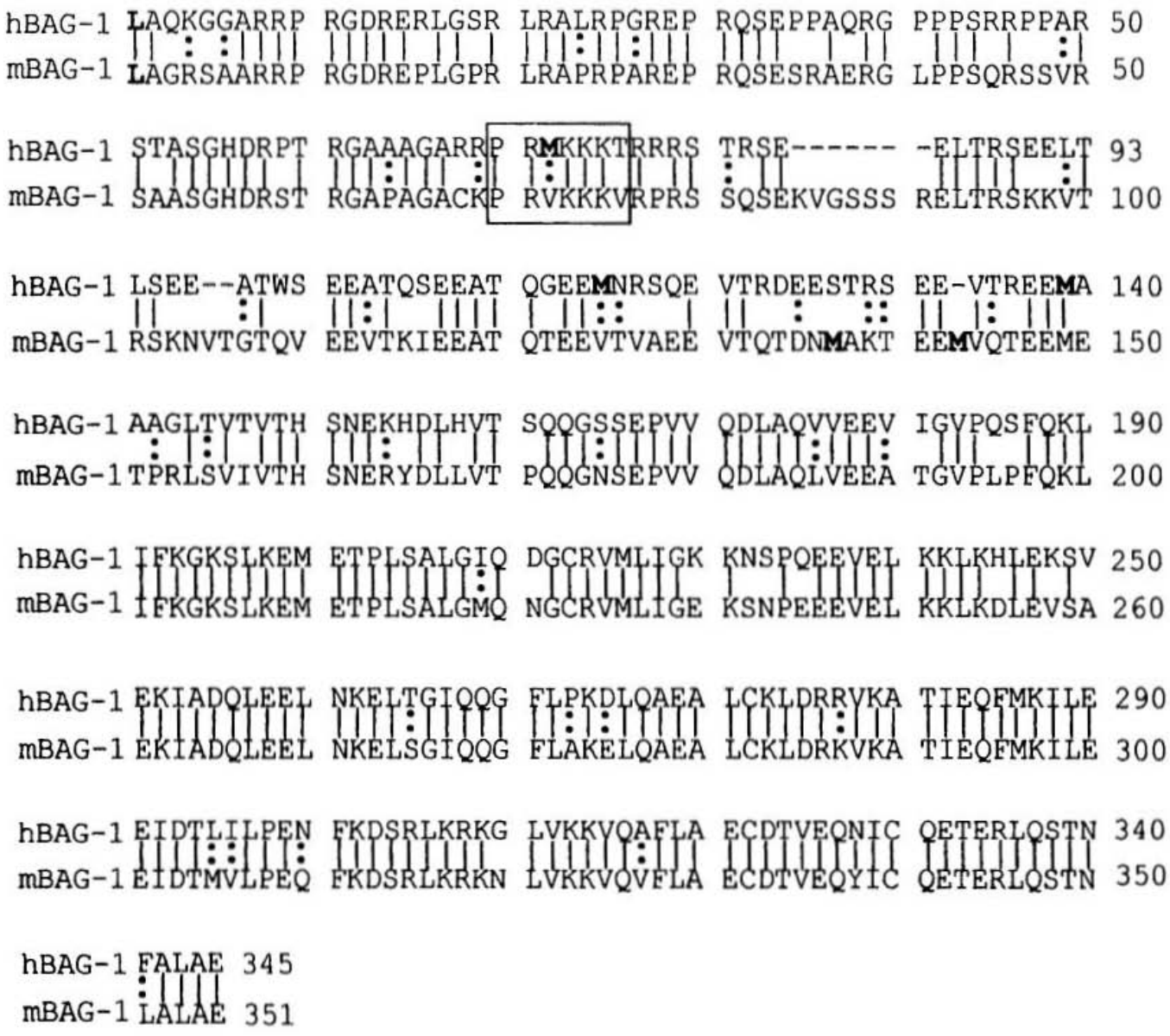
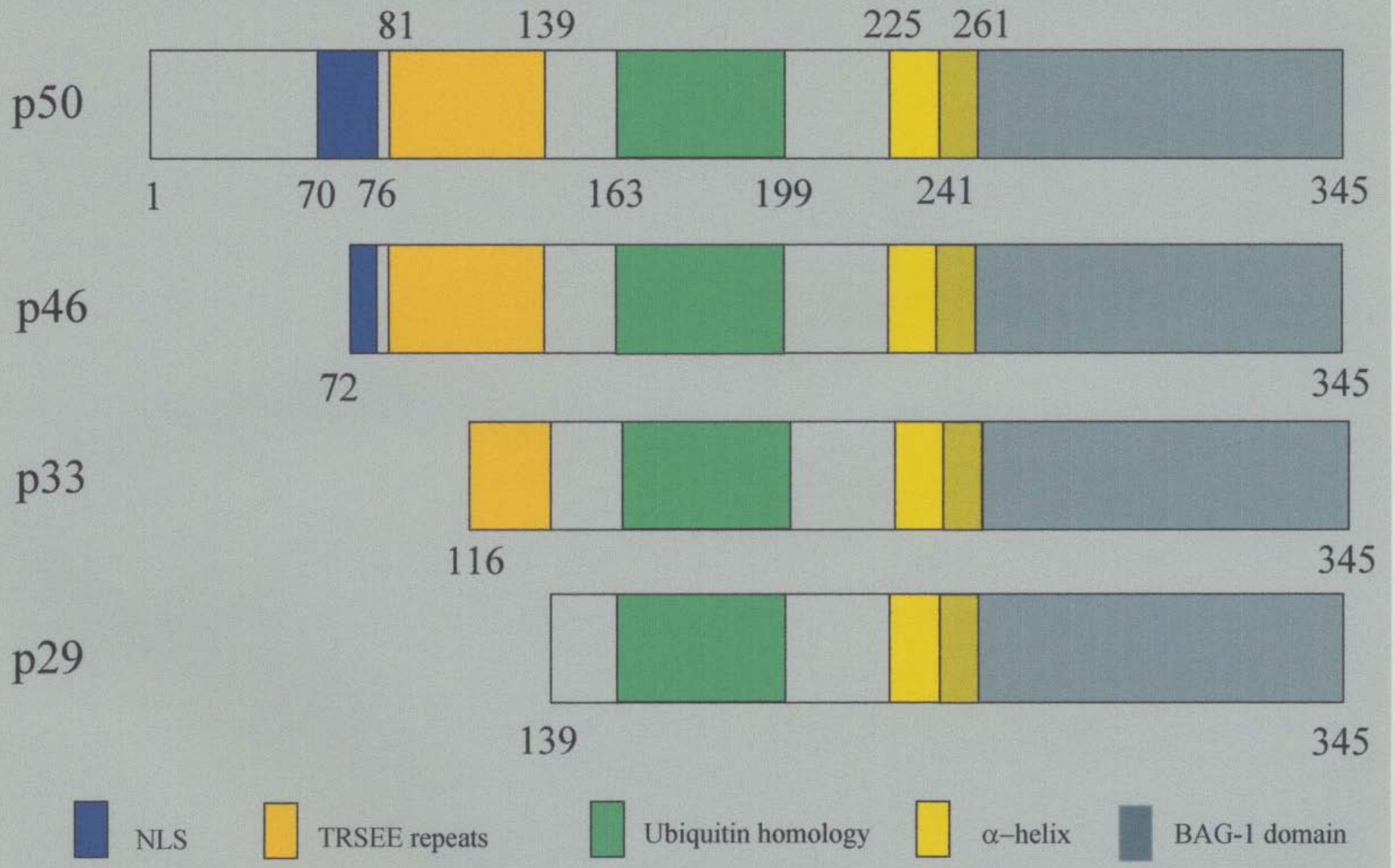

hBAG-1 LAQKGGARRP RGDRERLGSR LRALRPGREP RQSEPPAQRG PPPSRRPPAR 50
mBAG-1 LAGRSAARRP RGDREPLGPR LRAPRPAREP RQSESRAERG LPPSQRSSVR 50
hBAG-1 STASGHDRPT RGAAAGARRP RMKKKTRRRS TRSE------ -ELTRSEELT 93
mBAG-1 SAASGHDRST RGAPAGACKP RVKKKVRPRS SQSEKVGSSS RELTRSKKVT 100
hBAG-1 LSEE--ATWS EEATQSEEAT QGEEMNRSQE VTRDEESTRS EE-VTREEMA 140
mBAG-1 RSKNVTGTQV EEVTKIEEAT QTEEVTVAEE VTQTDNMAKT EEMVQTEEME 150
hBAG-1 AAGLTVTVTH SNEKHDLHVT SQQGSSEPVV QDLAQVVEEV IGVPQSFQKL 190
mBAG-1 TPRLSVIVTH SNERYDLLVT PQQGNSEPVV QDLAQLVEEA TGVPLPFQKL 200
hBAG-1 IFKGKSLKEM ETPLSALGIQ DGCRVMLIGK KNSPQEEVEL KKLKHLEKSV 250
mBAG-1 IFKGKSLKEM ETPLSALGMQ NGCRVMLIGE KSNPEEEVEL KKLKDLEVSA 260
hBAG-1 EKIADQLEEL NKELTGIQQG FLPKDLQAEA LCKLDRRVKA TIEQFMKILE 290
mBAG-1 EKIADQLEEL NKELSGIQQG FLAKELQAEA LCKLDRKVKA TIEQFMKILE 300
hBAG-1 EIDTLILPEN FKDSRLKRKG LVKKVQAFLA ECDTVEQNIC QETERLQSTN 340
mBAG-1 EIDTMVLPEQ FKDSRLKRKN LVKKVQVFLA ECDTVEQYIC QETERLQSTN 350
hBAG-1 FALAE 345
mBAG-1 LALAE 351

Fig. 2.7 Functional domains in human BAG-1 isoforms. The amino acid positions of each domain are shown either above or below the boxes. Different domains are indicated by distinct colors. The dark yellow color box represents overlapping region of α-helix and BAG-1 domains.


81
139
225
261
p50
1
70
76
163
199
241
345
p46
72
345
p33
116
345
p29
139
345
NLS
TRSEE repeats
Ubiquitin homology
α–helix
BAG-1 domain

Since four isoforms of human BAG-1 protein, namely p50, p46, p33 and p29, were produced by alternative translation initiation (see Chapter 3), the functional domains of each isoform were identified and are shown in Fig. 2.7. p50 isoform, the full-length BAG-1 protein, was found to have five functional domains including a nuclear localization signal (NLS; PRMKKKT), 10 copies of TRSEE (Thr-Arg-Ser-Glu-Glu) consensus homology repeats, a ubiquitin homology domain, an α-helix, and a BAG-1 domain. p46 contained all the other domains except five of eight NLS amino acids. No NLS and only four TRSEE consensus repeats were found in p33, whereas neither NSL nor TRSEE consensus repeats were contained in p29.

2.3.4 Tissue-specific expression of human BAG-1

The expression of human BAG-1 RNA in normal tissues was examined using a Multiple Tissue Northern Blot (MTN, Clontech) containing 2 μg per lane of poly(A)$^+$ RNA from different human tissue. BAG-1 RNA was found to be expressed in all the human tissues: heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Fig. 2.8). Relatively higher level of BAG-1 RNA was detected in heart and pancreas tissues.

2.4 DISCUSSION

Using mBAG-1 cDNA probe, the human homolog of mouse BAG-1 cDNA has been successfully isolated from a human cervical cell cDNA library. Surprisingly, human BAG-1 has 100% homology with a hormone receptor-binding protein, RAP46 (Zeiner and Gehring, 1995). RAP46 was isolated by protein-protein interaction screening of a human liver cDNA expression library with the glucocorticoid receptor. In addition to interacting with the glucocorticoid receptor, RAP46 also interacted with other human hormone receptors, including the estrogen, progesterone and thyroid receptors (Zeiner and Gehring, 1995). Therefore, BAG-1 is a hormone receptor-binding

Fig. 2.8 Expression of human BAG-1 RNA in different normal human tissues. An MTN blot containing 2 μg/lane of poly(A)$^+$ RNA was hybridized with ^{32}P-labelled human BAG-1 cDNA probe. Conditions and labels are as described for Fig. 2.4 B.

kb
Heart
Brain
Placenta
Lung
Liver
Skeletal muscle
Kidney
Pancreas
2.40
1.35
Actin

protein. Furthermore, human and mouse BAG-1 cDNA share 75% identity and 84% homology and are almost identical at their C-termini. Interestingly, both human and mouse BAG-1 mRNA were shown to be expressed as a single 1.35 kb transcript in mouse and human normal tissues (Fig. 2.8; Zeiner and Gehring, 1995), suggesting that BAG-1 is a ubiquitously expressed gene possibly involved in a common event in different cell types.

Sequence analysis identified an NLS (PRMKKKT) in BAG-1 similar to that identified in SV40 large T antigen (PKKKRKV) (Dingwall and Laskey, 1991). The NLS in human BAG-1 and mouse BAG-1 (PRVKKKV) were found to be conserved. However, the NLS was found to be full-length only in human p50, partially present in p46, and not in p33 and p29 isoforms, suggesting that different BAG-1 isoforms may localize in different subcellular compartments. In addition, 11 copies of consensus TRSEE pentameric motifs were identified in p50 and p46. Only four repeats were found in p33, whereas none were found in p29. The biological significance of these repeats remains unknown. Furthermore, a 37 amino acid sequence that has high homology with ubiquitin or ubiquitin-like proteins was also found in BAG-1 protein. Ubiquitin is a small 76 amino acid protein that is well conserved through evolution and can cause the degradation of target proteins also containing ubiquitin homology by covalent formation between its conserved lysine at position 48 and the C-terminal glycine carboxyl group of the target proteins (for review, see Hershko and Ciechanover, 1992). Therefore, this domain may be responsible for BAG-1 protein degradation. Furthermore, sequence analysis also identified an α-helix-like sequence from amino acid position 225 to 261. α-helix domains have been shown to be involved in protein-protein interactions (Muchmore et al., 1996). Therefore, an α-helical structure may be important for the interaction of BAG-1 with other proteins. Finally, the BAG-1 domain is a sequence that is well conserved between human and mouse BAG-1 (Fig. 2.6). This domain is important for some functions of BAG-1, as it will be described in Chapter 4.4.

CHAPTER 3

HUMAN BAG-1/RAP46 PROTEIN IS GENERATED AS FOUR ISOFORMS BY ALTERNATIVE TRANSLATION INITIATION

3.1 INTRODUCTION

To elucidate the role of BAG-1 in human cells, I isolated the human homolog of mBAG-1 by screening a HPV16-immortalized human cervical cell cDNA library with mBAG-1 cDNA as a probe (see Chapter 2). Recently, hBAG-1 was also been independently cloned by others (Zeiner and Gehring, 1995; Takayama *et al.*, 1996). Although the hBAG-1 cDNAs isolated by us and the other two groups have almost identical amino acid sequence, different molecular sizes of its protein products were noted. hBAG-1 or RAP46 was identified by Zeiner and Gehring (1995) as a protein with a molecular weight of 46 kDa, whereas it was detected by Takayama *et al.* (1996) as a protein with molecular weight of 34 kDa. In this study, three protein isoforms of hBAG-1 were observed *in vitro* that had molecular weights of 46 (p46), 33 (p33) and 29 kDa (p29). I examined the cDNA sequence of hBAG-1 and found three potential translation start codon ATGs (AUGs) at nucleotide position number (nt) 276 (AUG1), 411 (AUG2) and 477 (AUG3), respectively. It is possible that the distinct protein products of hBAG-1 identified by different groups may resulted from translation initiation from different start codon AUGs. To test my hypothesis, I conducted site-directed mutagenesis of hBAG-1 cDNA and analyzed its protein products *in vitro*. My results provided convincing evidence that p46, p33 and p29 are translated by alternative translation initiation from start codons AUG1, AUG2 and AUG3, respectively. In addition, an extra isoform of hBAG-1 with

a molecular weight of 50 kDa (p50) was also observed *in vitro* and *in vivo* by us and recently by Packham *et al.* (1997). p50 was found to be translated from CUG upstream of AUG1 (Packham *et al.*, 1997). The molecular mechanism of alternative translation initiation of hBAG-1 mRNA, and subcellular localization patterns of these four hBAG-1 isoforms were further investigated in this study.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture and transfection

HeLa and C33A cervical carcinoma cells (ATCC) used in this study were cultured in Dulbeco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS).

For cervical carcinoma C33A cell (an hBAG-1 negative cell line) transfections, wild type or mutated forms of hBAG-1 cDNA were cloned into an eukaryotic expression vector PCR3.1 (Invitrogen). Approximately 24 h prior to transfection, C33A were seeded at a density of 5×10^5 cells per 10-cm culture dish. Plasmid DNAs were stably transfected into C33A cells by calcium chloride or LipofectAMINE (GIBCO-BRL) followed by selection in medium containing 0.8 mg/ml G418 (GIBCO-BRL). The resulting transfectants were maintained as cell lines and harvested for protein subcellular fractionation or Western blot analysis.

3.2.2 Generation of anti-hBAG-1 mouse monoclonal antibody (mAb)

The coding region of hBAG-1 cDNA was cloned in-frame into pGEX-4T-3 vector (Pharmacia). The resulting plasmid was then transfected into BL21 bacteria and the GST-hBAG-1 fusion protein was induced with 0.1 mM isopropyl β-D-thiogalactoside at 30^0C for 5 h. hBAG-1

protein was purified by binding GST-hBAG-1 on glutathione-Sepharose-4B beads (Pharmacia) and cleavage with thrombin. The mAb was generated by immunization of mice with purified hBAG-1. After extensive screening, one clone named CC9E8, which recognized all the four isoforms of hBAG-1 and gave the best activity, was selected to produce anti-hBAG-1 mAb used for Western blot analysis.

3.2.3 Western blot analysis

Western blot analysis was performed as described (Yang *et al.*, 1997). In brief, proteins were extracted from 10^7 cells by lysis in 1 ml ice-cold extraction buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate] for 30 min and centrifuged at 14,000 × g at 4 ^{0}C for 10 min. A DC Protein Assay kit (Bio-Rad, Hercules, CA) was used to quantify the protein. Ten μg proteins were fractionated by 10% SDS-polyacrylamide gel electrophoresis, according to standard protocols. Stained protein markers (Amersham, Arlington Heights, IL) were included in each gel as molecular weight standards. The proteins were subsequently transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham) under semi-dry conditions. Immunodetection was performed by incubation with antibodies to apoptosis-associated genes and then with HRP-conjugated anti-IgG at 1:1,500 dilution. After detection of signals with the ECL system (Amersham), the blots were stripped and rehybridized with monoclonal anti-actin antibody and HRP-conjugated anti-IgG, and the signals were detected with the same system. The intensity of the signals was quantified by measuring the density of each band with an Eagle Sight Image Capture and Analysis system (Stratagene, La Jolla, CA). CC9E8 anti-hBAG-1 mAb and goat anti-mouse HRP-conjugated IgG were used as the first and the

secondary antibodies, respectively. For testing the specificity of anti-hBAG-1 mAb, the purified hBAG-1 protein was added to the hybridization reaction in 10-fold excess. The bands eliminated by the addition of hBAG-1 protein were regarded as genuine bands.

3.2.4 In vitro mutagenesis

Mutation of hBAG-1 ATG start codon into TTG was carried out by oligonucleotide-directed mutagenesis with QuikChange Site-Directed Mutagenesis kit (Stratagene). Deletion of hBAG-1 and optimization of sequence surrounding hBAG-1 start codon into the Kozak sequence (GCCACCATGG) were carried out by PCR. All mutations were confirmed by sequencing with Sequenase Version 2.0 kit (USB).

3.2.5 In vitro transcription/translation

Wild type and mutated hBAG-1 cDNA were transcribed and translated *in vitro* in the presence of [^{35}S]-methionine (Amersham) using a coupled transcription/translation system (TNT; Promega), as described by the supplier. In brief, 1 μg of hBAG-1 cDNA plasmids containing wild-type and mutated p46 were in *vitro*-transcribed/translated in the presence of [^{35}S]-methionine. The [^{35}S]-labelled protein products were separated by 10% SDS-PAGE and subsequently detected by fluorography. Reticulocyte lysate and luciferase were used as negative and positive controls for the reaction, respectively.

3.2.6 Indirect immunofluorescence analysis

For analysis of hBAG-1 subcellular localization, HeLa cells grown on multiwell slides in

monolayer culture were fixed in ice-cold 50% methanol/50% acetone for 30 min, air-dried and stored at room temperature until use. The fixed cells were consecutively immersed 3 times in 100% ethanol, 3 times in 70% ethanol, once in H_2O, and 3 times in 1 X PBS. The slides were incubated in 20% goat serum for 1 h, with mouse anti-hBAG-1 antibody in PBS at 1:200 dilution for 1 h, and with FITC-conjugated anti-mouse IgG diluted 1:40 in 20% goat serum for 1 h. Staining was visualized by mounting in 30% glycerol and examining under a Leitz Laborlux S Fluorescence Microscope (Germany) in the dark. For nuclear staining, the slides were hydrated as above and then stained with 3 μg/ml 4, 6-diamidino-2-phenylindole (DAPI) for 5 min. For control, anti-hBAG-1 antibody was omitted in the hybridization buffer.

3.2.7 Subcellular protein fractionation

About 10^7 HeLa cells were washed twice with PBS, lysed in 500 μl hypotonic lysis buffer (10 mM Hepes, pH 7.4, 38 mM NaCl, 25 μg/ml phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin), homogenized in a Dounce homogenizer and centrifuged at 900 x g to pellet the nuclei. The postnuclear supernatant was then centrifuged at 130,000 x g at 4 ^{0}C to pellet the membranes. Both the nuclear and membrane pellets were resuspended in 500 μl lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate). The cytosolic supernatant was adjusted to 150 mM NaCl, 1% NP-40 and 0.1% SDS. The relative levels of hBAG-1, Bcl-2, Lamin B and β-tubulin in the nuclear, cytosolic and membrane fractions were analyzed by Western blot using 10 μg protein for each fraction.

3.3 RESULTS

3.3.1 hBAG-1 protein was expressed as four isoforms by alternative translation initiation

To characterize hBAG-1 protein, I constructed the recombinant plasmid p46 by cloning the hBAG-1 coding region (nt 274-1113), which covering the region between the first start codon ATG1 and stop codon TGA, into a PCR3.1 vector. *In vitro*-transcription/translation of p46 cDNA resulted in two major bands of 46 kDa (p46) and 33 kDa (p33), and one minor band of 29 kDa, (p29) (Fig. 3.1B). No translation products were found when rabbit reticulocyte lysate was used as a negative control. A 61 kDa luciferase protein was detected as a positive control for the reaction.

Although the amino acid sequences of hBAG-1 and RAP46 are identical, different size of protein products was found by us and two other groups (Zeiner and Gehring, 1995; Takayama *et al.*, 1996). RAP46 mRNA was translated *in vitro* into a 46 kDa protein, whereas hBAG-1 isolated by Takayama *et al.* (1996) was detected as a protein with molecular weight of 34 kDa. Since there are three AUG start codons in hBAG-1 mRNA at nt 276, 411 and 477 (Fig. 3.2), the distinct protein products of hBAG-1, p46, p33 and p29, respectively, may have resulted from alternative translation initiation from each of these start codons (Fig. 3.2). To test this hypothesis, a series of cDNA constructs containing a single base mutation of each of the three ATGs in p46 cDNA were generated by site-directed mutagenesis (Fig. 3.1A). *In vitro* transcription/translation of these mutated constructs indicated that p46, p33 and p29 were produced by alternative use of start codon AUG1, AUG2 or AUG3 in hBAG-1 mRNA, respectively. Mutation of any one of these ATGs into TTGs would abolish its function as a translation initiation site and the production of the respective protein product. Mutation of ATG1 into TTG (ΔATG1) resulted in the loss of the p46 isoform and the generation of only p33 and p29; mutation of the second ATG (ΔATG2) led to the loss of p33 and the production of p46 and p29; whereas mutation of the third ATG (ΔATG3) caused the loss of p29 and the

Fig. 3.1 Expression of hBAG-1 p46, p33 and p29 isoforms from three alternative translational start sites. (A) Schematic representation of site-specific mutagenesis. The p46 cDNA, its mutated constructs and their expected molecular weights in kDa are shown. The mutated ATG start codons were boxed TTGs. The nt for each ATG start codon and the TGA stop codon is indicated below each construct. (B) *In vitro* transcription/translation of wild type and mutated hBAG-1. cDNAs containing wild type and mutated p46 were *in vitro*-transcribed/translated in the presence of [^{35}S]-methionine. The [^{35}S]-labelled protein products were separated by 10% SDS-PAGE and subsequently detected by fluorography. Reticulocyte lysate and luciferase were used as negative and positive controls, respectively, for the reaction. Molecular weights in kDa are shown on the left. The positions of p46, p33 and p29 were indicated on the right.

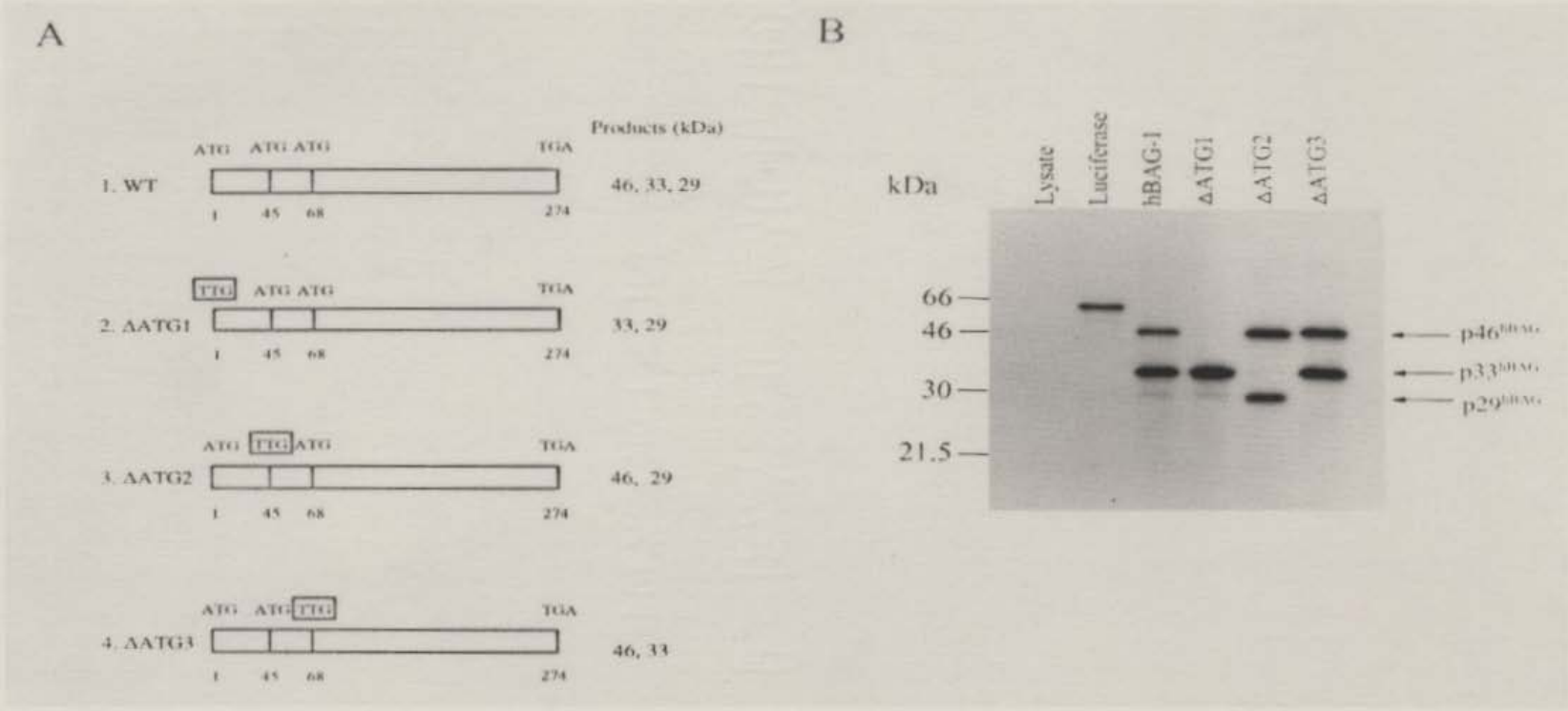
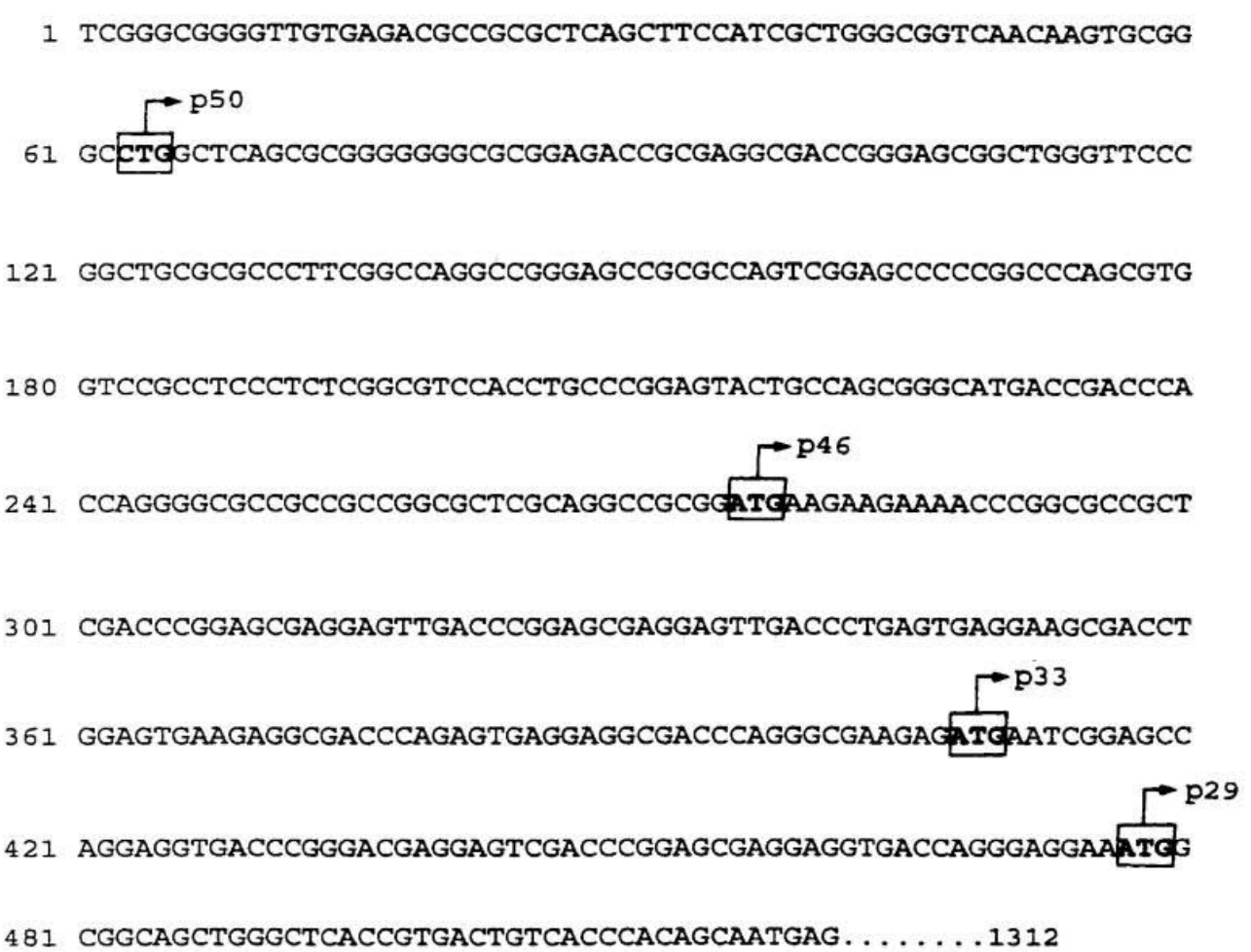

A
Products (kDa)
ATG ATG ATG
TGA
1. WT
46, 33, 29
1 45 68 274
TTG ATG ATG
TGA
2. ΔATG1
33, 29
1 45 68 274
ATG TTG ATG
TGA
3. ΔATG2
46, 29
1 45 68 274
ATG ATG TTG
TGA
4. ΔATG3
46, 33
1 45 68 274
B
kDa
Lysate
Luciferase
hBAG-1
ΔATG1
ΔATG2
ΔATG3
66
46
30
21.5
p46hBAG-1
p33hBAG-1
p29hBAG-1

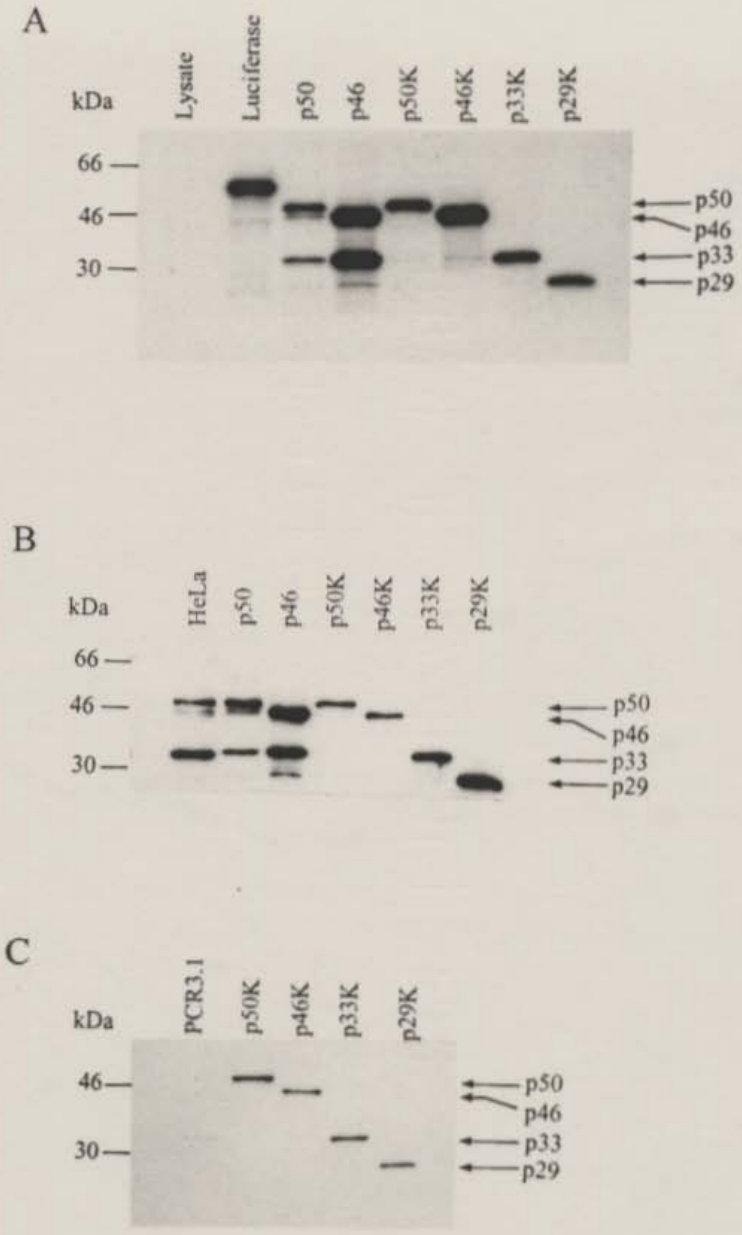
Fig. 3.2 Translation of hBAG-1 mRNA may be initiated from one upstream CTG and three potential start codon ATGs. Nucleotide sequence of 5'-end hBAG-1 cDNA is shown. The CTG start codon at nt 63 and three potential start codon ATGs at nt 276, 422 and 477, are boxed and in bold face. The respective protein products, p50, p46, p33 or p29, which were translated from each start codon, is indicated.


1 TCGGGCGGGGTTGTGAGACGCCGCGCTCAGCTTCCATCGCTGGGCGGTCAACAAGTGCGG
p50
61 GCCTGGCTCAGCGCGGGGGGGCGCGGAGACCGCGAGGCGACCGGGAGCGGCTGGGTTCCC
121 GGCTGCGCGCCCTTCGGCCAGGCCGGGAGCCGCGCCAGTCGGAGCCCCCGGCCCAGCGTG
180 GTCCGCCTCCCTCTCGGCGTCCACCTGCCCGGAGTACTGCCAGCGGGCATGACCGACCCA
p46
241 CCAGGGGCGCCGCCGCCGGCGCTCGCAGGCCGCGGATGAAGAAGAAAACCCGGCGCCGCT
301 CGACCCGGAGCGAGGAGTTGACCCGGAGCGAGGAGTTGACCCTGAGTGAGGAAGCGACCT
p33
361 GGAGTGAAGAGGCGACCCAGAGTGAGGAGGCGACCCAGGGCGAAGAGATGAATCGGAGCC
p29
421 AGGAGGTGACCCGGGACGAGGAGTCGACCCGGAGCGAGGAGGTGACCAGGGAGGAAATGG
481 CGGCAGCTGGGCTCACCGTGACTGTCACCCACAGCAATGAG.....1312

generation of p46 and p33 (Fig. 3.1B). Interestingly, when the second ATG was mutated (ΔATG2), the expression of p29 increased significantly.

To confirm our *in vitro* transcription/translation results *in vivo*, we produced anti-hBAG-1 mAb by immunizing of mice with purified p46 protein and examined the expression of hBAG-1 protein in human cervical carcinoma HeLa cells by Western blot. Consistently, p46, p33 and p29 were detected in the cells. However, an extra 50 kDa band, which migrated slower than p46 and was eliminated by competition of anti-hBAG-1 mAb with 10-fold excess purified hBAG-1 (data not shown), was also detected by Western blots (Fig. 3.3B). This protein is either a modified form of hBAG-1 protein or a protein translated from upstream translation start codon. Recently, hBAG-1 was found to be translated from a CUG upstream of AUG1. However, only two hBAG-1 protein products with molecular weight of 50 and 36 kDa were observed both *in vitro* and *in vivo* (Packham *et al.*, 1997). Since the cDNA used previously for *in vitro* transcription/translation by us and others did not contain the upstream CTG (Zeiner and Gehring, 1995; Takayama *et al.*, 1996), the absence of p50 in *in vitro* transcription/translation products may have been due to the absence of translation initiation from this upstream CTG. To test this possibility, I used the cDNA containing upstream CTG (p50) and compared its *in vitro* translation products with those from p46 cDNA. Interestingly, p50 cDNA produced four protein isoforms with molecular weights of 50, 46, 33 and 29 kDa, respectively (Fig. 3.3A). Deletion of the upstream CTG in p46 cDNA resulted in the loss of p50 isoform and the enhanced expression of p46, p33 and p29 (Fig. 3.3A). To correlate my *in vitro* translation results with those *in vivo*, I compared the expression pattern of hBAG-1 protein products in HeLa cells with those from *in vitro* transcription/translation of p50 and p46 cDNAs by Western blot. Consistently, four hBAG-1 isoforms of identical size were detected both in HeLa cells and in *in vitro* translated

Fig. 3.3 The four hBAG-1 protein isoforms were generated by the leaky scanning mechanism. The sequence contexts of CTG and three putative start codon ATGs were modified into the Kozak sequence, respectively. p50K, p46K, p33K and p29K correspond to p50, p46, p33 and p29 cDNA containing the Kozak sequence, respectively. Conditions and labels were as described in Fig. 3.1B. (**A**) *In vitro* transcription/translation of wild-type and mutated hBAG-1 cDNAs. (**B**) Detection of hBAG-1 protein in HeLa cells and *in vitro*-translated protein products by Western blot. Lysate containing 10 μg protein from HeLa cells or 2 μl *in vitro* translated products were subjected to 10% SDS-PAGE and transferred to an ECL nitrocellulose membrane. The membrane was subsequently incubated with anti-hBAG-1 mAb (CC9E8), and detected using a goat HRP-conjugated anti-mouse IgG antibody by the ECL method. (**C**) Expression of the four hBAG-1 isoforms *in vivo*. The vector control plasmid PCR3.1 and the plasmids containing p50K, p46K, p33K and p29K cDNA were stably transfected into C33A cells. The C33A cells expressing different cDNA were lysed in protein lysis buffer and 10 μg protein were analyzed by Western blot.


A
kDa
Lysate
Luciferase
p50
p46
p50K
p46K
p33K
p29K
66
46
30
p50
p46
p33
p29
B
kDa
HeLa
p50
p46
p50K
p46K
p33K
p29K
66
46
30
p50
p46
p33
p29
C
kDa
PCR3.1
p50K
p46K
p33K
p29K
46
30
p50
p46
p33
p29

products of p50 (Fig. 3.3A, 3.3B).

3.3.2 Alternative translation initiation resulted through the leaky scanning mechanism

Alternative initiation of translation is usually caused by the leaking past of the preinitiation scanning complex past the first AUG and the initiation of translation at a downstream start codon when the first AUG codon is not in a perfect Kozak sequence (Kozak, 1989). Since neither the CTG at nt 63 nor the first two AUG start codons of hBAG-1 mRNA are in a perfect Kozak sequence (Fig. 3.2), this leaky scanning mechanism may be the cause of alternative initiation of translation of hBAG-1 mRNA. To prove my hypothesis, I optimized each of the four putative start codons and its surrounding sequences into the Kozak sequence (GCCACCATGG) and analyzed the protein products from each mutated constructs both *in vitro* and *in vivo*. As expected, optimization of CTG-contexts or any of the three ATG-context into the Kozak sequence abolished the alternative initiation from its downstream ATGs: when CTG was optimized in Kozak sequence (p50K), only the p50 protein was produced, whereas when ATG1 or ATG2 or ATG3 was in Kozak sequence (p46K, p33K and p29K, respectively), only p46, p33 or p29 proteins, respectively, were translated *in vitro* (Fig. 3.3A). The identity of each *in vitro* translated product was further confirmed by Western blot using anti-hBAG-1 mAb (Fig. 3.3B). Consistent results were found *in vivo* when p50K, p46K, p33K or p29K cDNA was stably transfected into an hBAG-1 negative cervical cell line C33A cells (Fig. 3.3C).

3.3.3 Distinct isoforms of hBAG-1 protein are differentially localized in different subcellular compartments

hBAG-1 contains a putative bipartite nuclear localization signal (NLS) located in aa 149-165 (Zeiner and Gehring, 1995), suggesting that it may distribute in the nucleus. This is consistent with

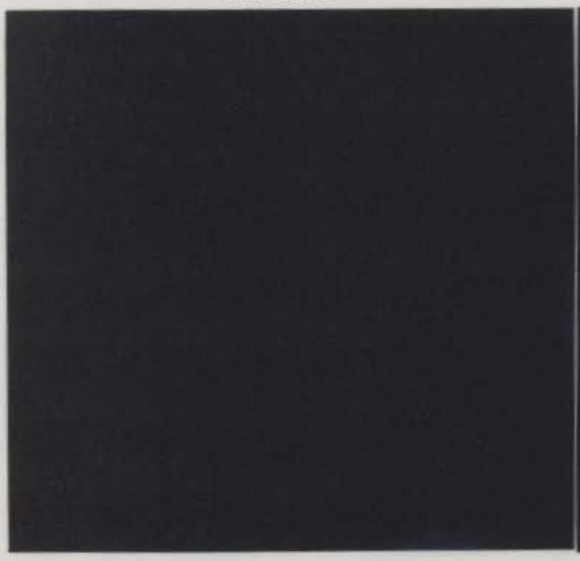
my finding that mBAG-1 functioned as a transcription factor (unpublished data). However, hBAG-1 also interacts with Bcl-2, which is predominantly localized in the nuclear and mitochondrial membranes (Krajewski *et al.*, 1993). Therefore, the subcellular localization of hBAG-1 in HeLa cells was studied by immunofluorescence and protein fractionation analysis. As shown in Fig. 3.4, hBAG-1 protein was localized in both cytoplasm and nucleus. Protein fractionation assay further indicated that hBAG-1 protein was distributed in nuclear, cytosolic and membrane fractions, whereas Bcl-2 was localized in nuclear and membrane fractions but not in the cytosolic fraction (Fig. 3.5A). Lamin B was used as the nuclear fraction marker and β-tubulin as the cytoplasmic fraction marker (Fig. 3.5A; Joh et al., 1997). Interestingly, the four isoforms of hBAG-1 were distributed differently in the cells. The p50 isoform was predominantly present in the nuclear and membrane fractions and p46 was in the fractions from all three compartments, whereas p33 and p29 were found mostly in the cytosolic fraction (Fig. 3.5A). To further confirm these results, mutated hBAG-1 cDNAs expressing only p50, p46, p33 or p29, were stably transfected into the hBAG-1-negative cervical carcinoma cell line C33A. Protein fractionation clearly demonstrated that the distinct isoforms of hBAG-1 were distributed differently in the cells (Fig. 3.5B).

3.4 DISCUSSION

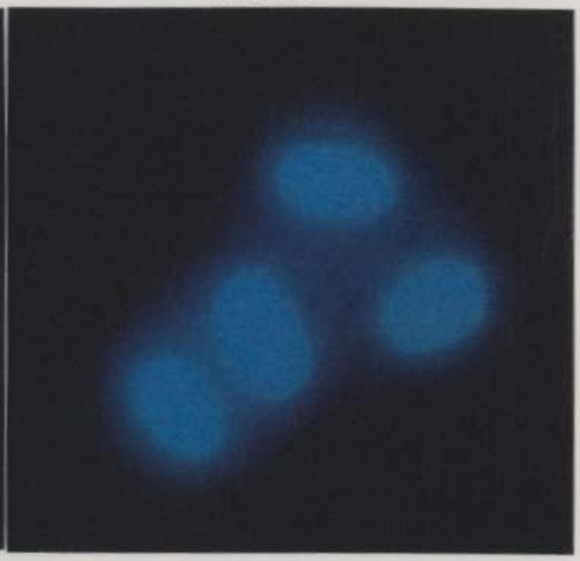
Using a mouse BAG-1 cDNA probe, I have successfully isolated the human homolog of mBAG-1 from a human cervical cell cDNA library (Chapter 2). Surprisingly, hBAG-1 has 100% homology with a novel hormone receptor-binding protein, RAP46, with molecular weight of 46 kDa (Zeiner and Gehring, 1995). Recently, hBAG-1 with a protein size of 34 kDa was also isolated from a human breast cell cDNA library by Takayama *et al.* (1996). However, the three groups identified different size of protein products for hBAG-1/RAP46. More recently, a 50 kDa hBAG-1 protein was also

Fig. 3.4 Subcellular localization of hBAG-1 protein. The subcellular localization of hBAG-1 was analyzed by immunofluorescence using CC9E8 mAb. Control panel shows the result of immunofluorescence analysis without CC9E8. DAPI stain was a control for nuclear staining. All panels are 400× magnification, except the indicated magnification for the bottom right panel.

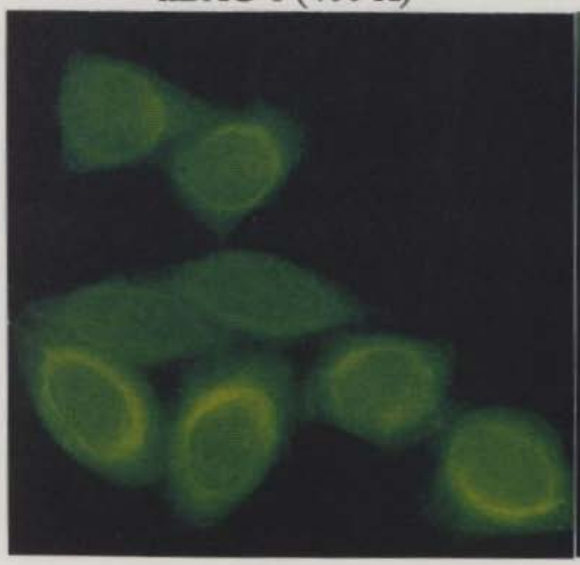
Control



DAPI

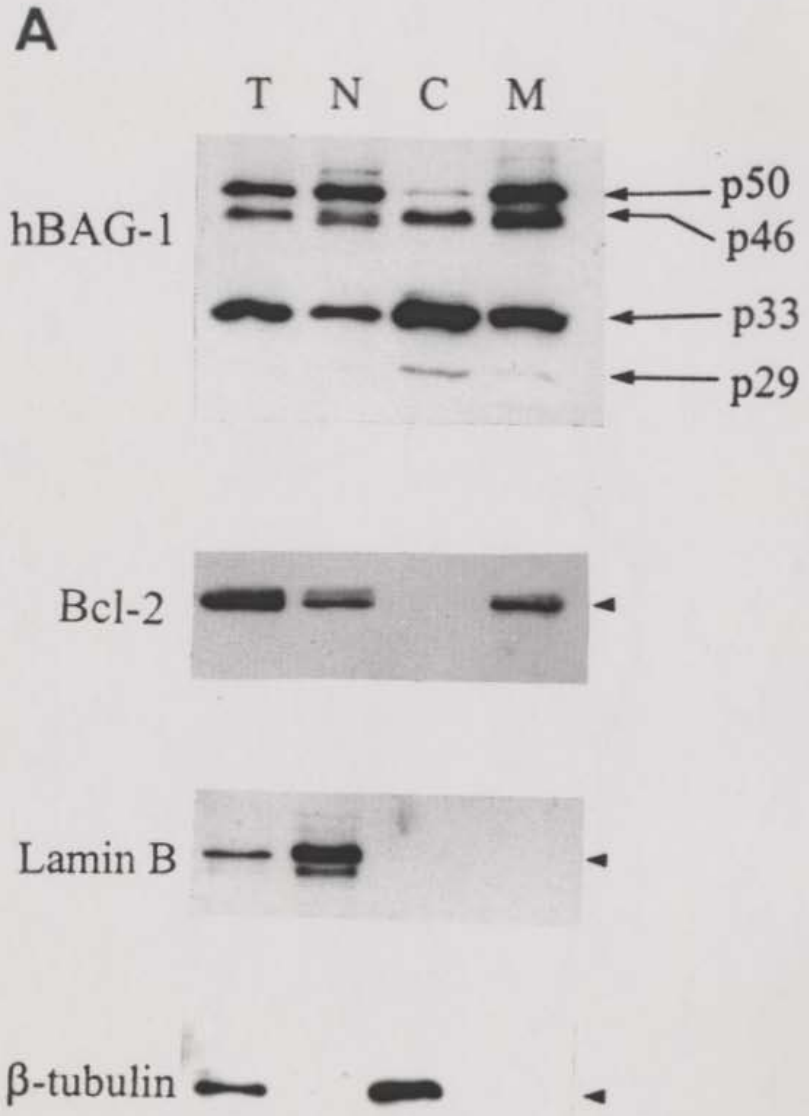


hBAG-1 (400 X)

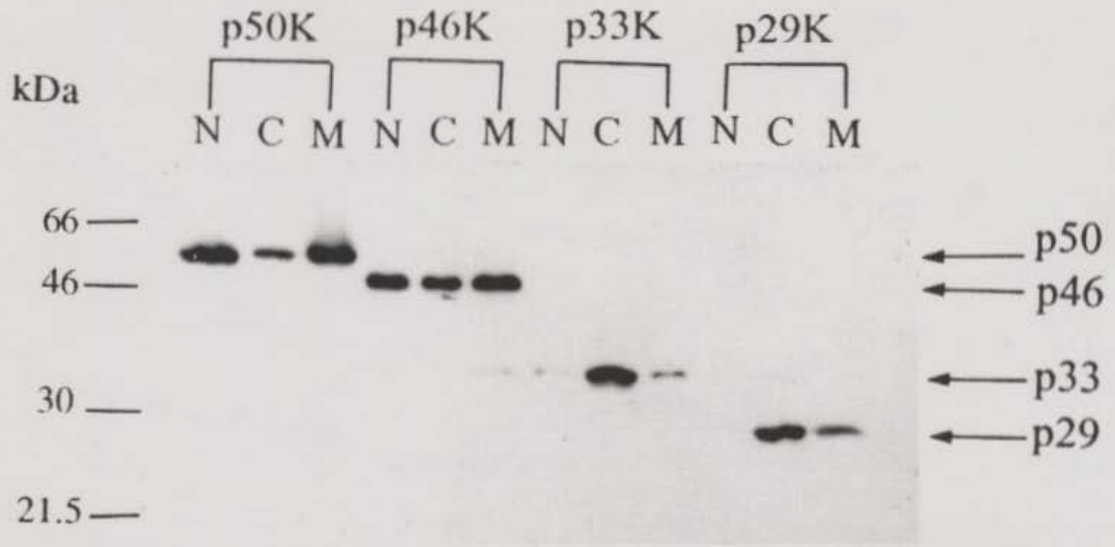


hBAG-1 (650 X)

Fig. 3.5 Distinct subcellular localization of the four hBAG-1 protein isoforms. Protein was either unfractionated (T) or fractionated into nuclear (N), soluble cytosolic (C) and high-speed membrane pellet (M) fractions by differential centrifugation. The unfractionated and fractionated protein samples were analyzed by Western blotting with anti-hBAG-1, anti-Bcl-2 and anti-β-tubulin mAbs, and lamin B polyclonal antibodies. **(A)** Protein fractionation of hBAG-1 in HeLa cells. The position of each isoform is indicated by an arrow. The positions of Bcl2, lamin B and β-tubulin are indicated by arrowheads. **(B)** Differential subcellular localization of the four isoforms of hBAG-1 in C33A cells. Lysates of C33A expressing different isoforms of hBAG-1 protein were fractionated into different subcellular fractions (N, C and M).


A
T N C M
hBAG-1
p50
p46
p33
p29
Bcl-2
Lamin B
β-tubulin

B


p50K
p46K
p33K
p29K
kDa
N C M N C M N C M N C M
66
46
30
21.5
p50
p46
p33
p29

observed by Packham *et al.* (1997). My results reconciled the discrepancies observed by others and clearly demonstrated that hBAG-1 was expressed as four isoforms of protein, p50, p46, p33 and p29, which were produced by alternative initiation of translation. Analogously, three species of mBAG-1 with molecular weights of 50 kDa (p50m), 30 kDa (p30m) and 28 kDa (p28m) may have been produced by alternative translation as well, since there are two AUG start codons on mBAG-1 mRNA, and the first AUG is not in perfect Kozak sequence. Most interestingly, the expression of mBAG-1 protein in mouse cell lines is also tissue-specific. Mouse P19 embryonal carcinoma cells expressed all the three products of mBAG-1, p50m, p30m and p28m, whereas no p28m was expressed in mouse fibroblast NIH 3T3 cells (data not shown).

Alternative initiation of translation usually results from a leaky scanning mechanism, in which the preinitiation scanning complex bypasses or "leaks" past the first AUG and initiates at a downstream codon when the first or second AUG codon is not in a perfect Kozak sequence (GCCA/GCCAUGG) for recognition by the scanning complex (Kozak, 1989). Since neither the upstream CUG nor the first AUG of hBAG-1 mRNA is in the optimal Kozak sequence, translation may start from the first, second or third AUG. By optimization of context of CTG and each of the three ATGs into the Kozak sequence, I provided both *in vitro* and *in vivo* evidence that alternative initiation of hBAG-1 is caused by the leaky scanning mechanism. Although not common, use of alternative translation initiation sites occurs in other genes such as c-*myc* (Spotts *et al.*, 1997), *cot* (Aoki *et al.*, 1993) and glutathione peroxidase (Pushpa-Rekha *et al.*, 1995). In addition, proteins translated by alternative initiation of translation from start codon AUGs were previously shown to have different transforming activity (Aoki *et al.*, 1993) or transactivation function (Spotts *et al.*, 1997). It would be of interest to study whether these four isoforms of hBAG-1 have distinct functions in the cells.

Although BAG-1 was originally identified as a Bcl-2-binding protein, its subcellular localization was apparently different from that of Bcl-2. Using immunofluorescence and protein fractionation, I demonstrated that hBAG-1 proteins were localized in all the cellular compartments, including the nucleus, cytosol and membranes, whereas Bcl-2 was only localized in the nuclear envelope and membranes (Krajewski *et al.*, 1993; Fig. 3.5A). Since hormone receptors are distributed in the cytosol and nucleus (Sackey *et al.*, 1996), hBAG-1 may interact with Bcl-2 on the membranes and with hormone receptors in the cytosol or nucleus. Most importantly, I found that p50 protein was predominantly localized in the nuclear and membrane fractions, whereas p33 and p29 proteins were mostly present in the soluble cytosol. Consistent with this result, analysis of functional domains in different BAG-1 isoforms revealed that only p50 has an SV40-like NLS (Chapter 2.3.3; Fig. 2.7). Differential subcellular localization of alternatively translated proteins was also found for other genes, such as FGF and *hck* (Bugler *et al.*, 1991; Lock *et al.*, 1991). Different subcellular localization of BAG-1 isoforms may be responsible for their distinct function in the cells (see Chapter 7.4).

SECTION 3

ROLE OF BAG-1 IN APOPTOSIS

CHAPTER 4

HUMAN BAG-1 SENSITIZES CERVICAL CELLS TO N-(4-HYDROXYPHENYL) RETINAMIDE-INDUCED APOPTOSIS

4.1 INTRODUCTION

N-(4-hydroxyphenyl) retinamide (4-HPR or fenretinide) is one of the most effective and promising synthetic retinoids used for human cancer chemotherapy (Costa, 1993; Formelli and Cleris, 1993; Sabichi *et al.*, 1998; Zou *et al.*, 1998). It has both preventive and therapeutic effects in experimental systems and clinical practice. In carcinogen-treated rodents, 4-HPR significantly decreases the incidence of bladder, breast, cervical, lung, prostate and skin cancer (Moon and Mehta, 1989; Pollard *et al.*, 1991; Kelloff *et al.*, 1994; Formelli and Cleris, 1993). In addition, 4-HPR is effective against established carcinogen-induced mammary tumors, and prostate and ovarian tumor cells transplanted in rats (Dowlatshahi *et al.*, 1989; Abou-Issa *et al.*, 1989; Pienta *et al.*, 1993; Formelli *et al.*, 1993). Unlike other retinoids, 4-HPR exhibits low normal tissue toxicity and long-term tolerability, in both animals and humans (Routmensz *et al.*, 1991; Moon *et al.*, 1992; Lotan, 1995; Abou-Issa *et al.*, 1997; Pienta *et al.*, 1997).

Recently, 4-HPR was shown to induce apoptosis in various human cancer cells, suggesting that this function may be important for its chemopreventive and chemotherapeutic effects (Delia *et al.*, 1995; Lotan *et al.*, 1995; Pellegrini *et al.*, 1995; Hsieh and Wu, 1997; Scher *et al.*, 1998; Zou *et al.*, 1998; Montaldo *et al.*, 1999). Most retinoids function by binding to their nuclear receptors, RAR α, β, and γ and retinoud X receptor α, β, and γ, whereas 4-HPR does not bind effectively in most cell lines (for review, see Chambon, 1996). Although several studies have shown that 4-HPR can induce apoptosis through interacting with RARs (Liu *et al.*, 1998b; Sun *et al.*, 1999), other reports suggested that 4-HPR can induce apoptosis via an RAR-independent mechanism (Delia *et al.*, 1993; Lotan *et al.*, 1995; Sheikh *et al.*, 1995; Clifford *et al.*, 1999; Sun *et al.*, 1999 a, b). In addition, in one study, 4-HPR could induce apoptosis in cells such as C33A containing mutated p53 without changing the levels of Bcl-2 and Bax, indicating that induction of apoptosis by 4-HPR is

independent of p53, Bcl-2 and Bax (Oridate *et al.*, 1995, 1997; Zou *et al.*, 1998). However, in another study, it was found that 4-HPR induced apoptosis by decreasing the Bcl-2:Bax ratio (Shen *et al.*, 1999). Further studies indicated that 4-HPR, rather than other retinoids such as all-trans RA, induces apoptosis by increasing reactive oxygen species (ROS) (Delia *et al.*, 1997; Oridate *et al.*, 1997). Treatment of cells with the antioxidant, pyrrolidine dithiocarbamate (PDTC), inhibits 4-HPR-induced apoptosis, suggesting that production of ROS may be important. However, although a number of studies have shown that ROS are required for apoptosis (Ross *et al.*, 1993; Lowe *et al.*, 1994; Symonds *et al.*, 1994), others have shown that apoptosis could be induced under hypoxic conditions in which ROS generation was inhibited (Schwartz and Bennett, 1995; Johnson *et al.*, 1996). Therefore, it is unclear what role is played by the retinoid receptors, apoptosis-regulating genes and ROS in 4-HPR-induced apoptosis.

BAG-1 is an anti-apoptotic gene that can function independent of Bcl-2 (Chapter 5). Recently, BAG-1 was shown to inhibit all-trans-RA-induced growth inhibition by interacting with RAR (Liu *et al.*, 1998a). Therefore, I examined whether BAG-1 can inhibit 4-HPR-induced apoptosis. Surprisingly, overexpression of BAG-1 enhanced apoptosis induced by 4-HPR. Further experiments indicated that distinct regions of BAG-1 protein are necessary for its functions as a pro-apoptotic protein in 4-HPR-induced and an anti-apoptotic protein in staurosporine-induced apoptosis.

4.2 MATERIALS AND METHODS

4.2.1 Construction of BAG-1 cDNA deletion mutations

PCR was employed to delete either N-terminal or C-terminal sequences from BAG-1 B5-2

cDNA (Fig. 4.1). Approximately 100 ng of BAG-1 cDNA plasmid were amplified by PCR using primers corresponding to different BAG-1 cDNA regions and a GeneAmp PCR System 2400 (Perkin Elmer). To produce single protein products, the Kozak sequence (GCCACCATGG) was included in-frame in each 5' primer (Yang *et al.*, 1998b). The conditions for PCR were as follow: 1× PCR buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.01% (w/v) gelatin], 0.5 mM dNTP, 0.2 mM of sense and antisense primers, and 2 U Taq polymerase (Promega) in 20 µl. The PCR program was: 94 °C for 3 min for the first cycle; 94 °C for 30 sec, 60 °C for 1 min and 72 °C for 1 min for a total of 35 cycles; 72 °C for 7 min for the last cycle; and 4 °C on hold. The PCR products were subsequently cloned into a eukaryotic expression vector, PCR3.1, using a TA cloning kit (Invitrogen). The identity of each PCR product was confirmed by sequencing.

4.2.2 Stable transfection of BAG-1 cDNA

Stable transfection of PCR3.1 vector control and BAG-1 cDNA into C33A cells was as described in Chapter 3.2.1.

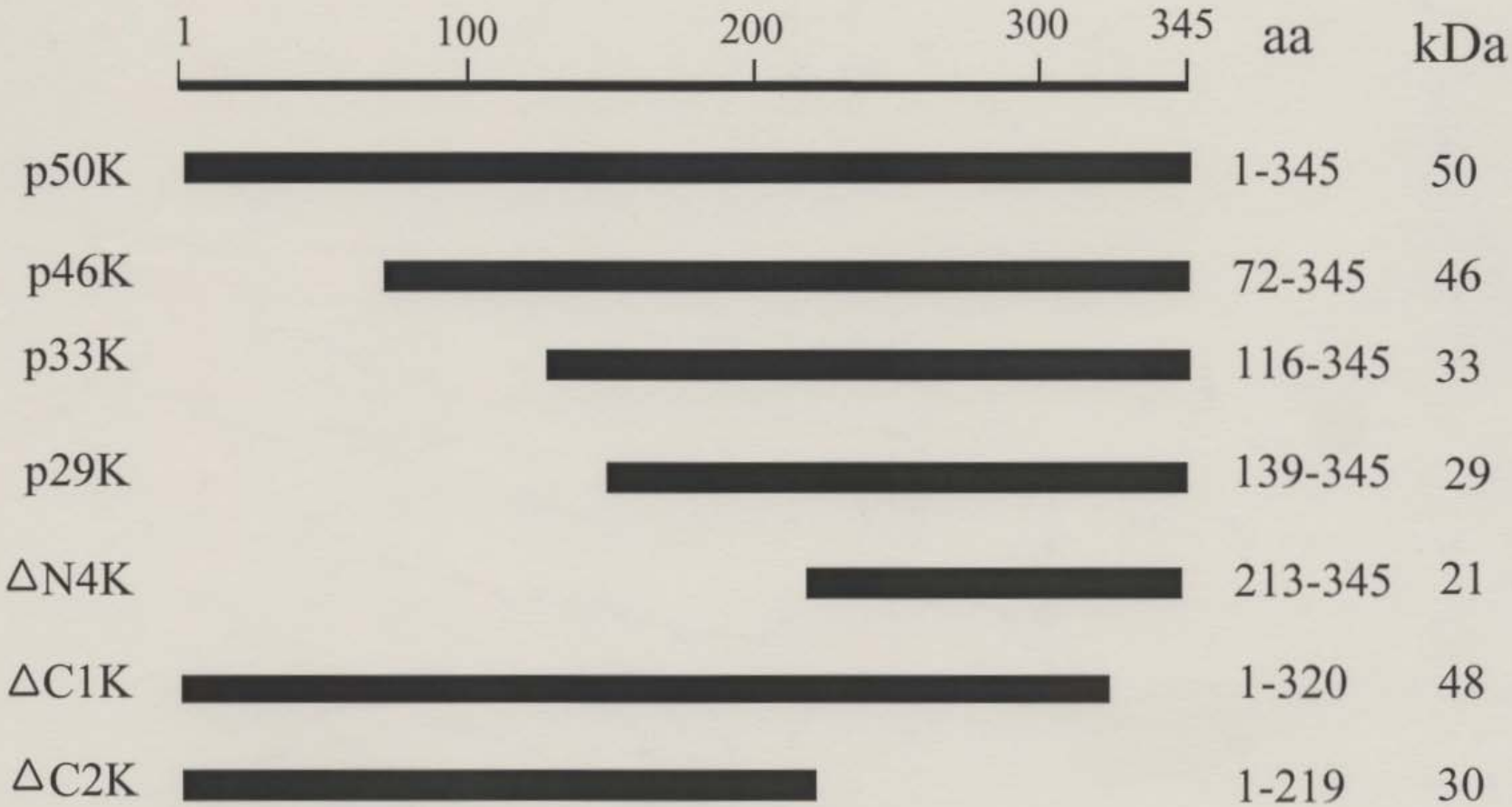
4.2.3 Cell death assays

4.2.3.1 Induction of cell death

To assay cell death induced by various apoptotic stimuli, exponentially growing C33A cells derived from clones overexpressing NEO or full-length or deletion mutated BAG-1 were seeded at 5×10^4 cells/well in 12-well plates and incubated for 24 h. Then, the cells were treated with various agents.

4.2.3.2 Cell viability assays

Fig. 4.1 Deletion mutations of full-length human BAG-1 cDNA. To produce single protein products, the Kozak sequence (GCCACCATGG) was constructed in frame 5' to each cDNA sequence during insertion into a eukaryotic expression vector. Amino acid (a.a) positions are shown on the top. The BAG-1 amino acid positions and apparent molecular weights in kDa (Fig. 4.6) are shown on the right.


1
100
200
300
345
aa
kDa
p50K
1-345
50
p46K
72-345
46
p33K
116-345
33
p29K
139-345
29
ΔN4K
213-345
21
ΔC1K
1-320
48
ΔC2K
1-219
30

Cell viability was determined with the trypan blue exclusion assay after the induction of cell death. In brief, equal volumes of cell suspensions and 4% trypan blue were mixed gently. The viability of the cells was examined immediately by microscopy. Viable cells were unstained, whereas dead cells stained blue. Cell viability represents the percentage of treated cells compared with untreated cells that excluded trypan blue dye. The statistical significance of the difference in cell death between control NEO- and full-length or deletion mutated BAG-1-overexpressing cells was analyzed using the Student t-test.

4.2.3.3 Flow cytometry apoptosis analysis by annexin V-fluorescein isothiocyanate (FITC) assays

To determine the effect of BAG-1 and 4-HPR, apoptosis was analyzed using an Annexin V-FITC Assay kit (Pharmingen), according to the manufacture's instruction. In brief, approximately 10^5 cells were plated in each well of 6-well plates and incubated for 24 h. Cells were treated with 0, 1, 5 or 10 μM 4-HPR for 1 day, and both floating dead cells and attached viable cells were harvested. 10^5 cells were incubated with annexin V-FITC and 5 μg/ml propiodium iodide (PI) in 1× binding buffer (10 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at room temperature in the dark for 15 min. Apoptotic cells were quantified using a Becton Dickinson FACStar Plus flow cytometer. Data analysis was performed with the standard Cell Quest software (Becton-Dickinson). In general, negative annexin V-FITC and negative PI signals represented viable cells; positive annexin V-FITC and negative or intermediate PI staining represented apoptotic cells; while positive annexin V-FITC and high PI staining represented necrotic cells. Apoptotic cells were calculated as the percentage of M1 (see Fig. 4.4) in total cells after subtraction of the percentage for untreated (0 μM) cells.

4.2.3.4 Caspase-3 activity

Cysteine protease caspase-3/CPP32 activity was assayed by colorimetry using an ApoAlert CPP32 protease assay kit (Clontech). In brief, 10^6 cells were plated in each well of 6-well plates and incubated for 24 h. Cells were treated with 10 μM 4-HPR for 0, 2, 4, and 6 h, harvested and lysed on ice for 10 min in 50 μl of ice-chilled lysis buffer. Lysates were incubated with 100 μM DEVD-pNA CPP32 substrate at 37 °C for 1 h and the casapase-3 activity was determined as the absorbance at 400 nm (A_{400}) using a spectrophotometer.

4.2.4 *In vitro* transcription/translation

In vitro transcription/translation of full-length or deletion mutated BAG-1 cDNA was as described in Chapter 3.2.5.

4.2.5 Western blot analysis

Western blot analysis of caspase-3 activation and BAG-1 expression in different stable translated clones was as described in Chapter 3.2.3. The experimental conditions were as described for caspase-3 activity assay (Chapter 4.2.3.4).

4.3 RESULTS

4.3.1 Inhibition of cell death by BAG-1

First, I examined the role of full-length human BAG-1 in cell death induced by various stimuli. Comparing cells overexpressing BAG-1 versus control NEO, cell viability was significantly and similarly enhanced by BAG-1 after inducing cell death by UV irradiation, heat shock,

staurosporine, cisplatin and doxorubicin (Fig. 4.2).

4.3.2 Enhancement of 4-HPR-induced cell death by BAG-1

4.3.2.1 Cell viability

Surprisingly, BAG-1 enhanced 4-HPR-induced cell death. After treatment of cells with 4-HPR, the viability of cells overexpressing BAG-1 was significantly lower than that of NEO vector control cells at 4-HPR concentrations higher than 1 μM (Fig. 4.3). The same results were obtained when other full-length BAG-1-overexpressing clones were examined (data not shown).

4.3.2.2 Apoptosis analysis by Annexin V-FITC assay

To confirm the cell death induced by 4-HPR was due to apoptosis, I analyzed apoptosis by annexin V-FITC staining and flow cytometry in both NEO control and BAG-1-overexpressing cells. Since PI staining in both types of cells for all concentrations of 4-HPR was either negative or intermediate (data not shown), all annexin V-FITC-positive signals represented apoptotic cells. The percentage of apoptotic cells was calculated as the percentage of the M1 in total cell population (Fig. 4.4). The M1 background annexin V-FITC signals detected for both untreated NEO control and BAG-1-overexpressing cells were subtracted from values for the respective treated cells. Consistent with the cell viability assay (Fig. 4.3), more cells died via apoptosis in BAG-1-overexpressing than NEO control cells. While no apoptosis was detected after the cells were treated with 1 μM 4-HPR, 21.5% of NEO control cells and 56.4% of BAG-1-overexpressing cells underwent apoptosis after they were treated with 5 μM 4-HPR. Treated with 10 μM 4-HPR, 54.7% of NEO control cells died through apoptosis, whereas 75.2% of BAG-1-overexpressing cells underwent apoptosis (Fig. 4.4).

Fig. 4.2 Effect of full-length BAG-1 on cell death of C33A cells treated with different apoptotic stimuli. The treatment conditions were as follows: UV irradiation (UV): 50 mJ/cm^2; heat shock (HS): 48 °C for 10 min; staurosporine (St): 4 μM for 2 days; cisplatin (Cis): 10 μM for 2 days; and doxorubicin (Dox): 1 μM for 2 days. Cell viability represents the percentage of treated cells compared with untreated cells that excluded trypan blue dye. *, $p < 0.05$, is the statistical significance of the difference in cell viability between control NEO- and full-length BAG-1-(p50K)-overexpressing cells after treatment.

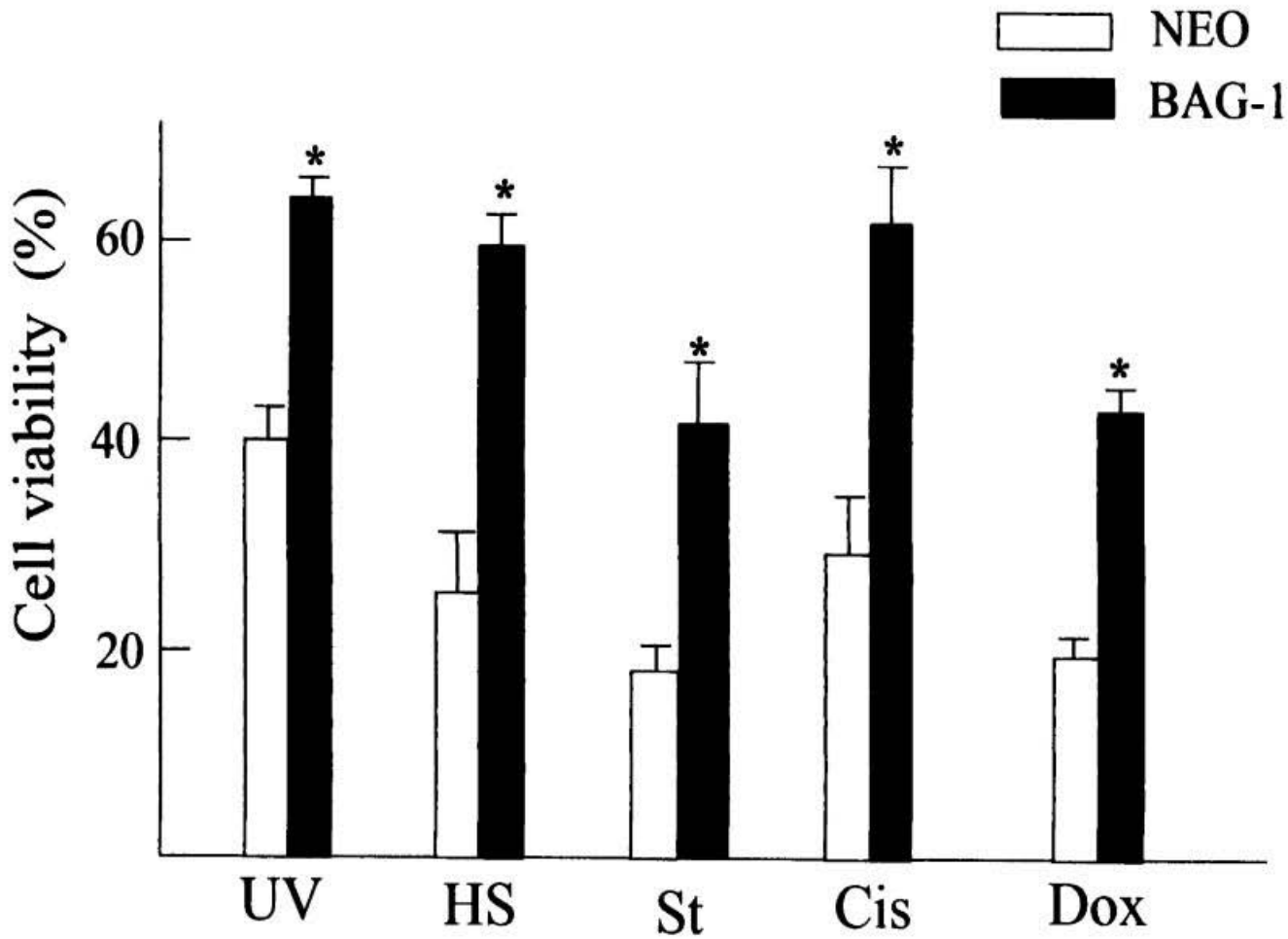

NEO
BAG-1
Cell viability (%)
60
40
20
*
*
*
*
*
UV
HS
St
Cis
Dox

Fig. 4.3 Effect of BAG-1 and 4-HPR concentration on cell death. NEO control cells and full-length BAG-1-overexpressing cells were treated with 0, 0.2, 1.0, 2.0, and 5.0 μM 4-HPR for 2 days. Cell viability represents the percentage of treated compared with untreated cells that excluded trypan blue dye.

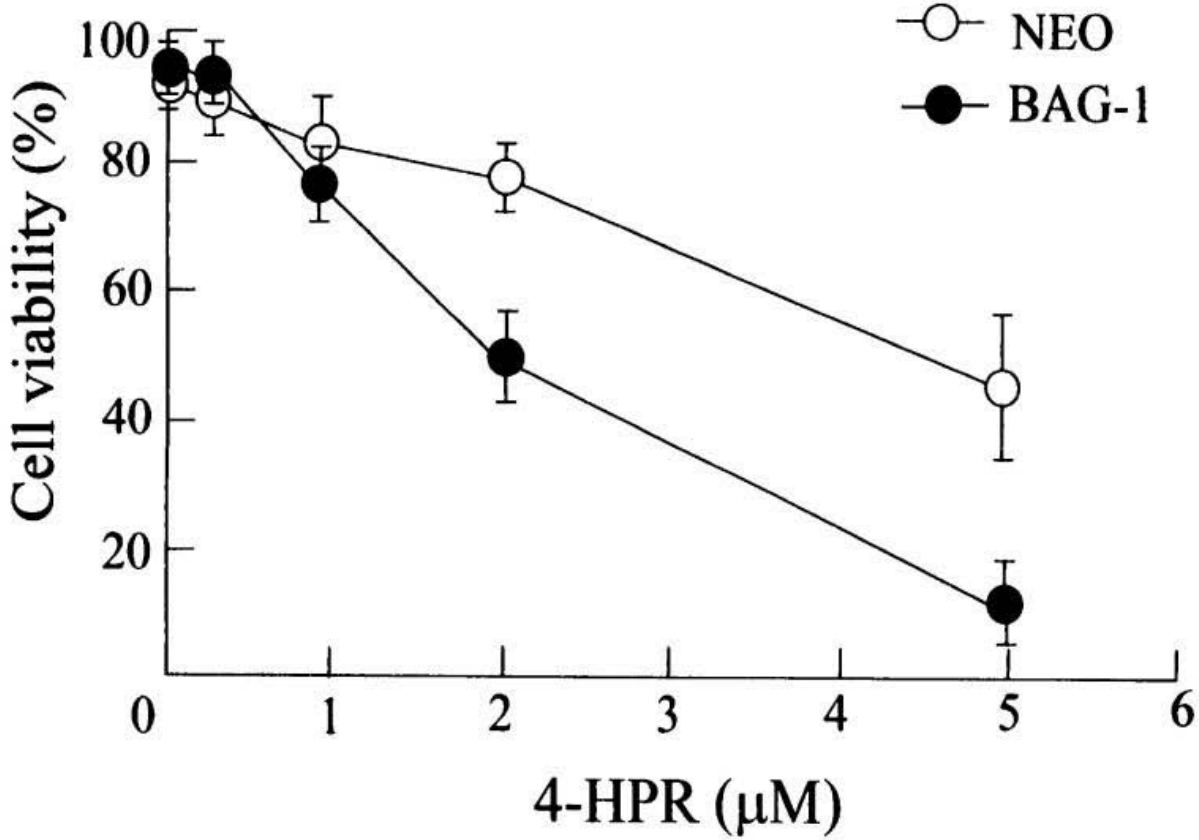
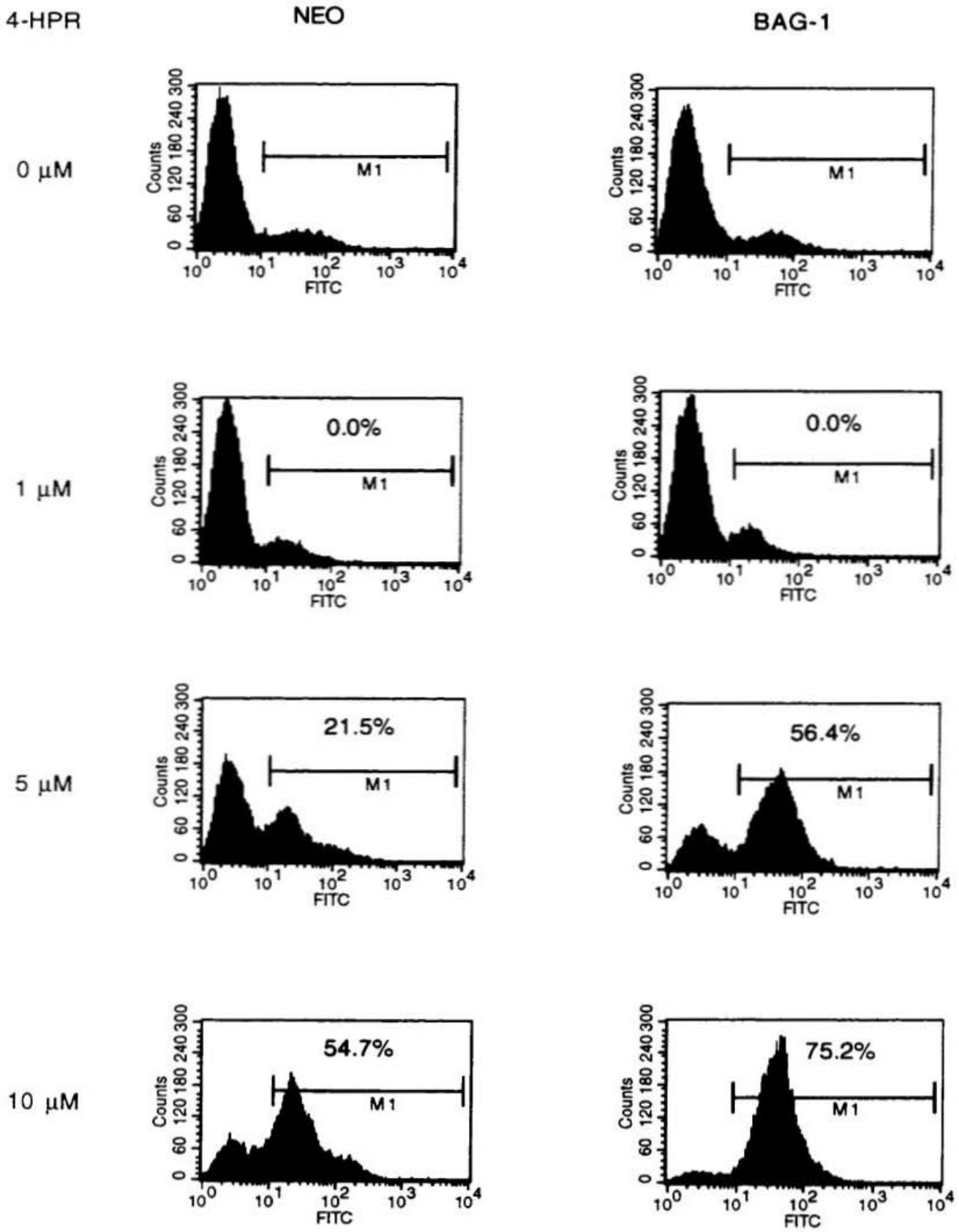

NEO
BAG-1
Cell viability (%)
100
80
60
40
20
0
1
2
3
4
5
6
4-HPR (μM)

Fig. 4.4 Annexin V-FITC assays of the effect of full-length BAG-1 and 4-HPR concentration on apoptosis. Cells were treated with 0, 1.0, 5.0, and 10.0 μM 4-HPR for 1 day. Values were calculated as the percentage apoptotic FITC-positive (M1) of total cell populations after subtraction of the percentage for untreated (0 μM) cells.


4-HPR
NEO
BAG-1
0 μM
1 μM
5 μM
10 μM
Counts
0 60 120 180 240 300
M1
0.0%
21.5%
54.7%
56.4%
75.2%
10^0 10^1 10^2 10^3 10^4
FITC

4.3.2.3 Caspase activity and activation

It has been shown that activation of effector caspases, especially caspase-3, is one of the most important events in apoptosis (for reviews, see Cryns *et al.*, 1998; Nunez *et al.*, 1998). To test whether BAG-1 caused more cell death stimulated by 4-HPR by enhancing caspase-3 activity, caspase-3 activity was compared in BAG-1-overexpressing and NEO control cells after treating the cells for 0-6 h with 10 μM 4-HPR. Maximum caspase-3 activity was detected 4 h after cell treatment. Consistent with the apoptosis assay, caspase activity was significantly higher in BAG-1-overexpressing compared with NEO control cells, indicating that BAG-1 sensitizes cells to apoptosis by a pathway that activate caspase-3 (Fig. 4.5A).

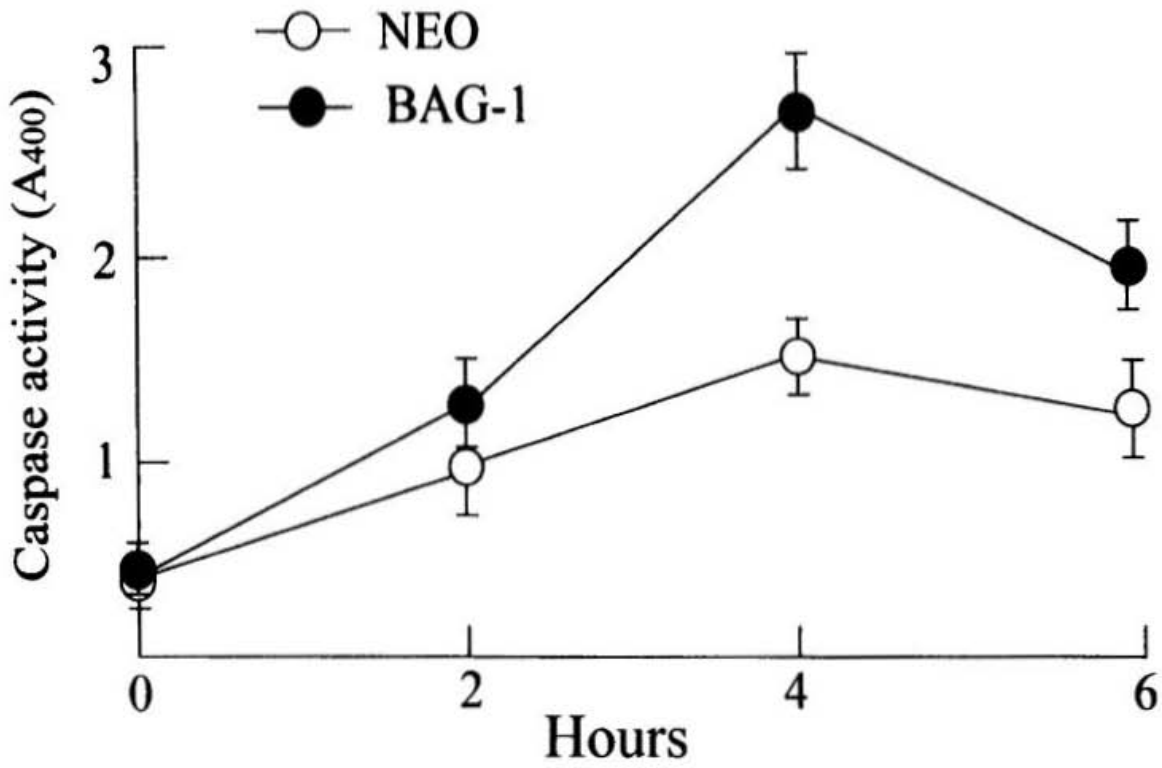
Since the 17 kDa caspase-3 was activated by proteolytic cleavage of 32 kDa procaspase-3 (Stennicke *et al.*, 1998), it was examined whether the enhancement of 4-HPR-induced caspase activity by BAG-1 was due to increased procaspase-3 processing. Correlating well with caspase-3 activity, Western blot analysis showed that a higher level of 17 kDa caspase-3 in BAG-1-overexpressing than NEO control cells (Fig. 4.5B). The highest level of 17 kDa caspase-3 was produced after treatment of both NEO and BAG-1-overexpressing cells with 10 μM 4-HPR for 4 h, as observed for caspase-3 activity (Fig. 4.5A, B). These results indicated that caspase-3 activity in 4-HPR-stimulated BAG-1-overexpressing cells was enhanced and the enhancement was due to increased cleavage of procaspase-3.

4.3.3 Effects of distinct BAG-1 protein functional domains on cell death induced by 4-HPR and staurosporine

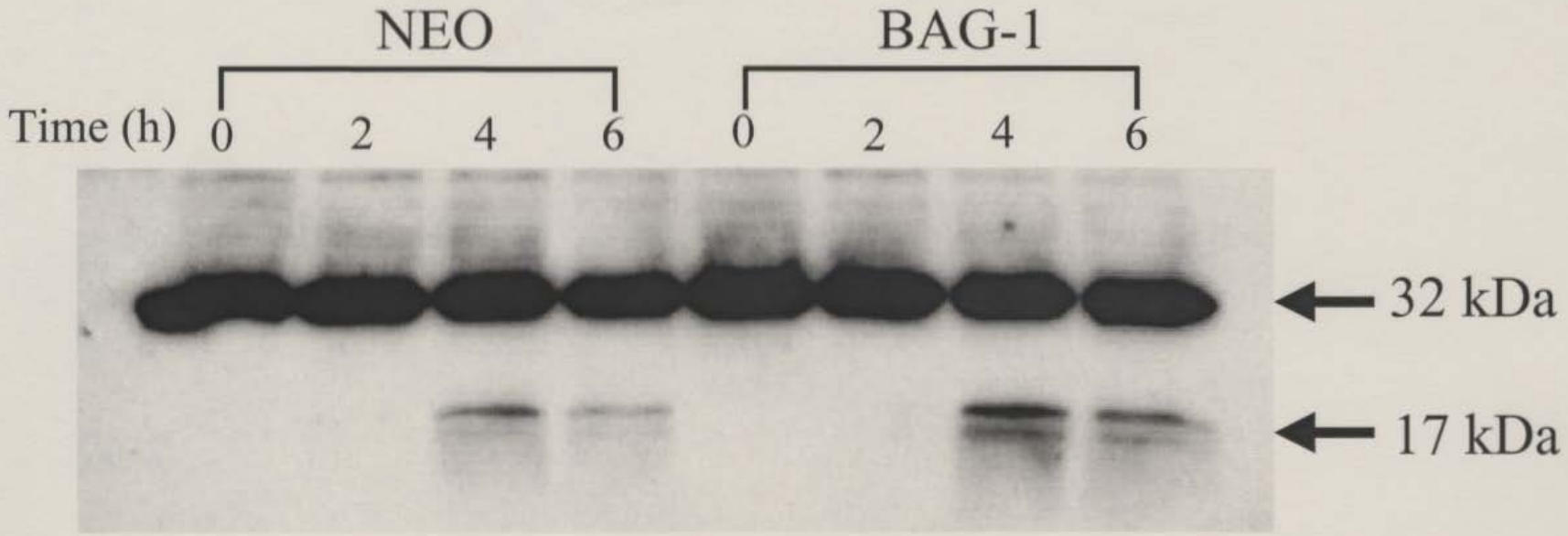
To identify the functional domains of BAG-1 responsible for enhancement of 4-HPR-induced

Fig. 4.5 Time-dependent effect of full-length BAG-1 on caspase-3 activity and proteolytic activation after treatment of cells with 4-HPR. Cells were treated with 10 μM 4-HPR for 0, 2, 4, and 6 h. **A**.Caspase-3 activity was assayed by spectrometry. **B**. Caspase-3 activation. Western blot analysis was used. The indicated 32 kDa protein represents pro-caspase-3, whereas the 17 kDa doublet represents activated caspase-3.

A



B



cell death, two series of plasmids containing nested deletions from N- or C-termini were generated from full-length (p50K) BAG-1 cDNA (Fig. 4.1). Single protein products were produced when each cDNA eukaryotic expression plasmid was *in vitro* transcribed/translated in the presence of [^{35}S]-methionine (Fig. 4.6A). Fig. 4.6B shows the Western blot analysis of BAG-1 proteins from C33A cell clones overexpressing each full-length or deletion mutated BAG-1 protein. Since the EE8C9 anti-BAG-1 monoclonal antibody (Yang *et al.*, 1998b) does not recognize ΔC2, overexpression of ΔC2 was confirmed *in vivo* by Northern blot (data not shown).

In the presence of 4-HPR, full-length BAG-1 p50K significantly decreased cell viability compared with the NEO vector control ($p < 0.05$). Deletion of N-termini in the three other isoforms, p46, p33 and p29, did not alter this inhibition (Fig. 4.1, 4.7). However, further deletion of the central BAG-1 cDNA region in ΔN4K abolished the enhancement of 4-HPR-induced cell death by BAG-1 ($p > 0.05$). Deletion of C-terminal 25 or 124 amino acids did not eliminate the BAG-1-induced enhancement. Instead, cell death was greater ($p < 0.01$). Interestingly, in contrast to cell death due to 4-HPR, staurosporine-induced cell death was unaffected by the ΔN4K deletion of the central BAG-1 region (Fig. 4.8). Moreover, the C-terminal deletions in ΔC1K and ΔC2K BAG-1 proteins abolished the inhibition of cell death induced by staurosporine.

4.4 DISCUSSION

Although 4-HPR has been extensively used for the treatment of human cancers, the molecular mechanism of its action and differential response of tissues to this drug is still unclear. Recent studies suggested that 4-HPR inhibits tumor growth through activation of apoptosis (Delia *et al.*,

Fig. 4.6 *In vitro* and *in vivo* expression of full-length and deletion mutated BAG-1 cDNA. All cDNA expression constructs are described in Fig. 4.1. Molecular weight markers in kDa are shown on the left. **A**. *In vitro* expression of BAG-1 cDNA. BAG-1 cDNA was *in vitro*-transcribed/translated in the presence of [^{35}S]-methionine. The [^{35}S]-labelled protein products were separated by 10% SDS-PAGE and subsequently detected by fluorography. Luciferase was used as a positive control for the reaction. **B**. *In vivo* expression. The vector control plasmid PCR3.1 and BAG-1 cDNA expression plasmids were stably transfected into C33A cells and 10 μg protein were analyzed by Western blot.

A
kDa
66
46
30
21.5
Luciferase
p50K
p46K
p33K
p29K
ΔN4K
ΔC1K
ΔC2K
B
kDa
66
46
30
21.5
p50K
p46K
p33K
p29K
ΔN4K
ΔC1K
ΔC2K
NEO

Fig. 4.7 Effect of BAG-1 functional domains on cell death induced by 4-HPR. Cell viability represents the percentage of 4-HPR-treated cells compared with untreated cells that excluded trypan blue dye. *, $p < 0.05$, and **, $p < 0.01$, are the statistical significance of the difference in cell viability between NEO control cells and cells overexpressing full-length and deletion mutated BAG-1 cDNA treated with 5 μM 4-HPR for 2 days.

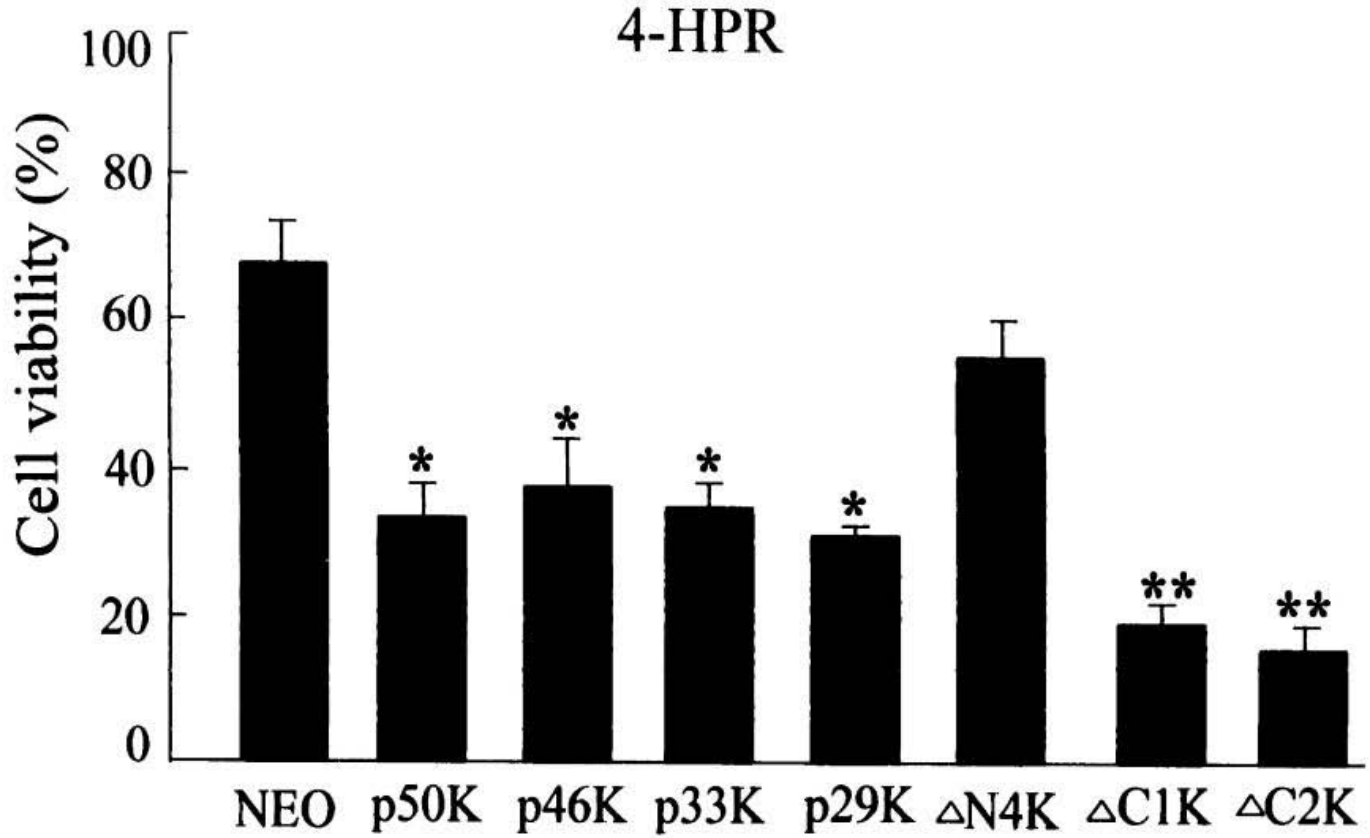
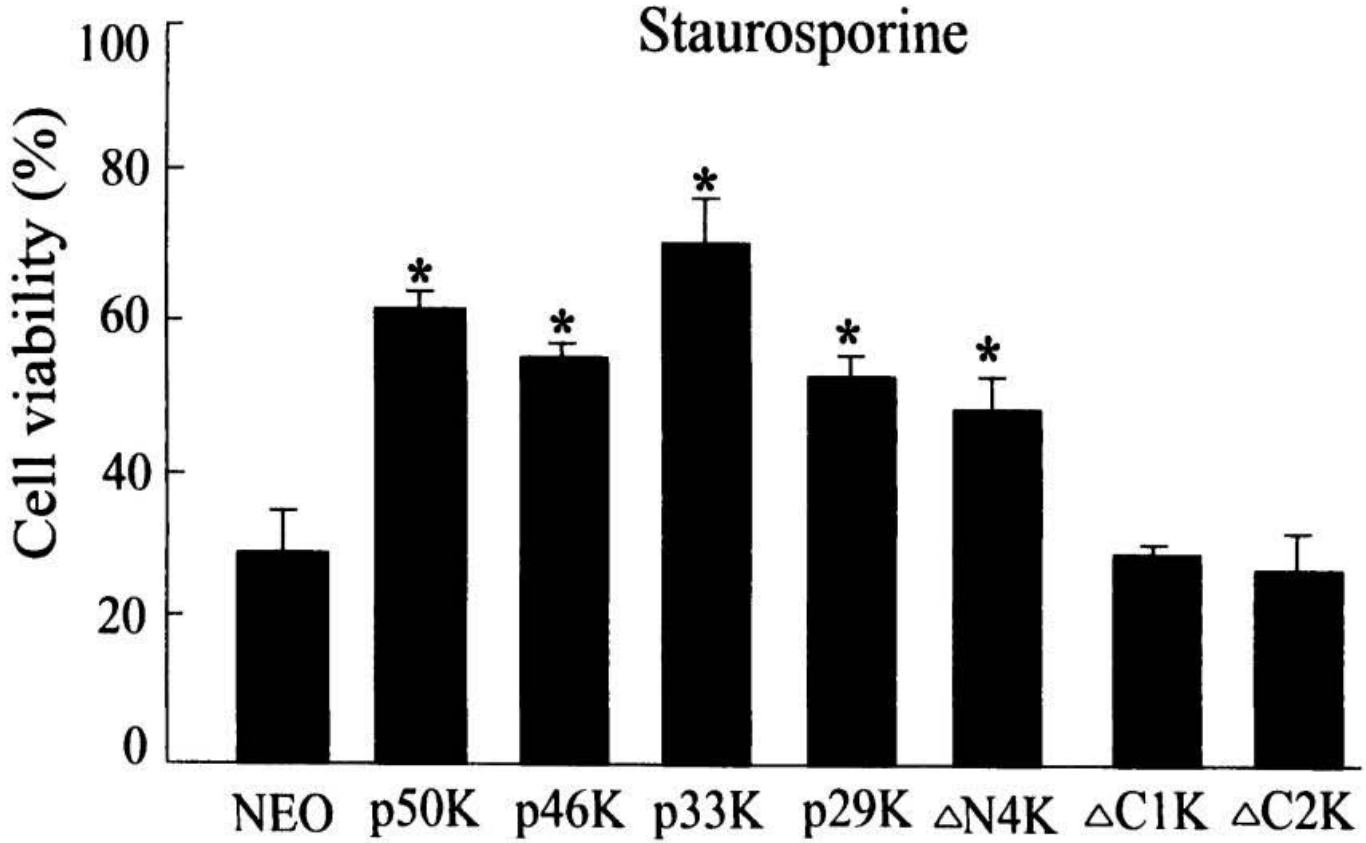

4-HPR
Cell viability (%)
100
80
60
40
20
0
*
*
*
*
**
**
NEO
p50K
p46K
p33K
p29K
ΔN4K
ΔC1K
ΔC2K

Fig. 4.8 Effect of BAG-1 functional domains on cell death induced by staurosporine. Cell viability represents the percentage of staurosporine-treated cells compared with untreated cells that excluded trypan blue dye. *, $p < 0.05$, is the statistical significance of the difference in cell viability between NEO control C33A cells and cells overexpressing full-length and deletion mutated BAG-1 cDNA treated with 0.5 μM staurosporine for 2 days.


Staurosporine
Cell viability (%)
100
80
60
40
20
0
*
*
*
*
*
NEO
p50K
p46K
p33K
p29K
△N4K
△C1K
△C2K

1995; Lotan *et al.*, 1995; Pellegrini *et al.*, 1995; Hsieh and Wu, 1997; Scher *et al.*, 1998; Zou *et al.*, 1998; Montaldo *et al.*, 1999). Therefore, it is possible that the level of apoptotic proteins may affect the sensitivity of cells to 4-HPR. However, previous studies showed that overexpression of Bcl-2, an anti-apoptotic protein that can inhibit apoptosis induced by a variety of apoptosis stimuli (Reed, 1994; 1998a), failed to protect C33A cervical carcinoma cells from apoptosis induced by 4-HPR treatment (Oridate *et al.*, 1997).

Since BAG-1 was originally identified as a Bcl-2-binding protein and can inhibit apoptosis when Bcl-2 was downregulated (Takayama *et al.*, 1995; Yang *et al.*, 1999a), I examined whether BAG-1 can inhibit 4-HPR-induced apoptosis in C33A cells. Surprisingly, although overexpression of BAG-1 inhibited apoptosis induced by a variety of apoptotic stimuli, cell viability studies showed that it sensitized C33A cells to apoptosis induced by 4-HPR. These results were confirmed by Annexin V-FITC flow cytometry assay, which examines early stage apoptosis using annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for the membrane phosphatidylserine (PS). In apoptotic cells, PS is translocated from the plasma membrane inner to outer outer leaflet, where it can tightly bind to annexin V. Similar results were also observed using the U87 glioblastoma cell line (data not shown). In agreement with my results, resistance to 4-HPR-induced apoptosis was found in normal human cervical epithelial cells (Oridate *et al.*, 1997), in which no BAG-1 protein was detected (Chapter 5). Furthermore, overexpression of BAG-1 and Bcl-2 was shown to enhance apoptin-induced apoptosis through a p53-independent pathway (van Oorschot *et al.*, 1997). Since p53 was mutated in C33A cells (Scheffner *et al.*, 1991), apoptosis induced by 4-HPR in this cell line should be p53-independent. Therefore, BAG-1 may enhance p53-independent apoptosis induced by this effective chemotherapeutic retinoid.

Previous and present studies indicated that BAG-1 can inhibit apoptosis induced by apoptotic

stimuli other than 4-HPR such as staurosporine through suppressing caspase-3 activity (Schulz *et al.*, 1997; data not shown). The reason for pro- and anti-apoptotic activity of BAG-1 is unknown. However, the studies reported here found that distinct regions of BAG-1 were responsible for either pro- or anti-apoptotic activity. The C-terminal BAG-1 homology domain, which is highly conserved during evolution (Chapter 2.3.3) and required for interacting with other proteins such as hsp70/hsc70 (Takayama *et al.*, 1998), appears to be additionally important for inhibiting apoptosis induced by both 4-HPR and staurosporine. However, the central ubiquitin homology domain, which might be important for protein-protein interaction (Toniolo *et al.*, 1988) or protein stability (Hershco and Ciechanover, 1992), may support an even greater apoptosis enhancing function that is induced by 4-HPR, but not staurosporine. Therefore, it is possible that BAG-1 may modulate, in a stimulus-specific manner, apoptosis through interaction of different proteins with its ubiquitin-like versus BAG-1 domains.

Although many studies suggest that 4-HPR induces apoptosis through a specific signal transduction pathway such as production of ROS (Delia *et al.*, 1997; Dmitrovsky, 1997; Oridate *et al.*, 1997; Sun *et al.*, 1999), the pathway for 4-HPR-induced apoptosis is still controversial. In this study, 4-HPR was found to induce apoptosis through activating caspase-3, a proteolytic cascade effector in many apoptosis pathways (Fig. 1.2; Nunez *et al.*, 1998; Porter and Janicke, 1999). Similar results were also found by others in different cell systems (Piedrafita and Pfahl, 1997; DiPietrantonio *et al.*, 1998). Therefore, activation of caspase-3 is an important step for 4-HPR-induced apoptosis. However, the increased 4-HPR-induced activation of caspase-3 by BAG-1 was not due to ROS, because activation of caspase-3 after 4-HPR treatment was not inhibited by the ROS inhibitor PDTC (data not shown). Similar findings were also observed by others studying hydrogen peroxide-induced apoptosis (Gotz *et al.*, 1999). Therefore, BAG-1 enhanced 4-HPR-

induced caspase activation through an ROS-independent pathway, possibly through RARs (Sun *et al.*, 1999b).

Cervical cancer is the third most common malignancy in women worldwide and is especially common in developing countries (Parkin *et al.*, 1999). 4-HPR has been used for the treatment of premalignant and malignant cervical neoplasia (Mitchell *et al.*, 1995). The finding that overexpression of BAG-1 enhanced 4-HPR-induced apoptosis may have an important significance in cervical cancer chemotherapy. First, since the level of BAG-1 is relatively higher in human cancer than in normal cervical cells (Chapter 4; Yang *et al.*, 1999a), 4-HPR will probably induce more apoptotic cell death in cancer than normal cells. This may be why 4-HPR has not only high tumor tissue cytotoxicity through apoptosis but also lower normal tissue cytotoxicity, than other drugs (Rotmensz *et al.*, 1991; Moon *et al.*, 1992; Lotan, 1995; Abou-Issa *et al.*, 1997; Pienta *et al.*, 1997). Second, BAG-1 may be useful to assess the efficacy of 4-HPR for individual cervical carcinomas, because this protein was found at various levels in different cervical tumor biopsies (Chapter 4, 5, 6). Third, it may be possible to enhance 4-HPR chemotherapy by introducing a BAG-1-expressing adenovirus vector into 4-HPR-resistant cervical cancer cells. To test the relevance of the *in vitro* results presented here *in vivo*, the correlation of BAG-1 levels with the response to 4-HPR needs to be examined in normal and cancer tissues from patients.

SECTION 4

ROLE OF HUMAN BAG-1 AND OTHER ANTI-APOPTOTIC PROTEINS IN TUMORIGENESIS

CHAPTER 5

OVEREXPRESSION OF ANTI-APOPTOTIC GENE BAG-1 IN HUMAN CERVICAL CANCER

5.1 INTRODUCTION

Cervical cancer is the third most frequent malignancy among women in the world (Parkin *et al.*, 1999). In the past 10 years, compelling evidence confirmed the hypothesis that HPVs, especially high risk HPVs such as HPV16 and HPV18, are the major contributing factor of this cancer. Furthermore, *in vitro* and *in vivo* studies strongly suggest that HPV infection is necessary but not sufficient for causing cervical cancer. Other factors, such as hormone, cigarette smoke and dysregulation of oncogenes and tumor suppressor genes, are also required for full malignant transformation (Herrington, 1995; Yang *et al.*, 1996b; Yang *et al.*, 1997). However, the molecular mechanism of the multistep, multifactor oncogenesis of cervical cells remains unclear.

Recently, overexpression of Bcl-2 was found in cervical carcinoma cell lines and premalignant and malignant tissues (Liang *et al.*, 1995; ter Harmsel *et al.*, 1996; Pillai *et al.*, 1996), suggesting that enhanced level of anti-apoptosis proteins may underlie the development of cervical cancer. However, the expression of apoptosis-associated proteins other than Bcl-2 and p53 has not been examined in cervical cancer. In this report, I investigated the expression of BAG-1 in cervical oncogenesis. The results show that both BAG-1 RNA and protein were overexpressed in human cervical carcinoma cell lines and tissues, indicating that BAG-1 may play an important role in the development of cervical cancer.

5.2 MATERIALS AND METHODS

5.2.1 Containment for biohazards

Level 2 biohazard containment procedures recommended by the MRC of Canada were followed for all tissue culture and experiments using human cervical biopsies. The experiments were approved by the Memorial University of Newfoundland Biosafety Committee.

5.2.2 Cell culture and establishment of BAG-1-overexpressing transfectant

Human endocervical cells (HEN) and ectocervical cells (HEC) were obtained after dissection of pathologically normal cervical tissues derived from hysterechtomies performed for benign conditions, and were maintained in serum-free media for keratinocytes (GIBCO BRL, Bethesda, MD). All cervical carcinoma cell lines were obtained from ATCC and cultured in DMEM containing 10% FCS.

The transfection of p50K (BAG-1) and a control PCR3.1 plasmid (NEO) into C33A and the establishment of a transfectant line was as described Chapter 3.2.1.

5.2.3 Cell viability assays

To assay cell viability of cervical cells after treatment with a DNA damaging reagent, staurosporine, exponentially growing cells were seeded at 5×10^4 cells/well in 12-well plates and incubated for 24 h. Then the cells were treated with either 0.2 μM staurosporine for the indicated times for the experiments comparing primary and cancer cells or 0.5 μM staurosporine for the indicated times for the experiments with stably BAG-1-transfected C33A cells. Cell viability was determined with the trypan blue exclusion assay as described in Chapter 4.2.3.2.

5.2.4 RNA and protein extraction from human cervical cell lines or biopsies

Total RNA was extracted from cervical normal and carcinoma cells at 70% confluence using the CsCl gradient centrifugation method (Sambrook et al., 1989). Protein was extracted from 10^7 cells or 0.1 g biopsies by lysis in 1 ml ice-cold extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 100 μg/ml of PMSF, 50 μg/ml of aprotinin) for 30 min and centrifugation at 12,000 g for 10 min.

5.2.5 Northern, Western and Southern blot analysis

Northern blot and Western blot analysis of cellular gene expression was as described in Chapters 2.2.4 and 3.2.3. CC9E8 anti-hBAG-1 mAb was generated, as described in Chapter 3.2.1. Anti-Bcl-2 and anti-β-actin mAbs, and anti-Bcl-X_L and anti-GR polyclonal antibodies, were purchased from Santa Cruz Inc. (Santa Cruz, CA).

DNA extraction and Southern blot analysis of high molecular weight genomic DNA from cervical cells was as described (Sarma *et al.*, 1996).

5.3 RESULTS

5.3.1 Overexpression of BAG-1 protein in cervical carcinoma cell lines and tissues

I first examined the expression of BAG-1 protein in normal and cervical carcinoma cell lines using Western blot analysis to determine if BAG-1 is involved in the oncogenesis of cervical cells. While no BAG-1 protein was detected in primary HEN and HEC, three (p50, p46 and p33) of the four isoforms of BAG-1, were overexpressed in all the cervical carcinoma cell lines. The shortest

isoform of BAG-1, p29, was only expressed in SiHa and HeLa cells, in which the highest protein level of p50, p46 and p33 was found (Fig. 5.1). Since synergistic effect of BAG-1 and Bcl-2 in anti-apoptosis was found (Takayama *et al.*, 1995), I examined whether the level of BAG-1-interacting proteins, Bcl-2, Bcl-X_L and GR, were also enhanced in cervical carcinoma cell lines. As was seen for BAG-1, no or low expression of Bcl-2, Bcl-X_L and GR was found in primary HEN and HEC, whereas Bcl-2, Bcl-X_L and GR were overexpressed in three (CaSki, HeLa and C33A), five (SiHa, HeLa, C4-I, C33A and HT-3) and six (CaSki, SiHa, HeLa, C4-I, C33A and HT-3) of the six cervical carcinoma cell lines (Fig. 5.1). The expression levels of Bcl-X_L and GR were correlated with that of BAG-1. However, the expression level of Bcl-2 and BAG-1 did not correlate. For example, the highest expression of BAG-1 was found in SiHa cells, whereas no Bcl-2 was detected in this cell line. On the other hand, the lowest level of BAG-1 protein was found in C33A cells, while the highest level of Bcl-2 was detected in this cell line (Fig. 5.1).

To compare my results for cell lines with those *in vivo*, I examined the expression of BAG-1 and its interacting proteins in primary normal and cervical carcinoma tissues. In general, BAG-1 protein was low or not detected in normal cervical tissues, but it was overexpressed in most of the cervical carcinomas. Each isoform of BAG-1 displayed distinct pattern in these tissues. p50 was detected in only two of eight (25%) normal cervical tissues, whereas it was overexpressed in six of eight (75 %) cervical carcinomas; p46 was not expressed in any of the normal tissues, whereas it was detected in two of eight (25 %) cervical carcinomas; p33 was not present in any of the normal tissues, whereas it was overexpressed in seven of eight (88 %) cervical carcinomas; p29 was not detected in any of the tissues examined (Fig. 5.2). On the other hand, in contrast to the cultured cells, all three BAG-1-interacting proteins, Bcl-2, Bcl-X_L and GR, were expressed on average at comparable levels in both cervical normal and carcinoma tissues (Fig. 5.2).

Fig. 5.1. Overexpression of BAG-1 and its interacting proteins in cervical carcinoma cell lines. For Western blots, 10 μg of proteins from normal HEN and HEC and cervical carcinoma cells were separated in a 10% SDS-PAGE gel and transferred to an ECL nitrocellulose membrane. Each protein was detected by the ECL system using primary antibodies to BAG-1, Bcl-2, Bcl-X_L and GR, and then secondary HRP-conjugated anti-mouse IgG. β-actin was used as an internal control.

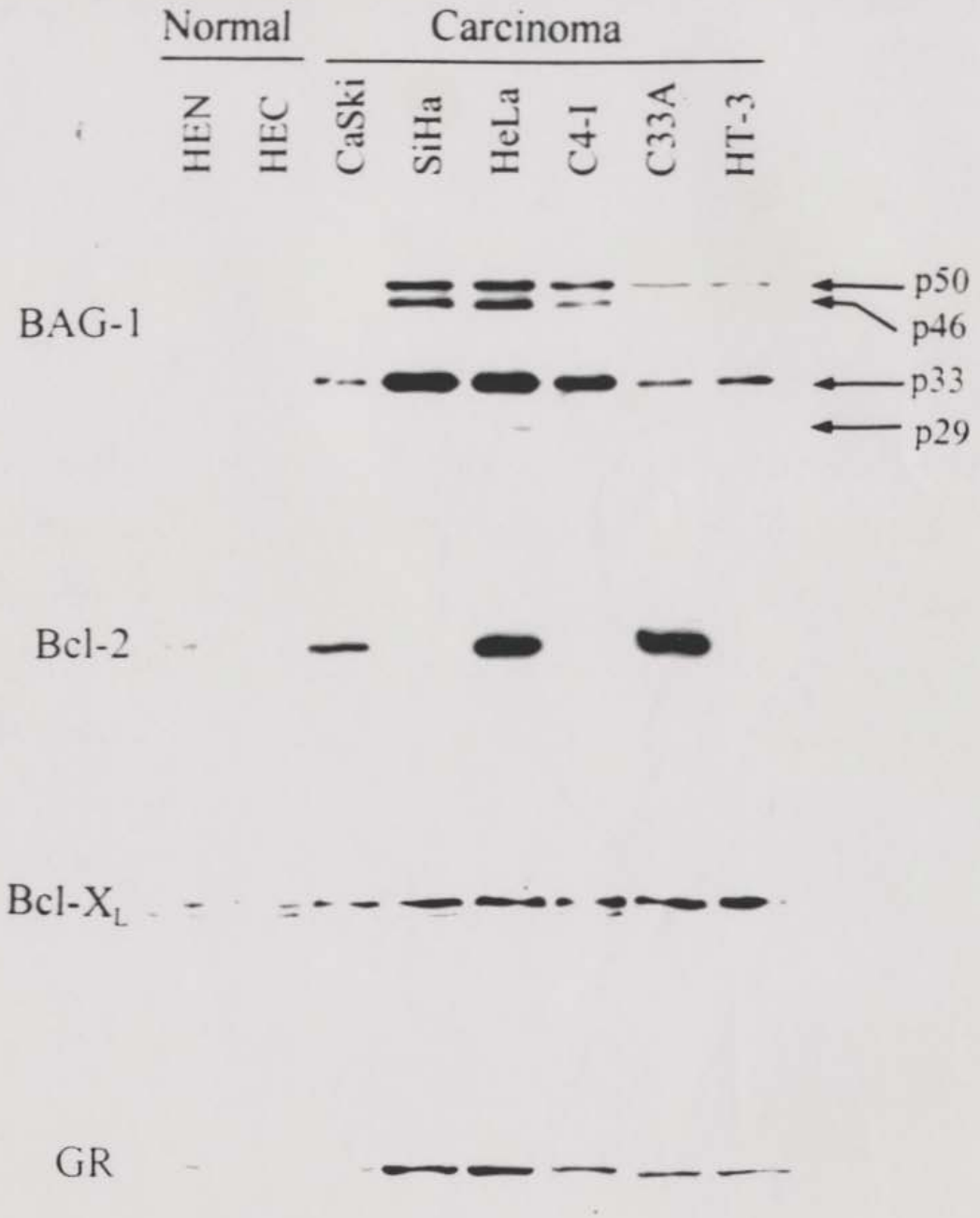
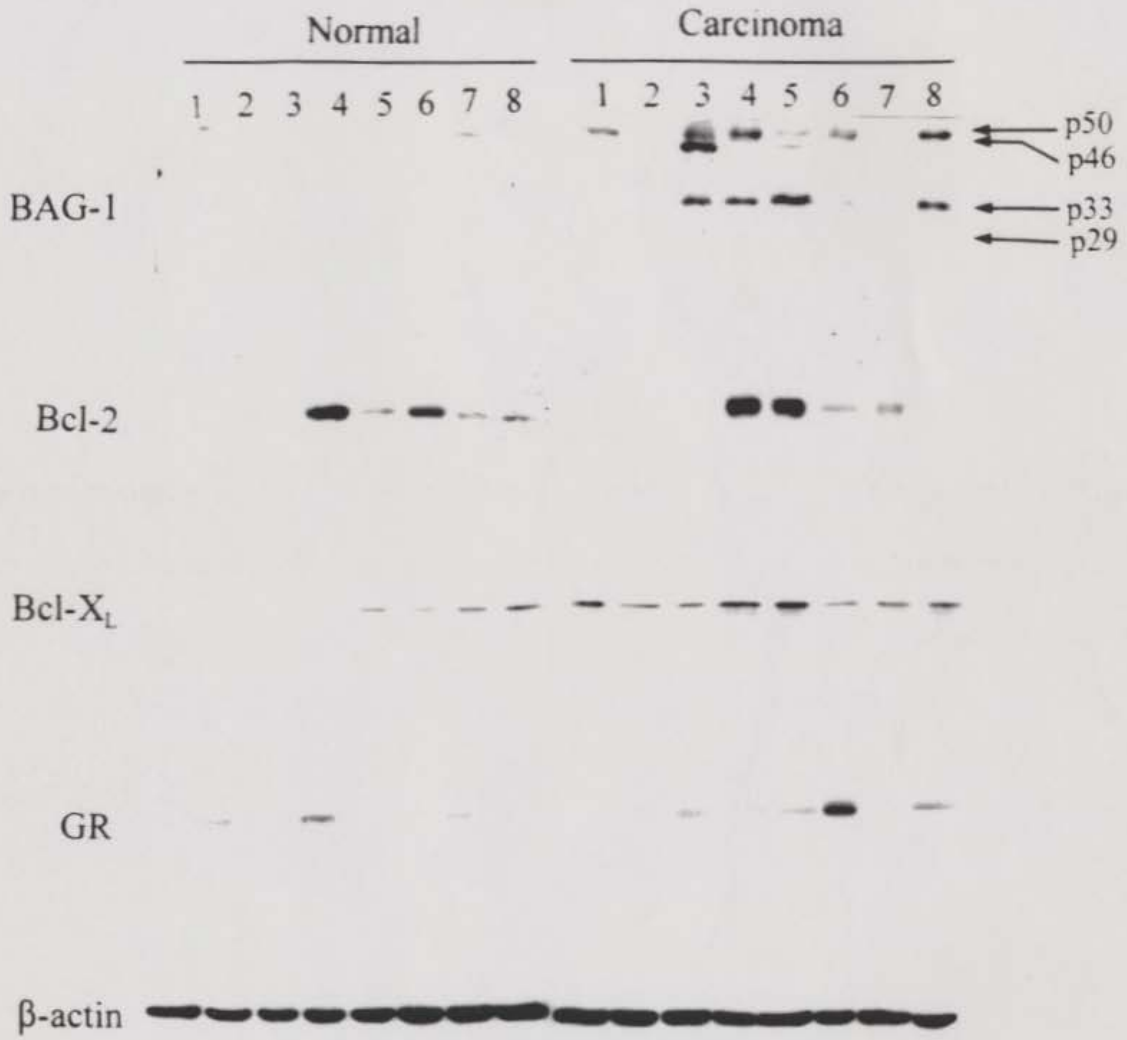

Normal
Carcinoma
HEN
HEC
CaSki
SiHa
HeLa
C4-1
C33A
HT-3
BAG-1
p50
p46
p33
p29
Bcl-2
Bcl-X$_L$
GR

Fig. 5.2. Overexpression of BAG-1 protein in cervical carcinoma tissues. Western blot methods and labels were as described in Fig. 5.1. Each lane numbered 1-8 represents different cervical normal or carcinoma tissues.


Normal
Carcinoma
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
p50
p46
p33
p29
BAG-1
Bcl-2
Bcl-X$_L$
GR
β-actin

5.3.2 RNA expression and integrity of the BAG-1 gene in cervical normal and carcinoma cell lines

To investigate whether the overexpression of BAG-1 protein in various cancer cell lines is caused by enhanced transcription, I analyzed the expression of BAG-1 RNA from cervical cultured normal and cancer cells by Northern blot. My results showed that the expression of BAG-1 RNA (Fig. 5.3) correlated well with that of BAG-1 protein (Fig. 5.1), suggesting that the overexpression of BAG-1 protein is caused by increased transcription of BAG-1 gene.

Since high level of *bcl*-2 expression in follicular lymphoma is thought to be a consequence of the t(14;18) chromosomal translocation of the *bcl*-2 gene (Graninger *et al.*, 1987), I used Southern blot analysis to examine whether a genomic rearrangement might underlie the increased transcription of BAG-1 RNA in cervical carcinoma cells. When DNA from cervical normal and carcinoma cells was digested with *Eco*RI, four BAG-1 DNA fragments with molecular weights of 2.0, 3.0, 8.1 and 15.2 kb were present in all the cell types (Fig. 5.4). When the DNA was cleaved with *Pst*I, four BAG-1 DNA fragments with molecular weights of 0.7, 1.8, 5.0 and 10.0 kb were found from all the samples. Compared to the band pattern of HEN and HEC DNA, no loss or gain of BAG-1 bands were detected in any of the cervical carcinoma cell lines.

5.3.3 Effect of BAG-1 overexpression on the resistance of cervical carcinoma cells to apoptosis induced by staurosporine

BAG-1 was originally identified as an anti-apoptotic protein (Takayama *et al.*, 1995). Therefore, I examined whether enhanced resistance to apoptosis in cervical carcinoma cells was correlated with the overexpression of BAG-1. As expected, decreased cell viability or enhanced

Fig. 5.3. Overexpression of BAG-1 RNA in cervical carcinoma cell lines. For Northern blots, 20 μg of total RNA from normal HEN and HEC and cervical carcinoma cells were size-fractionated on a 1.0% agarose formaldehyde gel, transferred to nitrocellulose membranes and hybridized with [^{32}P]-labelled BAG-1 cDNA probe. β-actin was used as an internal control.

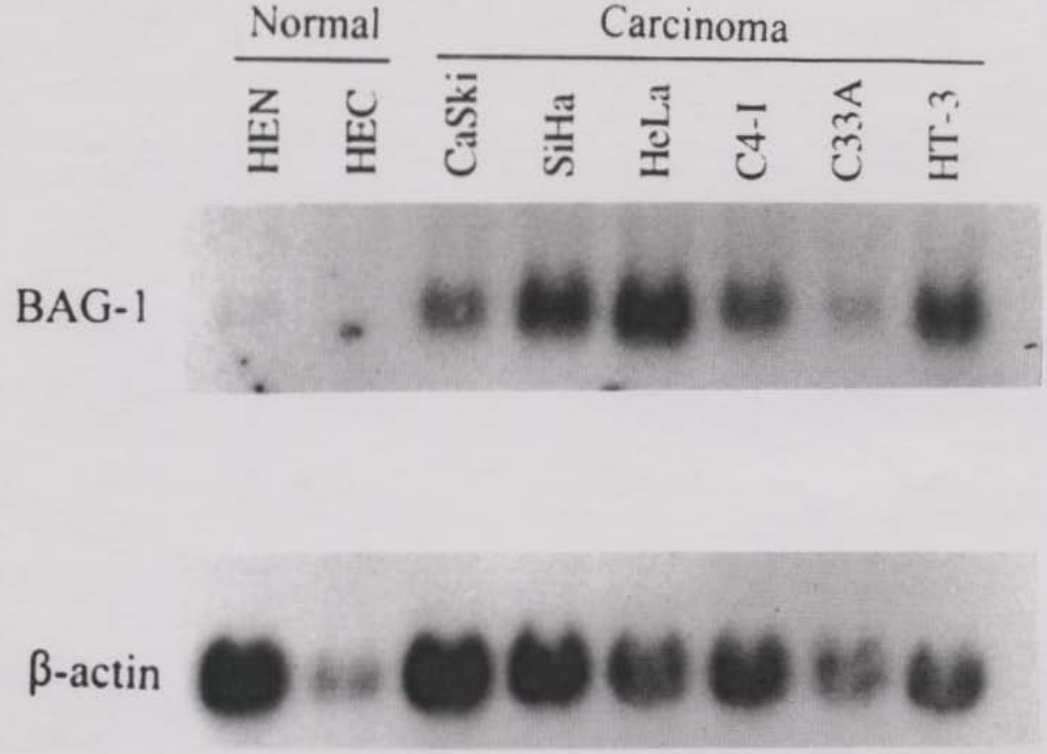
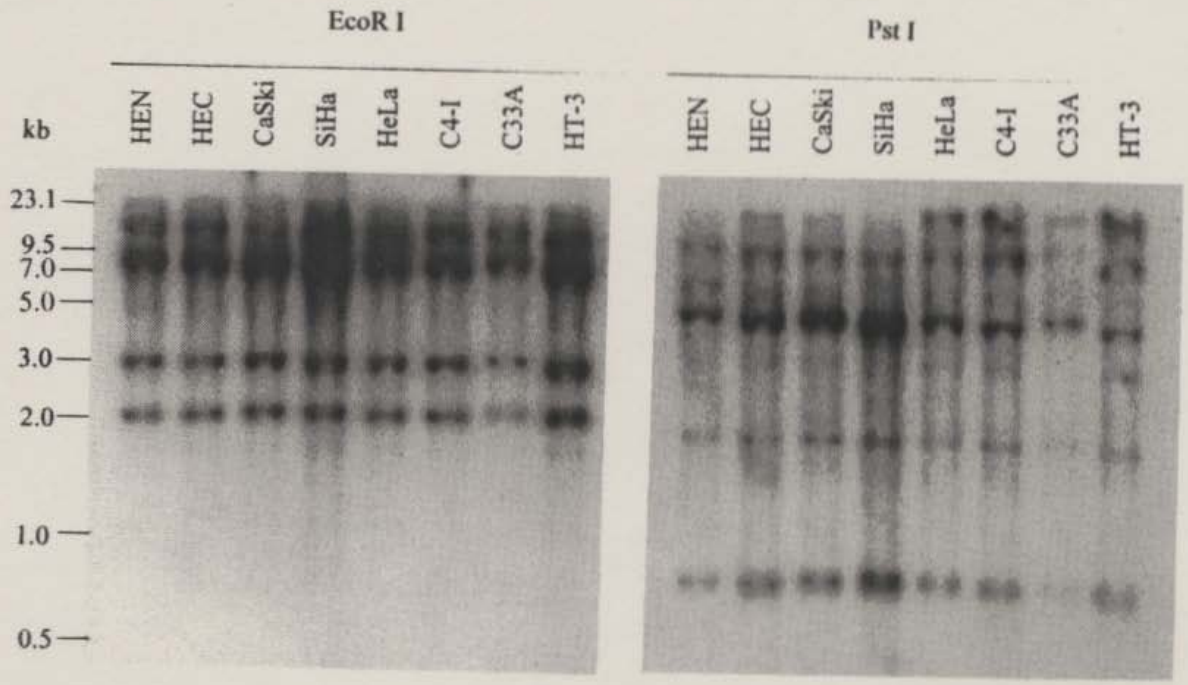

Normal
Carcinoma
HEN
HEC
CaSki
SiHa
HeLa
C4-1
C33A
HT-3
BAG-1
β-actin

Fig. 5.4. Southern blot analysis of BAG-1 in genomic DNA from cervical normal and carcinoma cells. Ten µg of high molecular weight genomic DNA was digested with *Eco*RI or *Pst*I, separated in a 1% agarose gel and transferred to a Hybond-NX nylon membrane. BAG-1 DNA was detected by probing the blot with [^{32}P]-labelled full-length human BAG-1 cDNA. Molecular weight markers are shown on the left.


EcoR I
Pst I
kb
HEN
HEC
CaSki
SiHa
HeLa
C4-I
C33A
HT-3
23.1
9.5
7.0
5.0
3.0
2.0
1.0
0.5

resistance to apoptosis correlated well with overexpression of BAG-1 p50. As shown in Fig. 5.5, while all the HEN and HEC cells died after treatment with 0.2 μM staurosporine for 24 h, the viability of cervical carcinoma cells (CaSki, SiHa, HeLa, C4-I, C33A and HT-3) was over 80% after 48 h treatment.

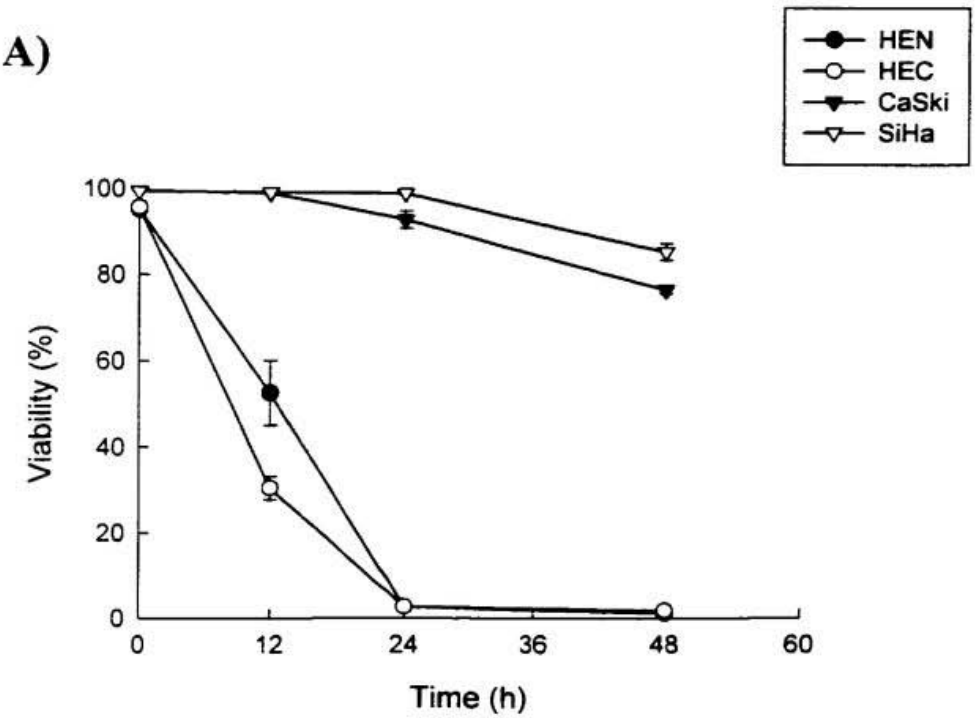
To examine whether BAG-1 is directly involved in the resistance of cervical carcinoma cells to apoptosis, I stably transfected BAG-1 cDNA into the C33A cervical carcinoma cell line, which expressed low level of BAG-1 (Fig. 5.1). Since C33A was relatively resistance to apoptosis induced by 0.2 μM staurosporine, I assayed apoptosis after treatment of the cells with 0.5 μM staurosporine. Because distinct isoforms of human BAG-1 have similar function in anti-apoptosis (unpublished data), only the result for p50 was shown in Fig. 5.6. The viability was significantly higher in the cells overexpressing BAG-1(BAG-1) than in the cells expressing vector plasmid (NEO) after treatment with 0.5 μM staurosporine for 6 days ($p < 0.05$; Fig. 5.6), indicating that overexpression of BAG-1 enhanced the resistance of cervical cells to apoptosis.

5.4 DISCUSSION

A number of studies support the concept that HPVs, a family of small double-stranded circular DNA viruses, are the major cause of cervical cancer (zur Hausen, 1991; zur Hausen and de Villiers, 1994; zur Hausen, 1996). High risk HPVs, in particular HPV16 and HPV18, have been detected in 90% of cervical carcinoma biopsies (Riou *et al.*, 1990; zur Hausen, 1996). Since cooperation of the E6 and E7 proteins of high risk HPVs is sufficient to immortalize human cervical keratinocytes and epithelial cells, they have been considered to be oncogenic proteins important for cervical carcinogenesis (for review, see zur Hausen and de Villiers, 1994; MacDougall, 1994). E6 and E7 proteins were shown to bind to tumor suppressor proteins p53 and Rb, respectively, and to inactivate

Fig. 5.5. Resistance of human cervical carcinoma cells to apoptosis induced by staurosporine. Cervical normal (panel A) and carcinoma (panels A and B) cells were treated with 0.2 μM staurosporine for the indicated times. The percentage of viable cells was determined by trypan blue exclusion assays. The results represent the mean ± the standard deviation of three experiments.

A)


HEN
HEC
CaSki
SiHa
Viability (%)
100
80
60
40
20
0
0
12
24
36
48
60
Time (h)

B)

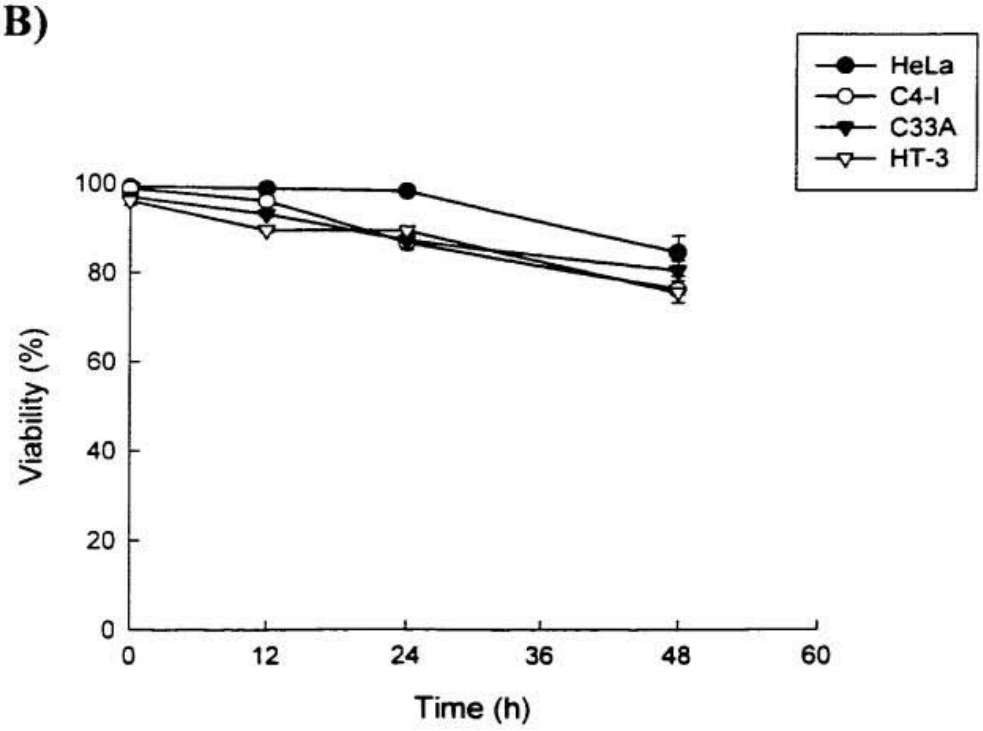
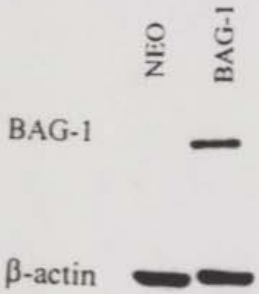
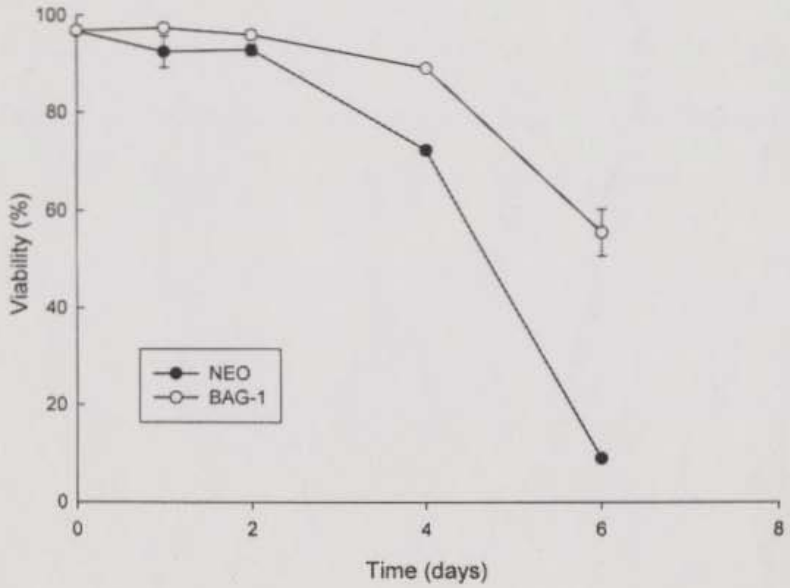

HeLa
C4-I
C33A
HT-3
Viability (%)
100
80
60
40
20
0
0
12
24
36
48
60
Time (h)

Fig. 5.6. Enhanced resistance to staurosporine-induced apoptosis by overexpression of BAG-1 cDNA in C33A cells. The vector control plasmid PCR3.1 (NEO) and the plasmid containing p50K cDNA (BAG-1) were stably transfected into C33A cells. (A) Overexpression of BAG-1 p50 in C33A cells. Western blot methods and labels were as in Fig. 5.1. (B) Resistance to apoptosis induced by stauroporine in BAG-1-overexpressing C33A cells. The cells were treated with 0.5 μM staurosporine for the indicated times. Detection of apoptosis and presentation of the data were as described in Fig. 5.5.

A


NEO
BAG-1
BAG-1
β-actin

B


100
80
60
40
20
0
Viability (%)
NEO
BAG-1
0
2
4
6
8
Time (days)

their tumor suppressor activity, leading to uncontrolled cell growth (Munger *et al.*, 1989; Werness *et al.*, 1990; McDougall, 1994; zur Hausen, 1998). Therefore, inactivation of p53 and Rb by HPV16 E6 and E7 was considered to be one of the necessary steps toward transformation of cervical cells.

p53 is a tumor-suppressor gene that functions as a participant in cell cycle control, DNA synthesis and repair, and maintenance of genomic stability (for review, see Levine, 1997). Deletions or mutations of *p53* have been detected in about 50% of human cancers (Hollstein *et al.*, 1991; Levine, 1997). Recently, *p53* was also found to modulate apoptosis by upregulating the expression of the pro-apoptotic gene *bax* and downregulating the anti-apoptotic gene *bcl*-2 (Miyashita *et al.*, 1994a, b; Miyashita *et al.*, 1995). Since p53 was inactivated in a variety of human cancers, it was consistent that enhanced expression of Bcl-2 was observed in these cancers (Tsujimoto and Croce, 1986; Hollstein *et al.*, 1991; Reed *et al.*, 1991; McDonnell *et al.*, 1992; Halder *et al.*, 1994; Ikegaki *et al.*, 1994; Liang *et al.*, 1995; Sinicrope *et al.*, 1995). In this study, I provided the first evidence that anti-apoptotic protein BAG-1, a Bcl-2-interacting protein, is overexpressed in human cervical carcinoma cell lines and tissues and that overexpression is due to increased transcription of the BAG-1 gene. On the other hand, overexpression of BAG-1-interacting proteins, Bcl-2, Bcl-X_L and GR, was found in only cervical carcinoma cell lines but not cervical carcinoma tissues. Enhanced expression of Bcl-2 in cervical cancer cell lines and tissues has also been reported previously (ter Harmsel *et al.*, 1996; Pillai *et al.*, 1996; ter Harmsel *et al.*, 1997). One likely explanation for the discrepancy between my results and those of others for the expression of Bcl-2 in cervical carcinoma tissues is that all the previous studies used immunocytochemistry in which the level of Bcl-2 represented the percentage of cells stained but not the total signal intensity from all the cells as presented in my data using Western blot analysis. However, my results suggest that BAG-1 may serve as a better molecular marker than Bcl-2, Bcl-X_L or GR for the oncogenesis of cervical cells *in*

vivo. Because only eight normal and eight cancerous samples were used in this study, analysis of a larger number of samples will be performed to address this suggestion.

The exact mechanism for the enhanced expression of BAG-1 in cervical cancers is unknown. Since p53 was inactivated by either its mutation (C33A and HT-3) or HPV (CaSki, SiHa, HeLa and C4-I) in cervical carcinoma cell lines (Scheffner *et al.*, 1991), the enhanced expression of BAG-1 may be the result of loss of transcriptional repression by p53. Consistent with this hypothesis, enhanced expression of BAG-1 was also found in HPV16-immortalized HEN in which p53 was reduced (Yang *et al.*, 1998a) probably through its degradation by HPV16 E6. I have cloned the promoter region of the BAG-1 gene (Chapter 8). However, the BAG-1 promoter was not suppressed by wild-type p53 (Chapter 8). Since p53 can suppress bcl-2 transcription through the bcl-2 5'-untranslated region (UTR) sequence (Miyashita et al., 1994b), it remains possible that p53 may negatively regulate BAG-1 transcription via its 5'-UTR sequence.

Although the role of BAG-1 in apoptosis has been extensively studied, it had not been examined in cervical cells. This study provided the first evidence that overexpression of BAG-1 in cervical carcinoma cells may partially contribute to their enhanced resistance to apoptosis induced by DNA-damaging reagents. The enhanced resistance to induction of apoptosis in cervical carcinoma cells is not due to a reduced expression of the pro-apoptotic proteins Bax and Bak, since no difference in Bax and Bak expression was found compared with that in HEN and HEC (unpublished data). Although the increase of Bcl-X_L in cervical carcinoma cells was not pronounced compared with that in normal cervical cells, enhanced expression of Bcl-X_L may also contribute to the enhanced resistance of cervical carcinoma cells to apoptosis. In addition, my results suggest that BAG-1 and Bcl-2 have important independent compensatory effects in apoptosis, apart from the synergistic effect found by Takayama *et al.* (1995). For example, overexpression of BAG-1 but not Bcl-2 was

found in SiHa cervical carcinoma cells that had enhanced resistance to apoptosis. On the other hand, much higher level of Bcl-2 than BAG-1 was detected in C33A cervical carcinoma cells that also exhibited enhanced resistance to apoptosis.

In conclusion, my study provided the first evidence that BAG-1 protein is overexpressed in human cervical carcinoma cell lines and tissues. Reduced apoptosis in cancer cells by upregulation of BAG-1 possibly plays an important role in the development of cervical cancer.

CHAPTER 6

ENHANCED EXPRESSION OF ANTI-APOPTOTIC PROTEINS IN HUMAN PAPILLOMAVIRUS-IMMORTALIZED AND CIGARETTE SMOKE CONDENSATE-TRANSFORMED HUMAN ENDOCERVICAL CELLS: CORRELATION WITH RESISTANCE TO APOPTOSIS INDUCED BY DNA DAMAGE

6.1 INTRODUCTION

I previously established an *in vitro* system in which primary human endocervical cells (HEN) were immortalized by HPV16 and subsequently transformed by treatment of immortalized cells with cigarette smoke condensate (CSC). This *in vitro* system mimics the multistage oncogenesis of cervical cells *in situ* (Tsutsumi *et al.*, 1992; Yang *et al.*, 1996). To elucidate the role of expression of genes regulating apoptosis in this *in vitro* multistage oncogenesis, I examined the protein expression of genes promoting or inhibiting apoptosis in primary, HPV16-immortalized and CSC-transformed HEN by Western blot analysis. My study showed that the protein levels of anti-apoptotic genes (Bcl-2, Bcl-X_L and BAG-1), but not pro-apoptotic genes, were progressively enhanced after immortalization and transformation of primary cervical cells. The enhanced protein levels of anti-apoptotic genes in HPV16-immortalized and CSC-transformed cells were correlated with enhanced resistance of these cells to apoptosis induced by staurosporine or cisplatin.

6.2 MATERIALS AND METHODS

6.2.1 Cell culture and treatment of cells with staurosporine or cisplatin.

HEN and HPV16-immortalized HEN-16 and HEN-16-2 were established and propagated in culture, as described previously (Sun *et al.*, 1992; Tsutsumi *et al.*, 1992). CSC-transformed cells, HEN-16T and HEN-16-2T, were established from HEN-16 and HEN-16-2, respectively, by treatment of immortalized cells with CSC (Yang *et al.*, 1996b). All the cells were maintained in serum-free media for keratinocytes (GIBCO BRL, Bethesda, MD). The growth rates of all cell types were similar in this culture condition (Yang *et al.*, 1996b). To assay the resistance of different cells to DNA damage, exponentially growing cells were treated with 0.2 μM staurosporine or 5 μM cisplatin for 12, 24 and 48 h. Cell viability was determined with trypan blue exclusion assays, as described in Chapter 4.2.3.2.

6.2.2 DNA fragmentation analysis

Cervical cells were incubated with 0.2 μM staurosporine or 5 μM cisplatin for 48 h and then 5×10^5 cells were lysed in 20 μl lysis buffer. After incubation with 300 μg/ml RNase A at 37 °C for 1 h, 10 μl proteinase K (20 μg/μl) were added and the mixture was incubated at 50 °C for 14 h. The DNA was resolved by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

6.2.3 Western blot analysis

Western blot analysis of cellular proteins was as described in Chapter3.2.3. Mouse anti-p53 and anti-actin mAbs were purchased from DAKO (Glostrup, Denmark) and Sigma (Oakville, Canada), respectively. Rabbit polyclonal antibodies for Bcl-X_L, Bax and BAG-1 and mouse mAbs for Bak and Bcl-2 were obtained from Santa Cruz Biotech. (Santa Cruz, CA). To identify the

specificity of bands bound by each antibody, peptide competitors of specific antibodies were added to the hybridization assays in 10-fold excess. The bands eliminated by addition of competitor peptide (data not shown) were regarded as genuine bands.

6.2.4 Reverse transcription-PCR (RT-PCR) analysis

To assay the expression of *bcl*-x mRNA, RT-PCR was performed, as described (Yang *et al.*, 1997). The PCR primers used for the amplification of *bcl*-X_L and *bcl*-X_S were: sense, 5'-TTGGACAATGGACTGGTTGA-3'; and antisense, 5'-GTAGAGTGGATGGTCAGTG-3'. The PCR program was: 94 ^{0}C for 3 min for the first cycle; 35 cycles of 94 ^{0}C for 1 min, 56 ^{0}C for 2 min and 72 ^{0}C for 2 min; and 72 ^{0}C for 7 min for the last cycle.

6.3 RESULTS

6.3.1 Levels of anti-apoptotic proteins were enhanced in HPV16-immortalized and CSC-transformed human endocervical cells

The levels of apoptosis-promoting proteins, p53, Bak, Bax and Bcl-X_S, were examined by Western blot analysis. The expression of p53 protein was high in HEN and decreased in HPV16-immortalized cells, HEN-16 and HEN-16-2. Its level increased more than 3-fold or was little affected in HEN-16-2T or HEN-16T, respectively, compared with that in their immortalized counterparts (Fig.6.1; Table 6.1). However, there was no significant difference for the high expression of Bak and Bax proteins among all five cell types examined (Fig. 6.1, Table 6.1). Since the expression of Bcl-X_S protein could not be detected by Western blot analysis in any cell type (data not shown), I employed RT-PCR to test if mRNA was expressed in the cells. While the 591 bp RT-PCR product of *bcl*-X_S

Fig. 6.1. Expression of p53, Bak and Bax apoptosis-promoting proteins in normal, HPV-immortalized and CSC-transformed human endocervical cells. Western blot analysis is shown using actin as an internal control.

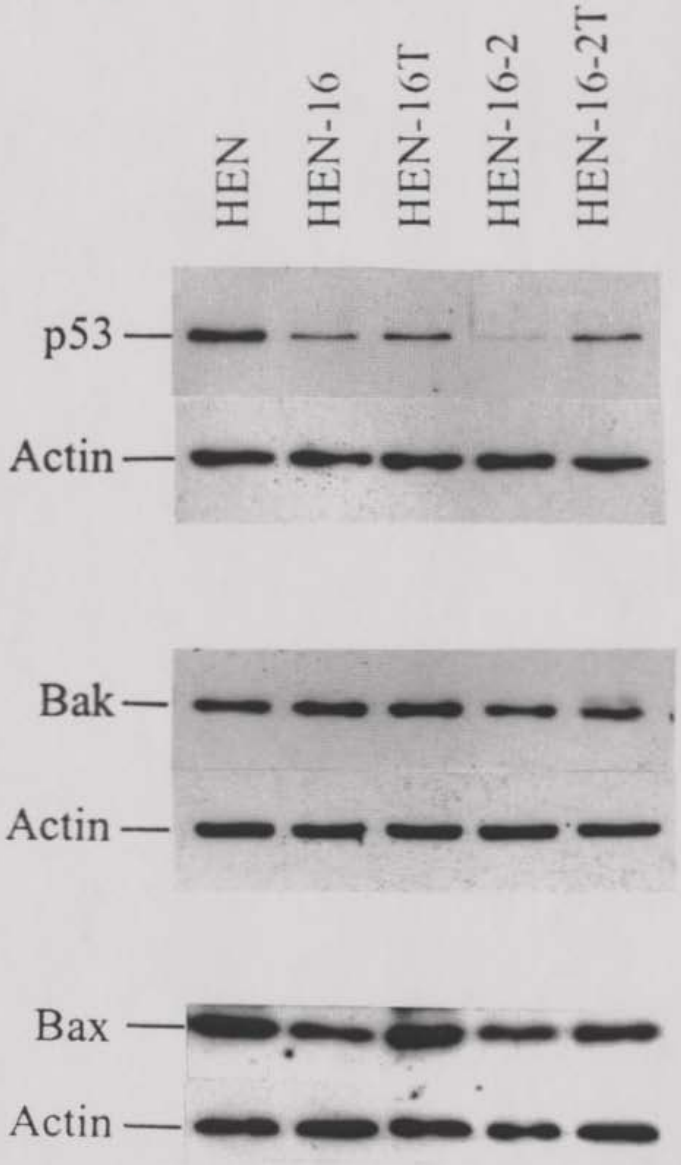

HEN
HEN-16
HEN-16T
HEN-16-2
HEN-16-2T
p53
Actin
Bak
Actin
Bax
Actin

Table 6.1. Relative Expression of Apoptosis-Regulating Proteins in Normal, HPV16-Immortalized and CSC-Transformed Human Endocervical Cells*

Cells	p53	Bak	Bax	Bcl-2	Bcl-X_L	BAG-1
HEN	1.0 (0)	1.0 (0)	1.0 (0)	1.0 (0)	1.0 (0)	1.0
HEN-16	0.4 (0.1)	1.3 (0.4)	0.6 (0.1)	5.9 (1.5)	1.8 (0.5)	7.3 (1.7)
HEN-16T	0.4 (0.2)	1.3 (0.5)	1.0 (0.2)	6.4 (2.7)	3.8 (1.7)	24.9 (8.1)
HEN-16-2	0.2 (0.1)	0.8 (0.2)	0.7 (0.2)	9.9 (3.6)	1.7 (0.5)	4.5 (1.8)
HEN-16-2T	0.6 (0.3)	1.0 (0.4)	0.7 (0.3)	26.8 (11.7)	2.1 (0.5)	11.1 (4.1)

* The levels of cellular protein were quantified relative to those of HEN after normalization to the actin control. Protein expression was quantified by measuring the optical density of bands at medium exposure on X-ray film. Data represents the mean of three experiments. Standard deviation of the mean is given in parenthesis.

was detected for all the cell types, the level was low relative to *bcl*-X_L and no major difference was observed in the level of its expression among the five cell types (Fig. 6.2B).

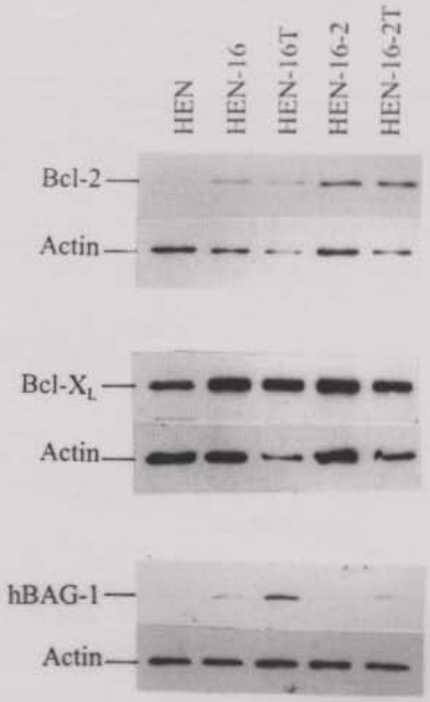
The expression of anti-apoptotic proteins, Bcl-2, Bcl-X_L and BAG-1, increased progressively from HEN to HPV16-immortalized cells (HEN-16 and HEN-16-2) and then to CSC-transformed cells (HEN-16T and HEN-16-2T) (Fig. 6.2A; Table 6.1). While the level of Bcl-2 was very low in HEN, it increased relative to HEN about 6- and 10-fold in HEN-16 and HEN-16-2 and 16- and 27-fold in HEN-16T and HEN-16-2T, respectively. A similar pattern was observed for the expression of BAG-1 protein. Compared with the expression of Bcl-2 and BAG-1 proteins, the increases in Bcl-X_L level were less pronounced. The two Bcl-X_L proteins (28 kDa and 30 kDa) were high in HEN. They increased about 2-fold in HPV16-immortalized cells and were further increased to about 2- to 4-fold in CSC-transformed cells relative to HEN (Fig. 6.2A; Table 6.1).

6.3.2 Resistance to Apoptosis Induced by Staurosporine or Cisplatin Was Correlated with the Enhanced Protein Levels of Anti-Apoptotic Genes

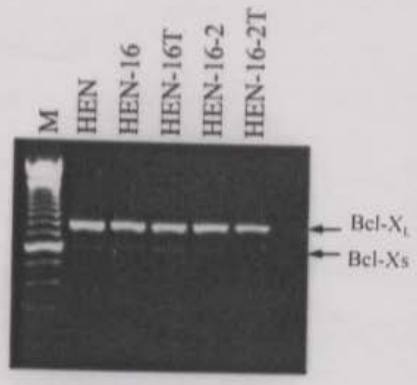
To examine whether there was a correlation of the expression of apoptosis-inhibiting genes with resistance to apoptosis, I assayed the induction of apoptosis by two DNA-damaging reagents, staurosporine and cisplatin, in primary, HPV16-immortalized and CSC-transformed HEN. The resistance to apoptosis increased greatly after immortalization of primary HEN. Similarly, the resistance to apoptosis was enhanced in HEN-16T and HEN-16-2T, compared with that in their immortalized counterparts, HEN-16 and HEN-16-2, respectively. After 48 h treatment of cells with 0.2 μM staurosporine, only 5% of HEN were viable, whereas 48% and 40% of HEN-16 and HEN-16-2, respectively, and 74% and 50% of HEN-16T and HEN-16-2T, respectively, were viable, as determined by trypan blue exclusion assays (Fig. 6.3). Consistent results were seen when all the

Fig. 6. 2. Expression of Bcl-2, Bcl-X_L and BAG-1 apoptosis-inhibiting proteins in normal, HPV16-immortalized and CSC-transformed human endocervical cells. A. Western blot analysis of Bcl-2, Bcl-X_L and BAG-1. B. RT-PCR analysis of Bcl-X_L and Bcl-Xs. Conditions for Western blot were as in Fig.6.1.

A


HEN
HEN-16
HEN-16T
HEN-16-2
HEN-16-2T
Bcl-2
Actin
Bcl-X$_L$
Actin
hBAG-1
Actin

B


M
HEN
HEN-16
HEN-16T
HEN-16-2
HEN-16-2T
Bcl-X$_L$
Bcl-Xs

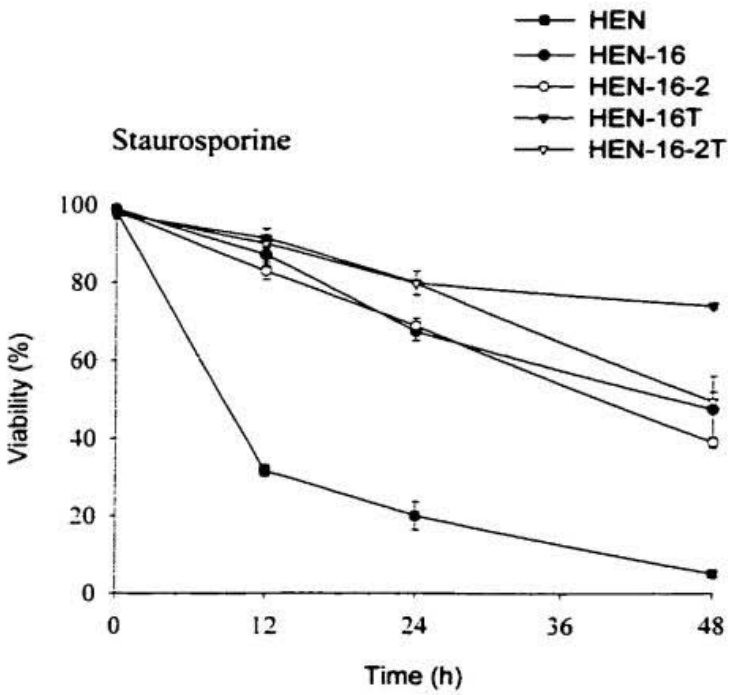
endocervical cells were treated with 5 μM cisplatin. For HEN, 59% were viable, whereas 70% and 68% of HEN-16 and HEN-16-2, respectively, and 81% and 71% of HEN-16T and HEN-16-2T, respectively, were viable after the cells were treated with cisplatin for 48 h (Fig. 6.3). A similar pattern was obtained when apoptosis was tested by DNA fragmentation analysis, confirming that the cell death was apoptotic (Fig. 6.4).

6.4 DISCUSSION

Recent clinical studies show a strong correlation between apoptosis and progression of premalignant cervical lesions (Isacson *et al.*, 1996; Shoji *et al.*, 1996). Cellular genes regulating apoptosis, such as *bcl*-2, but not HPVs, were considered to be important in this correlation (Shoji *et al.*, 1996; Pillai *et al.*, 1997). However, the exact role of the apoptosis-associated genes in this oncogenic progression is poorly understood. The purpose of this study was to determine whether the expression of the proteins of apoptosis-associated genes varied during multistage oncogenesis of human cervical cells. I found that the expression of anti-apoptotic proteins, Bcl-2, Bcl-X_L and BAG-1, increased both in HPV16-immortalized and CSC-transformed cells, indicating that their genes may be involved in the mutistage oncogenesis of cervical cells. In contrast, the apoptosis-promoting proteins, p53, Bak and Bax, varied little during multistage oncogenesis.

p53 was also found to play an important role in apoptosis. Loss of p53 function was reported to result in resistance to apoptosis induced by DNA-damaging reagents in various human cells (Zhan *et al.*, 1994). On the other hand, overexpression of p53 was shown to induce apoptosis in certain cell types (Yonish-Rouach *et al.*, 1991; Oren, 1994; Yonish-Rouach *et al.*, 1996). In cervical carcinoma cells, p53 is usually degraded by a ubiquitin-mediated proteolysis through interaction with E6 protein of HPVs (Scheffner *et al.*, 1994; Werness *et al.*, 1990). Consistently, in my study, decreased

Fig. 6.3. Resistance of human endocervical cells to apoptosis induced by staurosporine or cisplatin. Endocervical cells were treated with 2 μM staurosporine or 5 μM cisplatin for different period of time. The percentage of the viable cells was determined by trypan blue exclusion assays. The results represent the mean ± the standard deviation of three experiments.


HEN
HEN-16
HEN-16-2
HEN-16T
HEN-16-2T
Staurosporine
Viability (%)
100
80
60
40
20
0
0
12
24
36
48
Time (h)

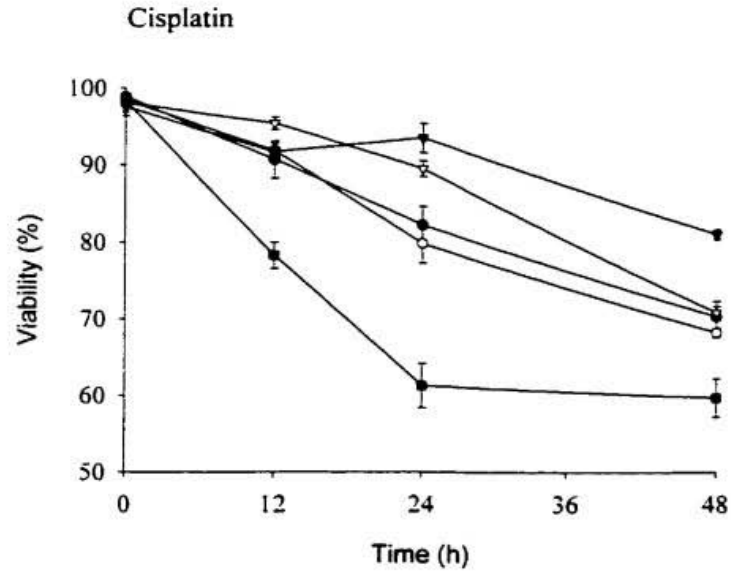
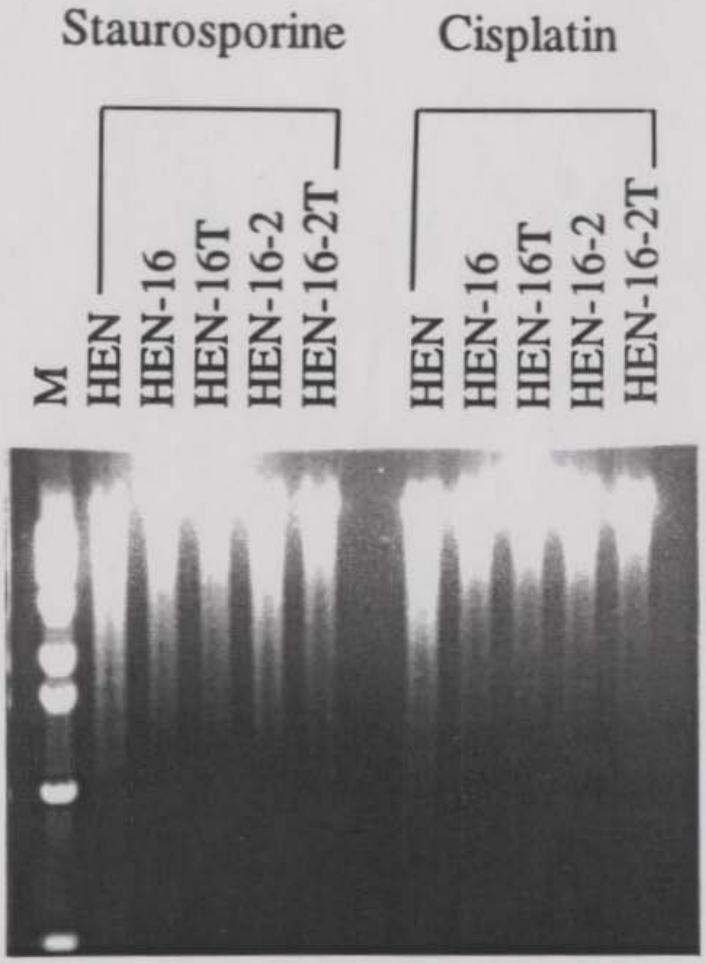

Cisplatin
Viability (%)
100
90
80
70
60
50
0
12
24
36
48
Time (h)

Fig. 6.4. Comparison of staurosporine- or cisplatin-induced DNA fragmentation in normal, HPV16-immortalized and CSC-transformed human endocervical cells. Cells were treated with 0.2 μM staurosporine or 5 μM cisplatin for 48 h. The low molecular weight DNA formed after DNA fragmentation was subsequently extracted and examined on an ethidium bromide agarose gel.

FIG-5


Staurosporine
Cisplatin
M
HEN
HEN-16
HEN-16T
HEN-16-2
HEN-16-2T
HEN
HEN-16
HEN-16T
HEN-16-2
HEN-16-2T

expression of p53 protein was detected in HPV16-immortalized cells (Fig. 6.1). Further, the reduced p53 level was in agreement with enhanced resistance to apoptosis induced by staurosporine or cisplatin. In contrast, due to enhanced expression of *p53* mRNA in these cells (Yang *et al.*, 1997), the expression of p53 protein increased after transformation of HPV16-immortalized cells by CSC. However, the resistance to apoptosis increased in CSC-transformed cells compared with their immortalized counterparts, indicating that the sensitivity to apoptosis in cervical cells also involves a p53-independent mechanism and that other genes regulating apoptosis may be important in this process. Inactivation of p53 by HPV E6 has been shown to enhance, reduce or have no effect on apoptosis induced by DNA-damaging reagents (Fan *et al.*, 1995; Labrecque *et al.*, 1995; Tsang *et al.*, 1995; Wahl *et al.*, 1996; Xu *et al.*, 1995). Therefore, the exact role of p53 in apoptosis of human cervical cells awaits full elucidation.

Bax and Bak are Bcl-2 family members that promote apoptosis by heterodimerizing with Bcl-2 (see Chapter 1.4.1.1). *bax* was also shown to be downstream of *p53* and transactivated by p53 (Miyashita *et al.*, 1994; Miyashita and Reed, 1995). Like *p53*, *bax* can function as a tumor suppressor gene by inducing apoptosis in tumor cells (Yin *et al.*, 1997). Although reduced expression of Bak or *bax* was detected in primary colorectal adenocarcinomas and breast cancers (Bargou *et al.*, 1995; Krajewska *et al.*, 1996), no changes were found in CSC-transformed human cervical cells. No changes in the expression of Bax or Bak proteins were also found in cervical carcinoma cells (see Chapter 5). This suggests that dysregulated expression of Bax or Bak may not be common in human cervical cancer.

Relative to normal keratinocytes, overexpression of Bcl-2 was found in human cervical carcinoma cell lines (Liang *et al.*, 1995). However, the role of *bcl*-2 in multistage oncogenesis of human cells has rarely been studied. Bcl-2 was found to be involved in an early event *in vivo* in

colorectal tumorigenesis (Sinicrope *et al.*, 1995). In this study, I found that the expression of Bcl-2 was progressively enhanced from primary HEN to HPV16-immortalized HEN and to CSC-transformed immortalized cells, suggesting that activation of the *bcl*-2 gene was involved in early and late events in cervical oncogenesis. Interestingly, enhanced expression of Bcl-2 was closely correlated with an increased resistance to apoptosis induced by DNA-damaging reagents in both immortalized and transformed cells (Fig. 6.2-6.4). This indicated that overexpression of Bcl-2 may play an important role in both apoptosis and multistage oncogenesis of human cervical cells, at least in our *in vitro* system. Previously, *bcl*-2 mRNA expression was shown to be suppressed by p53 through a p53-dependent negative response element (Miyashita *et al.*, 1994a, b). Therefore, an inverse correlation between the expression of *bcl*-2 and *p53* was found in breast cancer cells (Halder *et al.*, 1994). Since an inverse correlation in the relative expression of p53 and Bcl-2 was found after immortalization but not after transformation of human cervical cells, overexpression of Bcl-2 in human cervical cancers may be through p53-dependent and p53-independent pathways, as suggested previously (Liang *et al.*, 1995). Further, since p53 and Bcl-2 have opposing effects on apoptosis, the relative levels of p53 and Bcl-2 may be more important in apoptosis. For example, although HEN-16-2T had the highest expression of Bcl-2, it also had relatively higher level of p53. Possibly as a result, the resistance of HEN-16-2T to apoptosis induced by staurosporine and cisplatin after 48 hr treatment were similar to that for HEN-16 and HEN-16-2. Therefore, the sensitivity of cells to apoptosis induced by DNA-damaged reagents may be determined by the relative protein level of apoptosis-associated genes.

Two splice variants of *bcl*-X mRNA, Bcl-X_L and *bcl*-X_S, have different functions: Bcl-X_L is the longer form of *bcl*-X mRNA and has an anti-apoptotic effect, whereas *bcl*-X_S is the shorter *bcl*-X mRNA and promotes apoptosis by inhibiting Bcl-2 function (Boise *et al.*, 1993). Overexpression of

Bcl-X_L mRNAs and proteins was reported in many human cancers (see Chapter 1.4.1.2). However, there is no report on the expression of *bcl*-X in human cervical cells. In this study, I found that Bcl-X_L was the predominant form of *bcl*-X expressed in human cervical cells and it was expressed relatively higher in both HPV16-immortalized and CSC-transformed cell lines than in normal cervical cells. In contrast, the expression of Bcl-X_S protein was undetectable in all cells and a relatively low level of its transcript was detected only by RT-PCR. This study suggested that enhanced expression of Bcl-X_L may be correlated with the resistance to apoptosis of human cervical cells and oncogenesis.

Although BAG-1 was overexpressed in various human cancers, its role in multistage carcinogensis has not been examined. In this study, I found progressively increased expression of BAG-1 protein after immortalization of primary HEN by HPV16 and transformation by CSC. The synergistic enhancement of BAG-1 and Bcl-2 was closely correlated with the resistance of cells to apoptosis induced by staurosporine or cisplatin. These results suggest that BAG-1 plays an important role in both the early stage and late stage of oncogenesis of human cervical cells.

Taken together, my results provided the first evidence that anti-apoptotic gene proteins, Bcl-2, Bcl-X_L and BAG-1, were progressively increased during mutistage oncogenesis of human cervical cells. The enhanced expression of these genes in HPV16-immortalized and CSC-transformed cells closely correlated with their resistance to apoptosis induced by DNA-damaging staurosporine or cisplatin, indicating that reduced cell death by apoptosis due to progressive increase of anti-apoptotic proteins may play an important role in the multistage oncogenesis of human cancers.

CHAPTER 7

DIFFERENTIAL EXPRESSION OF ANTI-APOPTOTIC GENE BAG-1 IN HUMAN BREAST NORMAL AND CANCER CELL LINES AND TISSUES

7.1 INTRODUCTION

Recently, enhanced expression of BAG-1 in breast cancer tissues was found by Zapata *et al.* (1998). However, only one breast normal and three carcinoma tissues were used for Western blot in their study. In addition, I have recently shown that BAG-1 is expressed as four protein isoforms, p50, p36, p33 and p29, through alternative translation initiation (Yang *et al.*, 1998b). However, the role of different BAG-1 isoforms in tumorigenesis has not been elucidated. In this study, I showed that BAG-1 isoforms can be differentially expressed in cell lines *in vitro* compared with tissues *in vivo*. Further, I provided evidence that BAG-1 isoforms, p46 and p33, were overexpressed in both breast carcinoma cell lines and tissues and may play an important role, independent of Bcl-2 and Bcl-X_L, in breast carcinogenesis.

7.2 MATERIALS AND METHODS

7.2.1 Cell culture and breast biopsies

Three normal human mammary gland cell lines (HS574, HS578 and HS787) and nine human breast carcinoma cell lines (BT-20, BT-474, HS578T, MCF-7, SK-BR-3, MDA-MB-157, MDA-MB-231, MDA-MB-436 and MDA-MB-468) were used in this study. All the cell lines were obtained

from ATCC. Except for SK-BR-3 which was cultured in McCoy's 5a medium, all the cell lines were maintained in DMEM containing 10% heat-inactivated FCS.

The breast normal and carcinoma tissues were obtained from the General Hospital, the Health Science Center (St. John's, Canada) and NCIC Manitoba Breast Cancer Tumor Bank (Winnipeg, Canada). Tissue samples were excised fresh from operative specimens. Each specimen was either cut into 1 mm segments or sliced into 50 μm thick pieces and stored at -70^0 C before protein extraction. Sections were histologically classified with respect to the presence of breast normal and invasive carcinoma tissues. Care was taken to exclude all normal tissue from invasive tumor samples using histological examination.

7.2.2 RNA and protein extractions from human breast cell lines or biopsies

Extraction of RNA and protein from human breast cell lines or biopsies was as described in Chapter 5.2.4.

7.2.3 Northern and Western Blot Analysis

Northern and Western blot analysis of cellular RNA and protein was as described in Sections 2.2.4 and 3.2.3.

The statistical significance of the difference of protein expression between normal and tumor tissues was analyzed by the Student t-test.

7.3. RESULTS

7.3.1 Overexpression of BAG-1 RNA in human breast cancer cell lines

To investigate the role of BAG-1 in oncogenesis of human breast cells, I examined the expression of BAG-1 RNA in three cell types (HS574, HS578 and HS787) derived from normal breast epithelium and in nine breast cancer cell lines (BT-20, BT-474, HS787T, MCF-7, SK-BR-3, MDA-MB157, MDA-MB231, MDA-MB436 and MDA-MB468) by Northern blotting (Fig. 7.1). Whereas the expression of BAG-1 RNA was low in all the three normal breast cell lines, it was significantly higher (increased 3.75 to 7.76-fold) in seven of the nine breast cancer cell types. Only two breast carcinoma cell lines, HS578T and MDA-MB157, expressed relatively low level of BAG-1 RNA (0.70-fold and 1.78-fold, respectively, relative to BAG-1 RNA level in normal breast cell lines, Table 7.1).

7.3.2 Overexpression of four BAG-1 protein isoforms in breast cancer cell lines: correlation with the BAG-1-interacting proteins, Bcl-2 and Bcl-X_L

Consistent with BAG-1 RNA expression, the expression of the four BAG-1 protein isoforms, p50, p46, p33 and p29 (Yang *et al.*, 1998b), was low in the three normal breast cell lines. BAG-1 protein expression was higher relative to normal cell lines in seven of the nine breast carcinoma cell lines. Only HS578T and MDA-MB-157 expressed relatively low level of BAG-1 protein (0.65-fold and 0.75-fold, respectively, relative to the BAG-1 protein level in normal breast cell lines, Fig. 7.2; Table 7.1). Since BAG-1 binds and synergizes with Bcl-2 to suppress apoptosis (Takayama *et al.*, 1995), I examined whether the expression of BAG-1-interacting anti-apoptotic proteins, Bcl-2 and Bcl-X_L, was also higher in breast cancer cell lines than in normal cells. Interestingly, Bcl-2 protein level was higher in six of the nine breast cancer cell lines and the level of doublet Bcl-X_L was higher in five of the nine cancer cell lines (Fig. 7.2). The expression level of BAG-1, Bcl-2 and Bcl-X_L is summarized in Table 7.1. Overall, BAG-1 protein was overexpressed in carcinoma cell lines and its

Fig. 7.1 Overexpression of BAG-1 RNA in human breast carcinoma cell lines. For Northern blot, 20 μg of total RNA from human breast normal and carcinoma cells were size-fractionated on a 1.0% agarose formaldehyde gel, transferred to nitrocellulose membranes and hybridized with [^{32}P]-labelled BAG-1 cDNA probe. β-actin was used as an internal control.

β-actin
BAG-1
HS574
HS578
HS787
Normal
BT-20
BT-474
HS578T
MCF-7
SK-BR-3
MDA-MB157
MDA-MB231
MDA-MB436
MDA-MB468
Carcinoma

Fig. 7.2 Expression of BAG-1, Bcl-2 and Bcl-X_L proteins in breast normal and carcinoma cell lines. For Western blot, lysate containing 10 μg protein from normal and cancer cells was subjected to 10% SDS-PAGE and transferred to an ECL nitrocellulose membrane. The membrane was subsequently incubated with anti-human BAG-1 mAb (CC9E8), anti-Bcl-2 mAb or anti-Bcl-X_L polyclonal antibody and signals were detected using a goat HRP-conjugated anti-mouse IgG antibody by the ECL method. β-actin was used as an internal control. The four BAG-1 isoforms are indicated on the right.

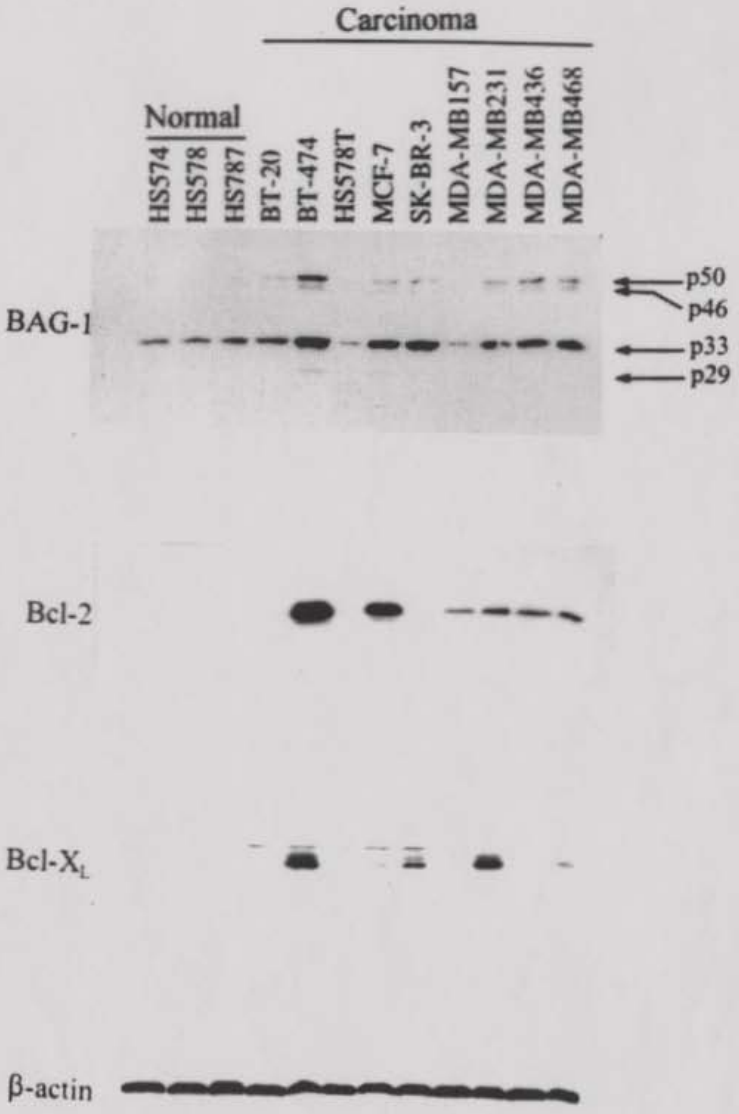

Carcinoma
Normal
HS574
HS578
HS787
BT-20
BT-474
HS578T
MCF-7
SK-BR-3
MDA-MB157
MDA-MB231
MDA-MB436
MDA-MB468
BAG-1
p50
p46
p33
p29
Bcl-2
Bcl-X$_L$
β-actin

Table 7.1. Relative expression of BAG-1, Bcl-2, and Bcl-X_L in breast normal and carcinoma cell lines*

Cell lines	Status	BAG-1		Bcl-2	Bcl-X_L
		RNA	Protein	Protein	Protein
HS574	Normal	0.90	0.92	0.90	0.93
HS578	Normal	0.92	0.91	1.08	1.02
HS787	Normal	1.20	1.23	1.10	1.11
BT-20	Adenocarcinoma	3.75	1.75	2.82	13.00
BT-474	Ductal Carcinoma	6.77	4.80	98.38	272.78
HS578T	Ductal Carcinoma	0.70	0.65	1.04	35.02
MCF-7	Adenocarcinoma	6.24	2.63	51.57	51.47
SK-BR-3	Adenocarcinoma	6.91	2.91	1.06	101.86
MDA-MB-157	Adenocarcinoma	1.78	0.75	11.00	1.11
MDA-MB-231	Adenocarcinoma	6.03	2.12	18.37	188.44
MDA-MB-436	Adenocarcinoma	7.24	2.94	19.24	34.91
MDA-MB-468	Adenocarcinoma	7.76	2.94	16.50	68.27

*Levels of cellular RNAs and proteins were quantified relative to the mean of three normal breast cell lines after normalization to the β-actin control. RNA and protein expression were quantified by measuring the optical density of bands at medium exposure on X-ray film. Note that the BAG-1 protein values represents the total band intensity of p50, p46, p33, and p29. All protein data are presented as the levels relative to those of β-actin.

level was statistically ($p < 0.05$) correlated with those of Bcl-2 and Bcl-X_L in breast normal and carcinoma cell lines.

7.3.3 Overexpression of BAG-1 protein in breast carcinoma tissues

To test the relevance of BAG-1 protein overexpression in breast carcinoma cell lines to the *in vivo* condition, I examined the expression of BAG-1 protein in 24 normal and 45 invasive carcinoma breast tissues. The expression level of each isoform of BAG-1 protein was quantified by normalizing the level of each band in Western blots with that of the β-actin control. Fig. 7.3 shows an example of the Western blots and Fig. 7.4 shows the distribution of protein level among individual samples. In general, both the mean level and percentage of sample positivity of p46 and p33 were markedly higher in breast carcinoma than normal tissues (Fig. 7.3, 7.4; Table 7.2). The mean level and percentage of p46-positive samples are 0.0% and 4.2%, respectively, in normal tissues, whereas they are 0.12% and 80.0%, respectively, in carcinoma tissues. Similarly, the mean level and percentage of p33-positive samples are 0.01% and 4.2%, respectively, in normal tissues, whereas they are 0.19% and 62.2%, respectively, in carcinoma tissues. Statistical analysis also shows that the expression of p46 and p33 in normal and breast cancer tissues is significantly different ($p < 0.001$; Table 7.2). Although the level of p50 in breast carcinomas was statistically higher than that in normal mammary tissues, its mean level or percentage of BAG-1-positive tissues was the same or almost the same (normal versus tumor tissue: mean, 0.99 versus 1.18, $p < 0.05$; % positive, 100% versus 100%; Fig. 7.4; Table 7.2). p29 was detected only in one breast carcinoma tissue (Fig. 7.3). While p33 was the predominant isoform of BAG-1 protein in breast normal and malignant cell lines (Fig. 7.2), p50 was the predominant isoform in breast normal and most carcinoma tissues (Fig. 7.3).

In contrast to my experiment using breast cell lines, Bcl-2 and Bcl-X_L were not overexpressed

Fig. 7.3 Expression of BAG-1, Bcl-2 and Bcl-X_L proteins in breast normal and carcinoma tissues. Western blot analysis and labels were as described for Fig. 7.2.

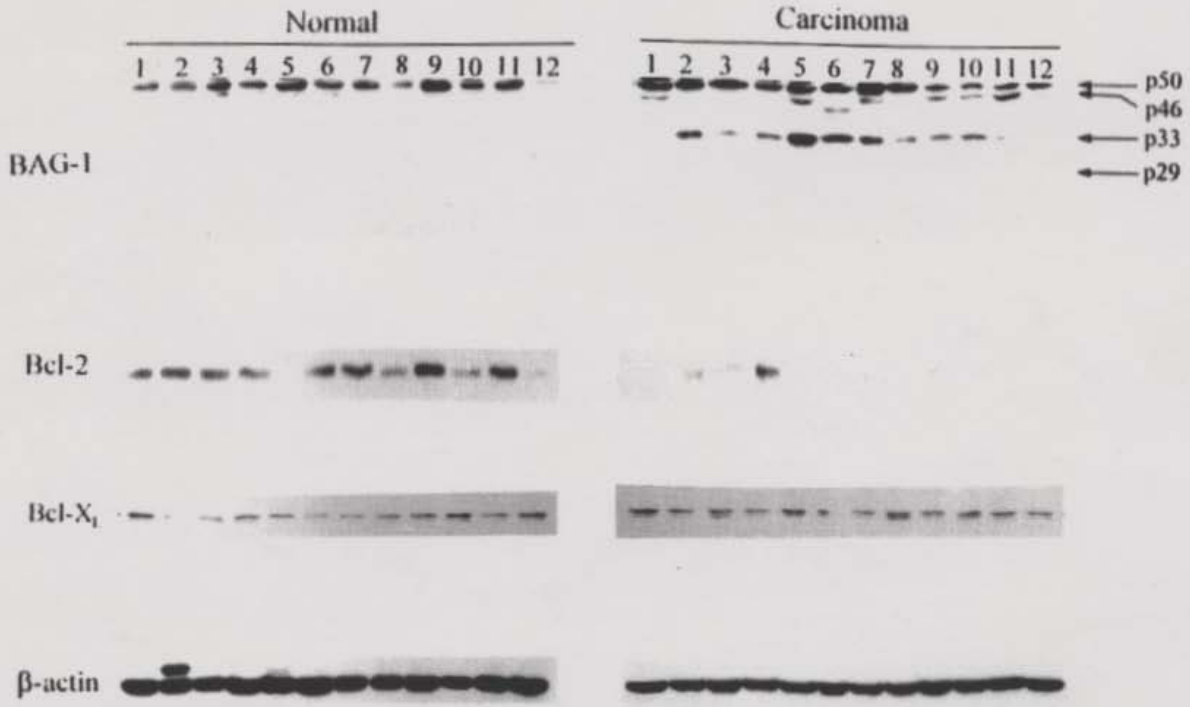
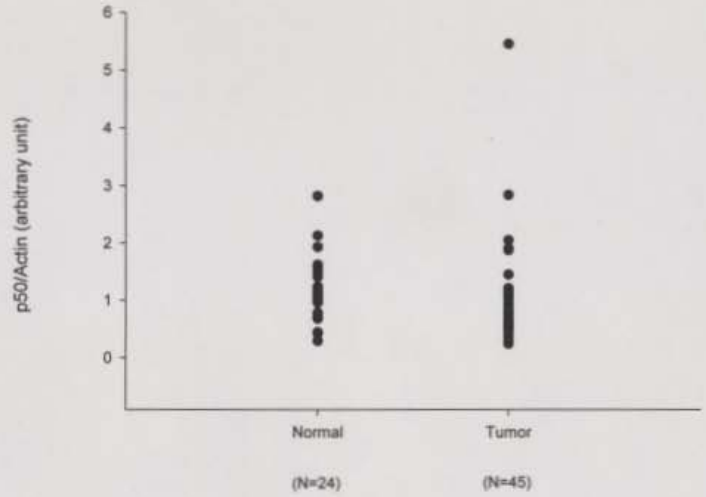
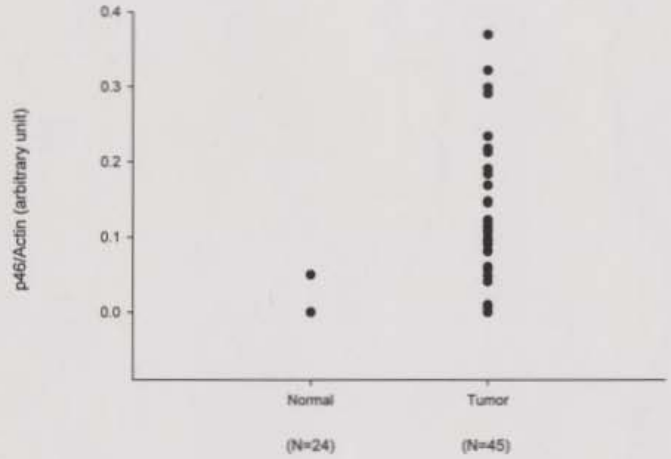

Normal
Carcinoma
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11 12
p50
p46
p33
p29
BAG-1
Bcl-2
Bcl-X$_L$
β-actin

Fig. 7.4 Normalized expression level of BAG-1 p50, p46 and p33 proteins in breast normal and carcinoma tissues. The level of each protein in Western blot was determined with a densitometer. The relative levels of p50, p46 and p33 were indicated for each tissue sample by the ratio of proteins to β-actin.


p50/Actin (arbitrary unit)
6
5
4
3
2
1
0
Normal
(N=24)
Tumor
(N=45)


p46/Actin (arbitrary unit)
0.4
0.3
0.2
0.1
0.0
Normal
(N=24)
Tumor
(N=45)

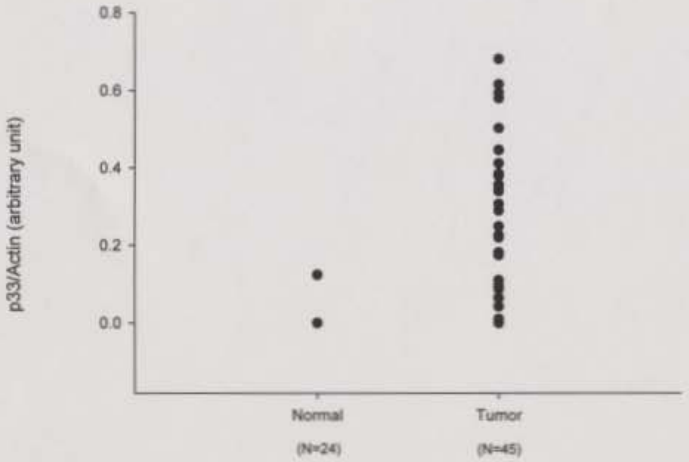

p33/Actin (arbitrary unit)
0.8
0.6
0.4
0.2
0.0
Normal
(N=24)
Tumor
(N=45)

Table 7.2 Summary of the expression of BAG-1, Bcl-2 and Bcl-X_L in breast normal and carcinoma tissues[a]

Proteins	Normal, N=24		Tumor, N=45		Statistical significance[b] (p-value)
	Mean ± S.D.	% positive	Mean ± S.D.	% positive	
p50	0.99 ± 0.85	100.0	1.18 ± 0.57	100.0	*, p = 0.018
p46	0.00 ± 0.00	4.2	0.12 ± 0.12	80.0	**, p<0.001
p33	0.01 ± 0.03	4.2	0.19 ± 0.21	62.2	**, p<0.001
Bcl-2	1.12 ± 0.97	91.7	0.11 ± 0.23	28.9	**, p<0.001
Bcl-X_L	0.10 ± 0.12	100.0	0.07 ± 0.07	93.3	No, p = 0.205

[a]The level of each protein in the Western blot was quantified by densitometry and normalized using the β-actin control. The mean ± the standard deviation of all normal or tumor samples was expressed in arbitrary units; % positive represents the percentage of samples with detectable BAG-1.

[b]No, *, and ** represent no, low ($p < 0.05$) and high ($p < 0.001$) statistically significant difference by Student t-test comparison of the level of each protein in normal and tumor samples.

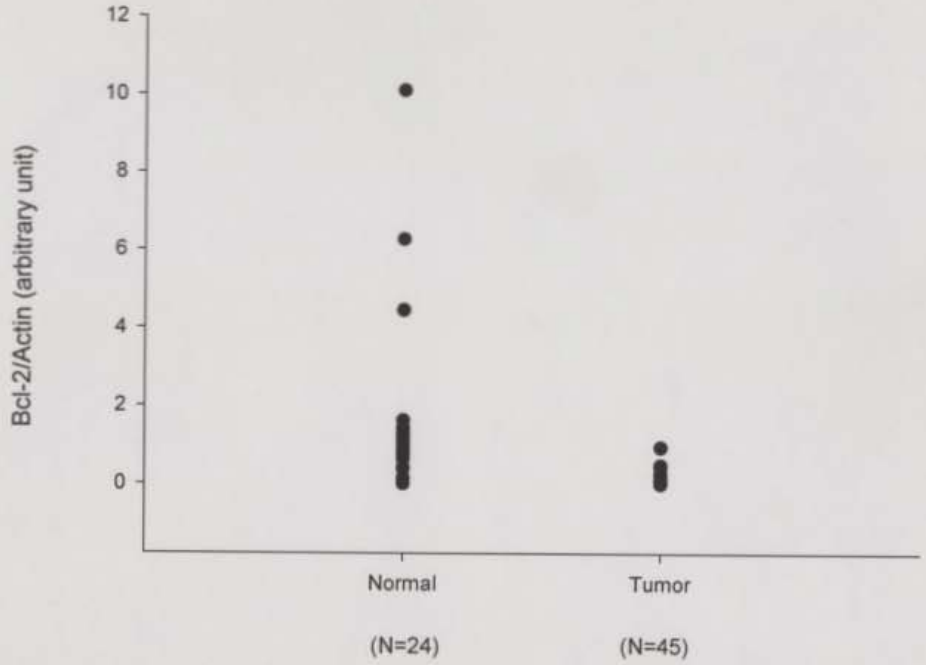
in breast cancer tissues (Fig. 7.3, 7.5). No difference was observed in the level of Bcl-X_L between breast normal and cancer tissues (Table 7.2). Moreover, the mean level of Bcl-2 was 10-fold lower in breast cancer than normal tissues.

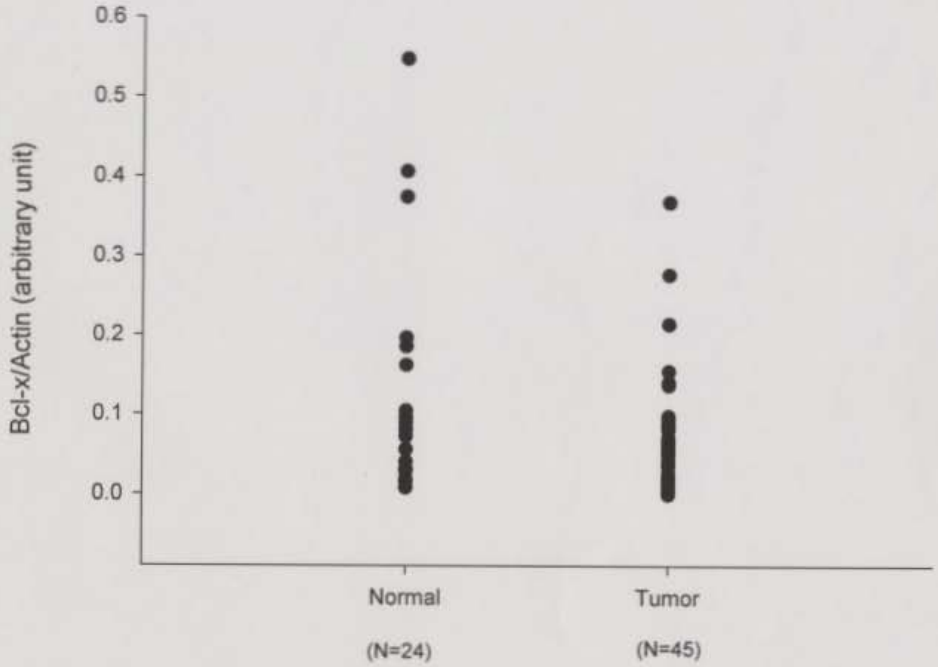
7.4 DISCUSSION

Although breast cancer is the second leading cause of cancer-associated death for women (Pisani *et al.*, 1999), the molecular mechanism for the carcinogenesis has not been fully understood. Recently, a number of observations have suggested that dysregulation of apoptosis plays an important role in the pathogenesis of human cancers (for reviews, see Williams, 1991; Orrenius, 1995; Thompson, 1995). While the expression of the apoptosis-associated genes, *p53*, *bcl-2* and *bax*, has been extensively studied in breast cancer, conflicting results have been reported in the literature (Haldar *et al.*; Bargou *et al.*, 1995; Bargou *et al.*, 1996; Steck *et al.*, 1996). Bcl-2 was found to be overexpressed in some breast carcinoma cell lines (Haldar *et al.*, 1994), but not changed or even lower in some other breast cancer cell lines or tissues (Bargou *et al.*, 1995; Bargou *et al.*, 1996; Leek *et al.*, 1994). Reduced expression of pro-apoptotic gene, *bax*, was observed in breast cancer cell lines and tissues and the expression of mRNA for the anti-apoptotic genes, *bcl*-2 and *bcl*-X_L, was similar in breast normal and cancer tissues (Bargou *et al.*, 1995; Bargou *et al.*, 1996). Heterogeneous expression of Bax was also detected in primary breast tumors (Krajewski *et al.*, 1995). To fully understand the role of apoptosis in the pathogenesis of breast cancer, it is necessary to examine the expression of genes other than *p53*, *bax* and *bcl*-2 that are involved in apoptosis.

In this study, I examined the expression of BAG-1, an anti-apoptotic protein, and its interacting proteins in breast normal and carcinoma cell lines and tissues using Western blot analysis. I provided convincing evidence that the expression of BAG-1 protein was increased in breast carcinoma versus

Fig. 7.5 Normalized expression level of Bcl-2 and Bcl-X_L in breast normal and cancer tissues. The level of each protein in Western blot was determined with a densitometer. The normalized levels of Bcl-2 and Bcl-X_L are indicated for each tissue sample by the ratio of proteins to β-actin.


Bcl-2/Actin (arbitrary unit)
12
10
8
6
4
2
0
Normal
(N=24)
Tumor
(N=45)


Bcl-x/Actin (arbitrary unit)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Normal
(N=24)
Tumor
(N=45)

normal cell lines and tissues. Although immunohistochemistry is not quantitative or able to distinguish isoforms, similar trends were observed when comparing BAG-1 staining in breast carcinoma with normal tissues using this method (unpublished data). My Western blot analysis of the four BAG-1 isoforms showed that there was a significant difference in normal versus cancer cells *in vitro* compared with tissues *in vivo*. While all the BAG-1 protein isoforms were overexpressed in breast carcinoma cell lines (Fig. 7.1), only p46 and p33 were higher in breast carcinoma tissues (Fig. 7.2). Since the levels of BAG-1 RNA and protein were closely correlated in breast cell lines, enhanced expression of BAG-1 protein isoforms in these carcinoma cell lines is likely due to increased production of BAG-1 RNA. However, all BAG-1 isoforms are translated from a single mRNA transcript (Yang *et al.*, 1998b). Therefore, higher level of p46 and p33 but not p50 in breast carcinoma tissues may be caused by relatively increased usage of the second and third initiation codons during translation of BAG-1 in breast cancer tissues. Similarly, a significant difference was also found for Bcl-2 and Bcl-X_L in breast cell lines and tissues. One possible cause for the differential expression of BAG-1, Bcl-2 and Bcl-X_L in breast normal and carcinoma cell lines versus breast normal and primary carcinoma tissues is the changes occurring in cell growth requirements or cell selection during initial or after prolonged culture of normal or tumor cells *in vitro*. In addition, changes for gene expression during the menstrual cycle in tissues (Sabourin *et al.*, 1994) but not cell lines may be another cause for the difference. Limited sample size could also contribute to the difference. Further experiments using a greater number of breast cell lines and tissues are needed to confirm the above possibilities.

Finding higher expression of p46 and p33 but not p50 in breast cancer tissues is of interest. My previous study indicated that different BAG-1 isoforms may have distinct subcellular localization (Yang *et al.*, 1998b), suggesting that they may have distinct function in the cells. Interestingly, p50,

but not p46 or p33, was recently found to enhance AR-mediated transactivating activity (Froesch *et al.*, 1998). Therefore, the four BAG-1 isoforms may have different roles in transcription regulation, apoptosis and tumorigenesis. Recently, enhanced expression of BAG-1 p33 in three breast primary tumors was observed by Zapata *et al.* (1998). Since other BAG-1 isoforms, p50, p46 and p29, were not examined in their study, the differential expression of BAG-1 isoforms in breast carcinoma was not clear. The molecular mechanism for the overexpression of p46 and p33 in breast carcinoma tissues is still unknown. Further experiments need to be performed to elucidate the role of BAG-1 isoforms in carcinogenesis of breast cells by transfecting different BAG-1 isoforms into human breast carcinoma cells and examining the tumorigenicity of BAG-1-overexpressing cells in nude mice.

A synergistic effect of BAG-1 and Bcl-2 on anti-apoptosis has been shown previously by Takayama *et al.* (1995). However, the expression level of BAG-1 and Bcl-2 was only correlated in breast cell lines but not tissues. While the expression level of BAG-1 increased in invasive breast carcinoma tissues, that of Bcl-2 decreased. Similar results were obtained by Zapata *et al.* (1998) using immunohistochemical assay for BAG-1 and Bcl-2. In addition, although a strong correlation between Bcl-2 immunostaining and ER positivity in invasive carcinoma of the breast was reported previously (Teixeira *et al.*, 1995), no such correlation was found between BAG-1 level and ER positivity (data not shown). In addition, BAG-1 did not correlate with conventional prognostic factors such as age, histology, stage, but its overexpression is associated with a shorter disease-free and overall survival (Tang *et al.*, 1999). Further, no correlation between BAG-1 and Bcl-X_L was found. Therefore, BAG-1 may serve as a molecular marker, independent of Bcl-2, Bcl-X_L and ER, for breast oncogenesis *in vivo*.

SECTION 5

TRANSCRIPTION REGULATION OF THE HUMAN BAG-1 GENE

CHAPTER 8

CLONING AND CHARACTERIZATION OF THE HUMAN BAG-1 GENE PROMOTER: UPREGULATION BY TUMOR-DERIVED p53 MUTANTS

8.1 INTRODUCTION

To study the molecular mechanism for the transcriptional regulation of the human BAG-1 gene, I have cloned its 5' flanking region from genomic DNA. Computer analysis and functional assays in different tumor cell lines were used to identify, localize and characterize the BAG-1 promoter. More importantly, I provided the first evidence that tumor-derived gain-of-function p53 mutants can upregulate the BAG-1 gene promoter activity.

8.2 MATERIALS AND METHODS

8.2.1 Cell culture

Human cell lines, HeLa (cervical carcinoma), C33A (cervical carcinoma), MCF-7 (breast carcinoma) and U87 (glioblastoma), were obtained from the American Tissue Culture Collection and cultured at 37°C in 5% CO_2 in DMEM supplement with 10% FCS.

8.2.2 Cloning and computer analysis of the human BAG-1 promoter region

Cloning of the BAG-1 promoter DNA was performed using a GenomeWalker kit (Clontech), according to the manufacture's instructions with minor modifications. In brief, two rounds of PCR were performed using five GenomeWalker "libraries", which are composed of uncloned, adaptor-ligated human genomic DNA fragments from DNA digested with *Eco*RV, *Sca*I, *Dra*I, *Pvu*II and *Ssp*I. The primary round of PCR used the outer adaptor primer (AP1: 5'-GTAATACGACTCACTATAGGGC-3') provided in the kit and an outer, BAG-1-specific primer (BGP1: 5'-CTGAGCCAGGCCCGCACTTGTTGACC-3'). The PCR conditions are as follows: 2 sec at 94°C and 3 min at 70°C for 7 cycles; 2 sec at 94°C and 3 min at 65°C for 32 cycles; and 65°C for an additional 4 min. The primary PCR reaction mixture was then diluted 1:50 and used as

a template for a secondary PCR using a nested adaptor primer (AP2: 5'-ACTATAGGGCACGCGTGGT-3') and a nested BAG-1-specific primer (BGP2: 5'-GGAAGCTGAGCGCGGCGTCTCACAAC-3'). The major PCR products were subsequently ligated into PCR3.1 vector (Invitrogen) and sequenced.

Homology searches were performed using BLAST (Basic Local Alignment Search Tool) from the National Centre for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov. A search for CpG islands was performed using GRAIL (Gene Recognition and Assembly Internet Link) at http://compbio.ornl.gov/Grail.1.3. Putative transcription factor binding elements in the BAG-1 promoter were analysed using the TESS (Transcription Element Search Software) program at: http://agave.humgen.upenn.edu/tess.index.html.

8.2.3 Construction of luciferase reporter gene constructs

All constructs of the BAG-1 promoter for luciferase assays were generated by PCR and ligated into the *Xho*I/*Hin*dIII sites of the promoterless pGL3-Basic (Promega) vector. The identity of each construct was confirmed by sequencing. The pGL3-Control (pGL3-C) postive control plasmid was obtained from Promega.

8.2.4 Transfection, luciferase assays and Northern blot analysis

All transfections were done in triplicate in 12-well plates. About 10^5 cells/well were seeded 24 h prior to transfection. Plasmids were transfected into cells using LipofectAMINE reagent (GIBCO-BRL). The cells were incubated in transfection mix for 5 h, and then harvested after 40 h in culture. Luciferase assays were performed using luciferase assay reagent (Promega) and Monolight 2010 Luminometer (Analytical Luminescence Laboratory). β-galactosidase reporter plasmid was cotransfected with luciferase reporter vector. Luciferase activity was normalized by β-

galactosiase activity. Each experiment was repeated at least three times. Purification of RNA from transfected cells and Northern blot analysis were as described previously (Yang *et al.*, 1997).

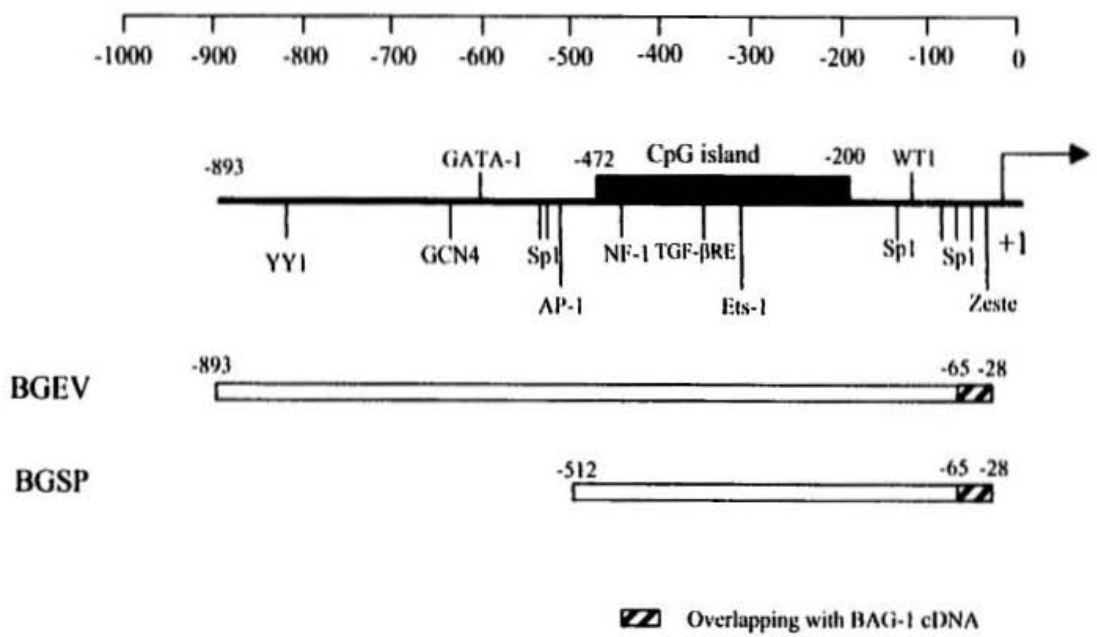
8.3 RESULTS

8.3.1 Cloning and sequence analysis of the human BAG-1 promoter

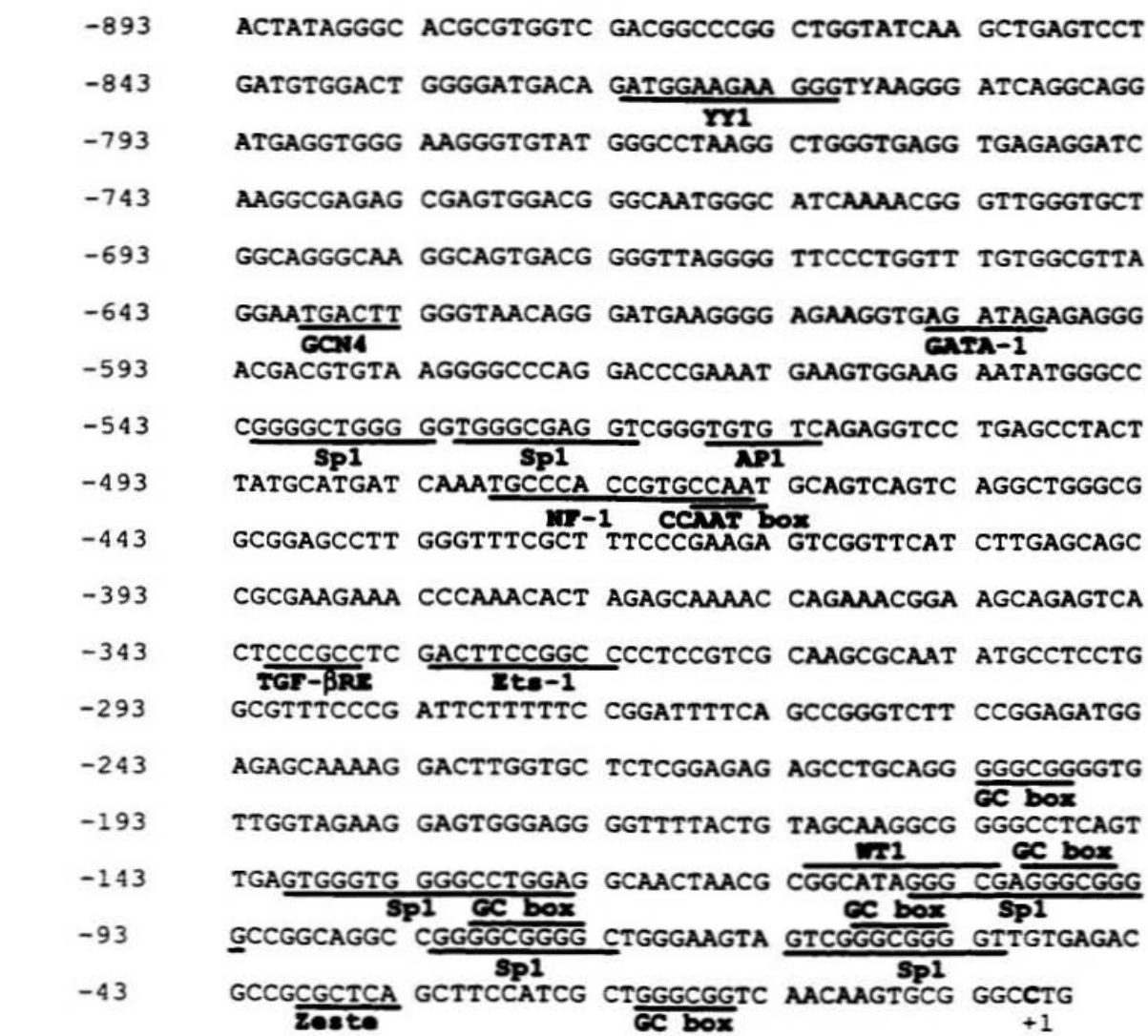
To clone the 5' flanking region of the BAG-1 gene, human genomic DNA was digested by *Eco*RV and *Ssp*I, and the fragments were amplified by nested PCR. Two 866 bp (BGEV) and 485 bp (BGSP) PCR products were generated from the DNA digested with *Eco*RV and *Ssp*I, respectively. DNA sequence analysis showed that BGSP is contained within the 485 bp 3' end of BGEV (Fig. 8.1A). A BLAST search for homology of BGEV DNA with sequences in the database showed that 37 bp at the most 5' end of the BAG-1 cDNA sequences reported in the literature are identical to the 3' end 37 bp of BGEV DNA (Fig. 8.1A). Since no other homology was found, this indicates that I have been the first to clone the 5' flanking region of the human BAG-1 gene. Fig. 8.1B shows the 893 bp sequence of the BAG-1 gene upstream of the first translation start codon (CTG) in the BAG-1 gene. No TATA box was present. However, a CCAAT box and five 3' end-proximal GC boxes were found. Further, I identified several GC-rich regions containing four Sp1 binding sites from 51 to 140 bp upstream of the first translation start codon CTG, three of which encompass three GC boxes (Fig. 8.1B). These features were also found in the promoter regions of other genes and required for basal and conditional expression (Ji *et al.*, 1996). DNA sequence analysis using GRAIL predicted a 272 bp CpG island located from nt -472 to -200. The CpG island has an average GC content of 60% and a CpG score of 0.89. Sequence analysis using TRANSFAC identified 15 putative binding sites for transcription factors, including zeste, Sp1, WT1, Ets, transforming growth factor (TGF)-β receptor, NF-1, AP-1, GATA-1, GCN4 and YY1.

Fig. 8.1. Nucleotide sequence of human BAG-1 gene 5'-flanking regions, putative transcription factor binding sites and CpG island. (A) Diagrammatic representation. The first nucleotide of the translation initiation site CTG (+1) is indicated by an arrow. The position of a CpG island is shown as a black rectangle. The locations of the two PCR products, BGEV and BGSP, are shown as striped boxes. The transcription factor binding sites are indicated. (B) Nucleotide sequence. The numbers of nucleotides upstream of the first nucleotide C (+1) of translational start codon (CTG) are shown on the left of the sequence. Sequence motifs for transcription factor binding are underlined and designated.

A


-1000
-900
-800
-700
-600
-500
-400
-300
-200
-100
0
-893
GATA-1
-472
CpG island
-200
WT1
YY1
GCN4
Sp1
NF-1
TGF-βRE
Sp1
Sp1
+1
AP-1
Ets-1
Zeste
BGEV
-893
-65 -28
BGSP
-512
-65 -28
Overlapping with BAG-1 cDNA

B


-893 ACTATAGGGC ACGCGTGGTC GACGGCCCGG CTGGTATCAA GCTGAGTCCT
-843 GATGTGGACT GGGGATGACA GATGGAAGAA GGGTYAAGGG ATCAGGCAGG
YY1
-793 ATGAGGTGGG AAGGGTGTAT GGGCCTAAGG CTGGGTGAGG TGAGAGGATC
-743 AAGGCGAGAG CGAGTGGACG GGCAATGGGC ATCAAAACGG GTTGGGTGCT
-693 GGCAGGGCAA GGCAGTGACG GGGTTAGGGG TTCCCTGGTT TGTGGCGTTA
-643 GGAATGACTT GGGTAACAGG GATGAAGGGG AGAAGGTGAG ATAGAGAGGG
GCN4
GATA-1
-593 ACGACGTGTA AGGGGCCCAG GACCCGAAAT GAAGTGGAAG AATATGGGCC
-543 CGGGGCTGGG GGTGGGCGAG GTCGGGTGTG TCAGAGGTCC TGAGCCTACT
Sp1
Sp1
AP1
-493 TATGCATGAT CAAATGCCCA CCGTGCCAAT GCAGTCAGTC AGGCTGGGCG
NF-1
CCAAT box
-443 GCGGAGCCTT GGGTTTCGCT TTCCCGAAGA GTCGGTTCAT CTTGAGCAGC
-393 CGCGAAGAAA CCCAAACACT AGAGCAAAAC CAGAAACGGA AGCAGAGTCA
-343 CTCCCGCCTC GACTTCCGGC CCCTCCGTCG CAAGCGCAAT ATGCCTCCTG
TGF-βRE
Ets-1
-293 GCGTTTCCCG ATTCTTTTTC CGGATTTTCA GCCGGGTCTT CCGGAGATGG
-243 AGAGCAAAAG GACTTGGTGC TCTCGGAGAG AGCCTGCAGG GGGCGGGGTG
GC box
-193 TTGGTAGAAG GAGTGGGAGG GGTTTTACTG TAGCAAGGCG GGGCCTCAGT
WT1
GC box
-143 TGAGTGGGTG GGGCCTGGAG GCAACTAACG CGGCATAGGG CGAGGGCGGG
Sp1
GC box
GC box
Sp1
-93 GCCGGCAGGC CGGGGCGGGG CTGGGAAGTA GTCGGGCGGG GTTGTGAGAC
Sp1
Sp1
-43 GCCGCGCTCA GCTTCCATCG CTGGGCGGTC AACAAGTGCG GGCCTG
Zeste
GC box
+1

8.3.2 BAG-1 promoter activity in different tumor cell lines

To test the promoter activity of the BAG-1 upstream sequences *in vivo*, the BGEV fragment was subcloned into a promoterless luciferase reporter vector, pGL3-basic (pGL3-B), in sense and anti-sense orientations. Then, the plasmids were transiently cotransfected with the β-galactosidase control vector into HeLa cervical carcinoma cells. Luciferase activity was 16-fold higher than that for the pGL3-B vector when BGEV was in the sense orientation (BGP-Luc) and was markedly lower than that for BGP-luc when BGEV was in antisense orientation (BGP-AS; Fig. 8.2). As a positive control, pGL3-Control (pGL3-C), which contains the SV40 promoter/enhancer, exhibited high luciferase activity. Since BAG-1 RNA was expressed at various levels (Takayama *et al.*, 1998; Yang *et al.*, 1998), I tested the cell specificity of the BAG-1 promoter in four different cancer cell lines. The luciferase activity from BGP-Luc was also high in another cervical carcinoma cell line, C33A, and a breast cancer cell line, MCF-7, but lower in a glioblastoma cell line, U87 (Fig. 8.3).

8.3.3 Location of the BAG-1 promoter region

To identify the functional region containing the BAG-1 promoter, a series of 5' deletion mutation constructs were produced using PCR with BGEV DNA as template (Fig. 8.4). The PCR products were subsequently cloned into the pGL3-B vector. Luciferase plasmids containing full-length or mutated BAG-1 upstream DNA were transiently cotransfected with β-galactosidase expression plasmid into HeLa cells. The plasmid containing the BAG-1 5' flanking sequence from -868 to -28 (-868/-28-Luc) had moderately high luciferase activity. Deletion of nucleotides from -868 to -702, containing a YY1 site, led to a 50% increase of luciferase activity. The highest promoter activity was observed in the -353/-28-Luc construct. Deletion of nucleotides from -353 to -240, containing a TGF-β response element (TGF-βRE), an Ets-1 motif and half the CpG island,

Fig. 8.2. Constitutive activity of the BAG-1 promoter. HeLa cells were transiently transfected with luciferase plasmid constructs containing the BAG-1 5'-flanking region in the sense (BGP-luc) or antisense (BGP-AS) orientation. The vector plasmid pGL3-Basic (pGL3-B) and the pGL3-Control plasmid (pGL3-C) were used as negative and positive controls, respectively. Following transfection, the cells were cultured for an additional 40 h and then lysed in luciferase lysis buffer. RLU indicates relative luciferase units. The values represent the mean of the β-galactosidase-normalized luciferase activity of three independent transfections and error bars represent the standard deviation.

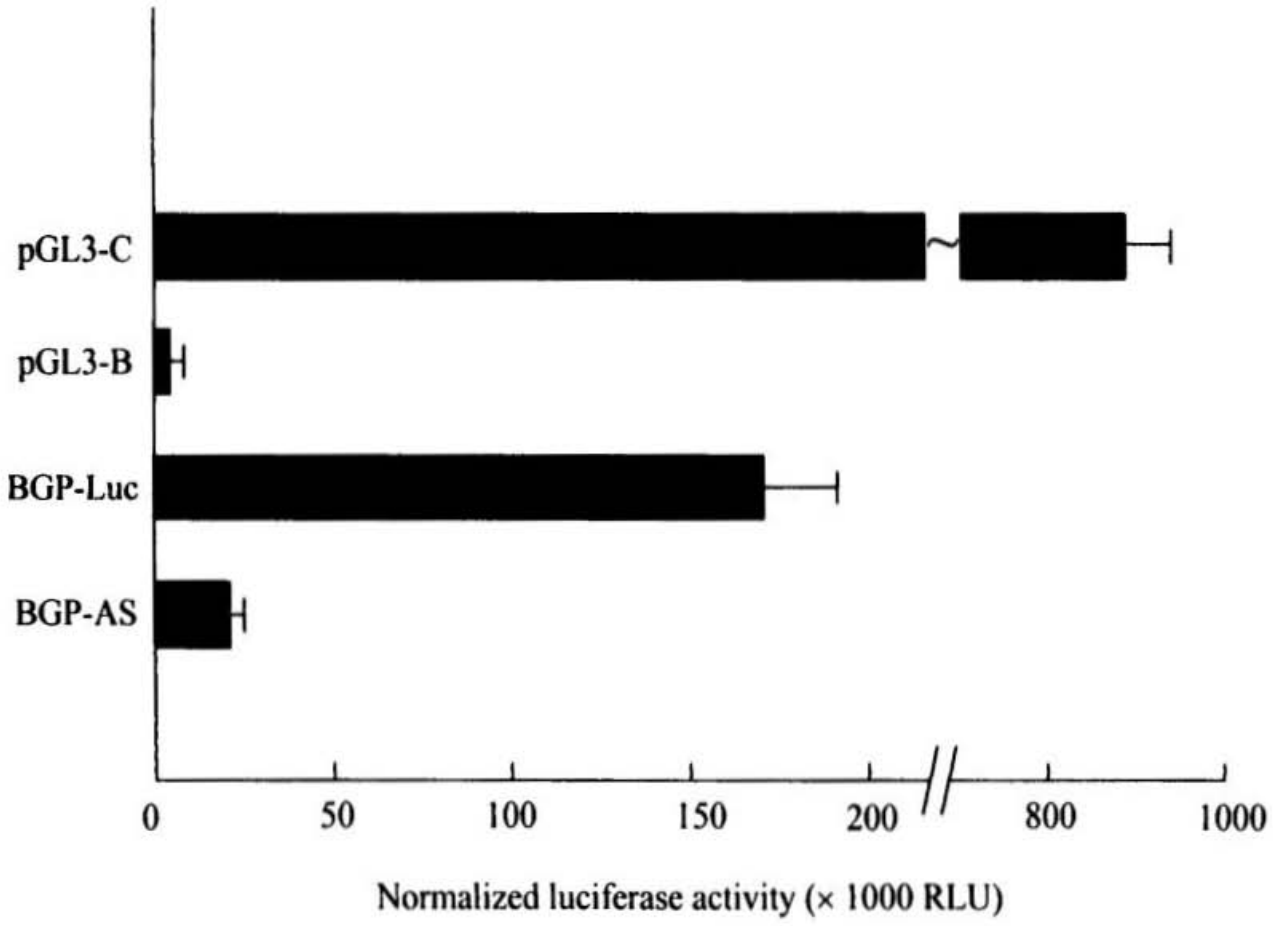

pGL3-C
pGL3-B
BGP-Luc
BGP-AS
0
50
100
150
200
800
1000
Normalized luciferase activity (× 1000 RLU)

Fig. 8.3. BAG-1 promoter activity in different human cancer cell lines. Experimental conditions and error bars are as described in the Fig. 8.2 legend. Luciferase activity is presented as the fold increase of luciferase activity from BGP-Luc relative to that from the pGL3-B vector control.

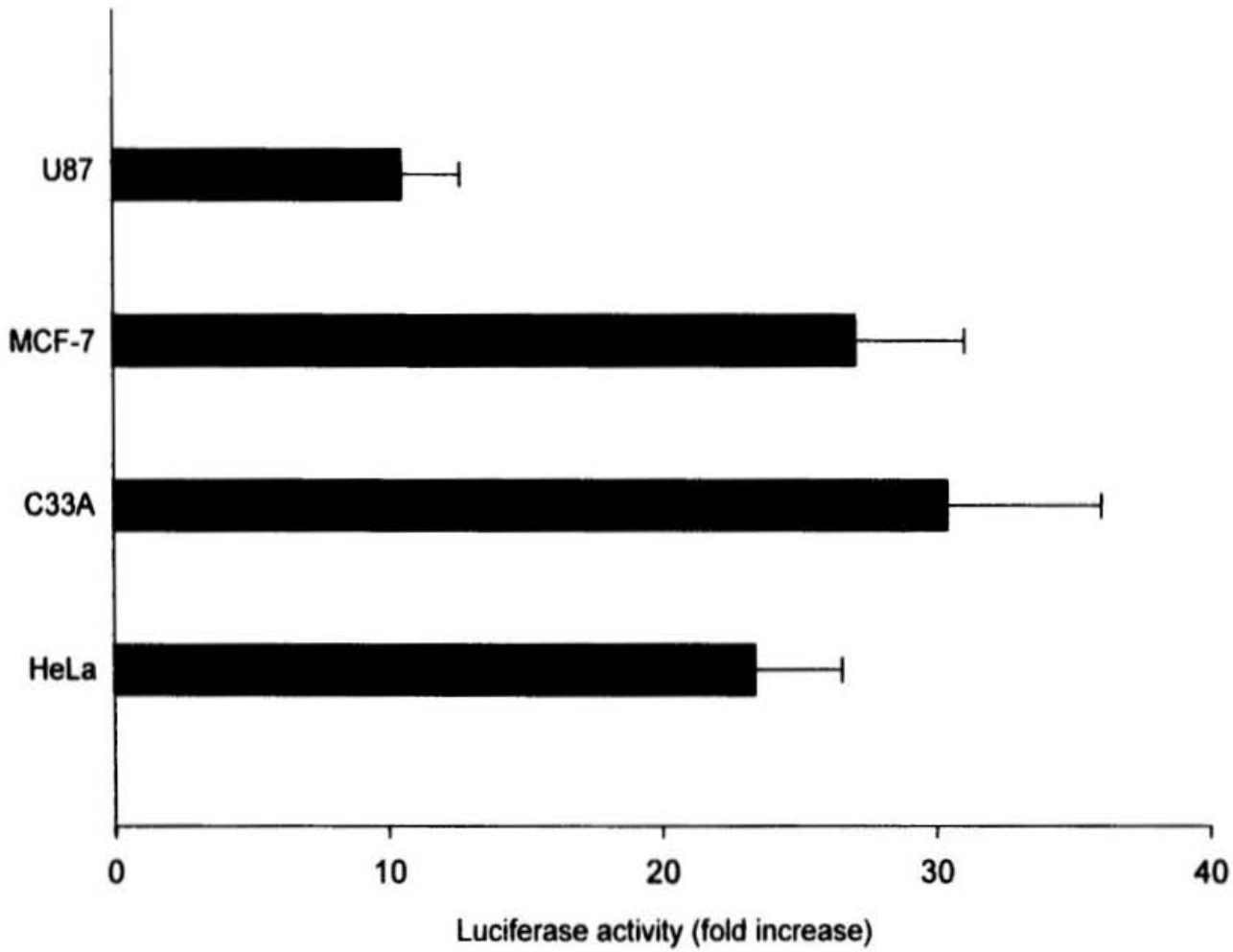
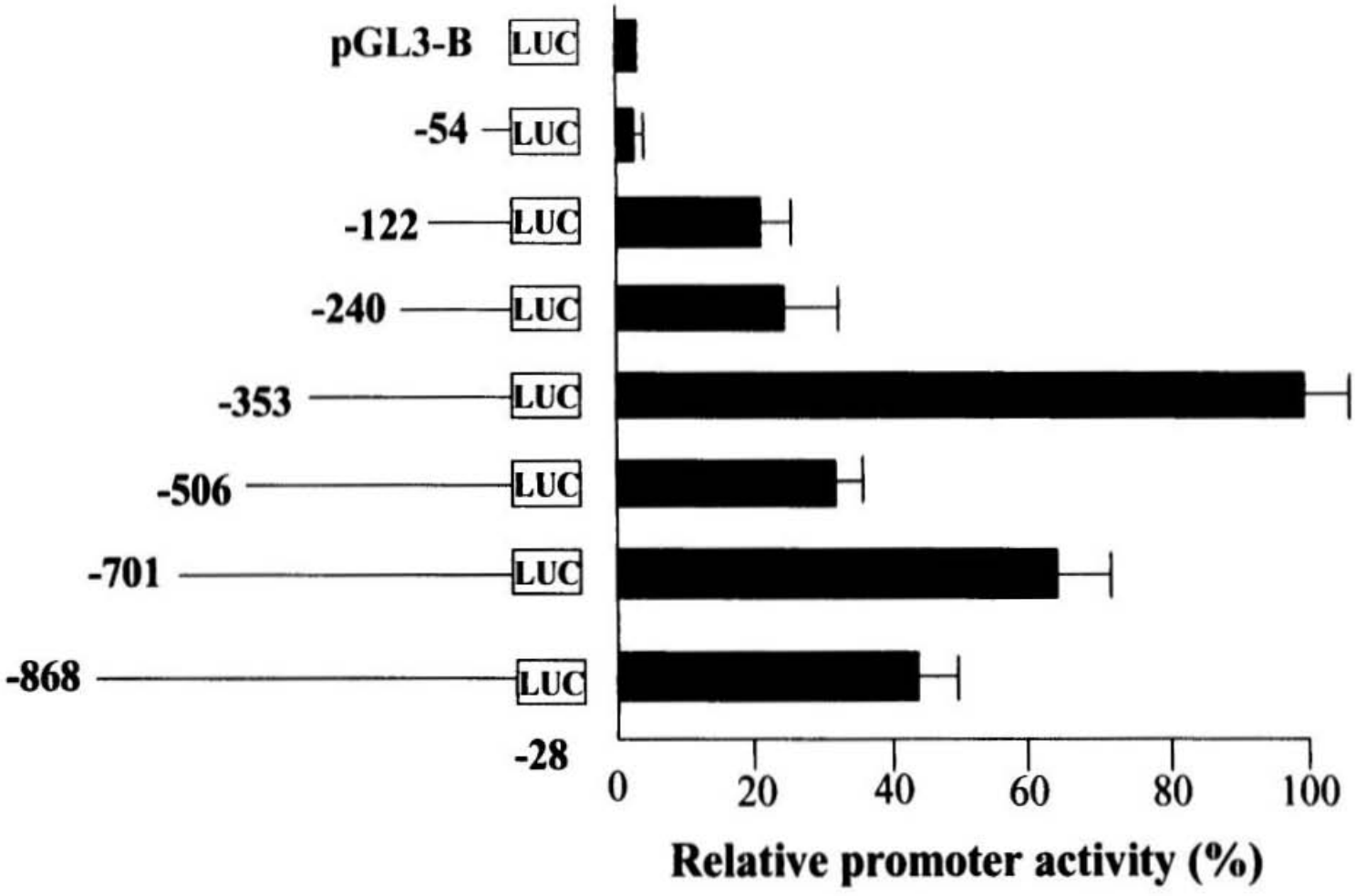

U87
MCF-7
C33A
HeLa
0
10
20
30
40
Luciferase activity (fold increase)

Fig. 8.4. Deletion analysis of the 5'-flanking region of the BAG-1 gene. The diagram on the left represents the luciferase (LUC) constructs made by a series of deletions extending from 28 bp upstream (-28) of the BAG-1 open reading frame. Values for each construct transfected into HeLa cells represent the percentage ± the standard deviation, relative to the construct -353/-28-Luc, which gave the highest (100%) activity. Conditions were as described for Fig. 8.2.


pGL3-B
LUC
-54
LUC
-122
LUC
-240
LUC
-353
LUC
-506
LUC
-701
LUC
-868
LUC
-28
0
20
40
60
80
100
Relative promoter activity (%)

dramatically decreased the luciferase activity (5.4-fold). Deleting nucleotides from -122 to -54, containing three Sp1 sites, three GC boxes and a WT1 site, abolished luciferase activity, leaving only control level activity for the plasmid containing the BAG-1 promoter 5' proximal 54 nucleotides (-54/-28-Luc). Taken together, the data show that the nt -353 to -54 region contains the maximum activity of the BAG-1 promoter.

8.3.4 Transactivation of the BAG-1 promoter by tumor-derived p53 mutants

Previous reports demonstrated that mutations of *p53* play a role in over 50% of human cancers (Hollstein *et al.*, 1991) and BAG-1 was overexpressed in variety of human cancers (Zapata *et al.*, 1998; Takayama *et al.*, 1998; Yang *et al.*, 1998). Mutant p53 derived from human cancer can exhibit gain-of-function and transactivate several genes associated with cell growth or tumorigenicity such as c-*myc* (Frazier *et al.*, 1998). Therefore, overexpression of BAG-1 in human cancer cells may be due to the transactivation of its promoter by mutant p53. To test this hypothesis, I first cotransfected an expression vector for mutant *p53*-143A with the BGP-Luc BAG-1 promoter reporter plasmid into HeLa human cervical carcinoma cells. When increasing amounts of *p53*-143A were cotransfected, progressive second order increases in luciferase activity from BGP-Luc were seen (Fig. 8.5). This is consistent with the requirement of the oligomerization domain of mutant p53 for some but not all promoters that are transactivated (Lanyi *et al.*, 1998). The luciferase activity expressed from BGP-Luc was increased 10-fold by 1 μg of *p53*-143A, whereas the luciferase activity of a control vector plasmid, pGL3-B, was unaffected by 1 μg of *p53*-143A (Fig. 8.5). This result indicated that the BAG-1 promoter was specifically transactivated by mutant p53. Furthermore, I showed by Northern blot that the increase of BAG-1 promoter activity caused by *p53*-143A was associated with a 2.5-fold increase in the level of BAG-1 RNA (Fig. 8.6). Consistent with an earlier report that c-*myc* transcription was transactivated by mutant p53 (Frazier *et al.*, 1998), an

Fig. 8.5. Transactivation of BAG-1 promoter by tumor-derived mutant *p53*-143A. BGP-luc was cotransfected with the indicated amounts of *p53*-143A in CMV expression vector. Conditions and labels are as described for Fig. 8.2 and 8.3. pGL3-B was used as a negative control. Luciferase activity is presented as the fold increase of β-galactosidase-normalized luciferase activity from HeLa cells cotransfected with BGP-luc and *p53*-143A, compared with BGP-Luc and equal amounts of CMV vector control.

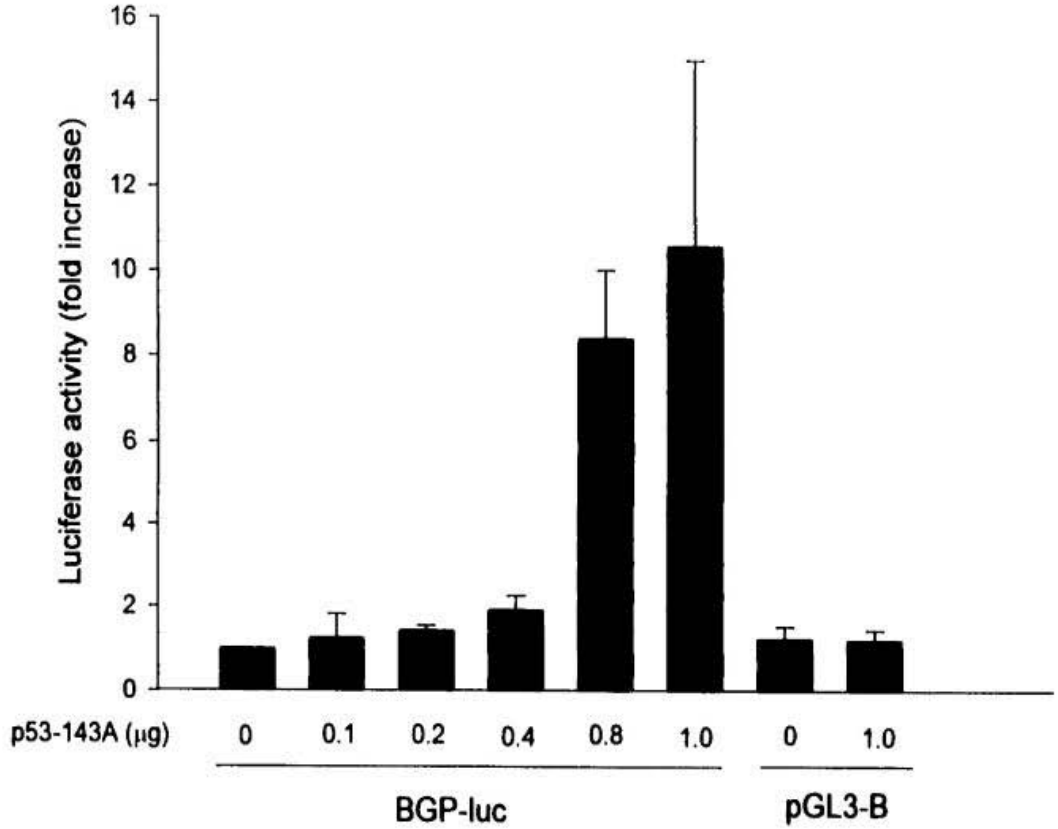

Luciferase activity (fold increase)
16
14
12
10
8
6
4
2
0
p53-143A (μg)
0
0.1
0.2
0.4
0.8
1.0
0
1.0
BGP-luc
pGL3-B

Fig. 8.6. Effect of mutant p53-143A on BAG-1 RNA transcription. CMV vector or *p53*-143A CMV expression plasmid was transiently transfected into HeLa cells. RNA was extracted from the cells incubated 40 h after transfection. Twenty μg of RNA were sized-fractionated on a 1.0% agarose formaldehyde gel, transferred to nitrocellulose membrane and sequentially hybridized with [^{32}P]-labeled human BAG-1 and c-*myc* cDNA probes. β-actin was used as an internal control.

CMV
p53-143A
BAG-1
c-myc
β-actin

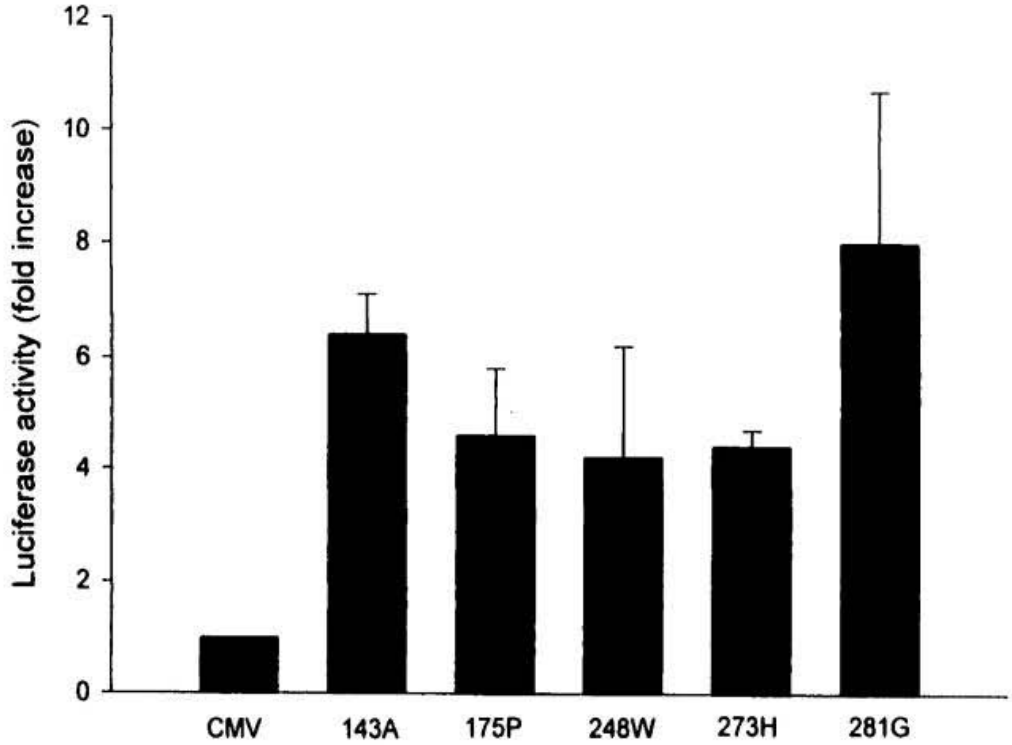
increase in the level of c-*myc* RNA was found when *p53*-143A was transfected into HeLa cells (Fig. 8.6). In addition, cotransfection of various levels of wild-type *p53* cDNA expression vector into HeLa cells had no effect on BGP-luc activity when normalized relative to the β-galactosidase internal control activity (data not shown).

To examine whether the effect of *p53*-143A on the BAG-1 promoter is common among other gain-of-function p53 mutants, I repeated the above experiment using 1 μg of different *p53* mutants derived from different human tumors (Hinds *et al.*, 1990; Frazier *et al.*, 1998; Ryan and Vousden, 1998). Consistent with the *p53*-143A results, 4- to 8-fold induction of BAG-1 promoter activity was found (Fig. 8.7). As a negative control, no induction was observed when the parental CMV expression vector was cotransfected with BGP-Luc into HeLa cells.

8.4 DISCUSSION

As a first step toward understanding the molecular mechanism of BAG-1 upregulation in human cancer, I cloned the promoter region of the human BAG-1 gene. Analysis of this 5' flanking region identified a number of putative transcription factor binding sites including consensus sequences for YY1, GATA-1, Sp1, AP-1, TGF-β receptor, Ets-1 and WT1. Interestingly, several similar features were found in the promoter region of BAG-1 and those of the genes, *bcl*-2 and *bcl*-X_L, of its interacting proteins. As for *bcl*-2 and *bcl*-X_L, the BAG-1 promoter has no TATA box, but several putative Sp1-binding motifs were found in GC-rich regions in all three promoters. Sp1-binding motifs were shown to be essential for the function of the SV40 promoter *in vivo* (Negrini *et al.*, 1987; Grillot *et al.*, 1997). Consistently, deletion of the three Sp1-binding sequences at the 3' end of the BAG-1 promoter abolished its activity (Fig. 8.1 and 8.4). Further, several other transcription factor binding sites (GATA-1, Ets and WT1) that were previously found in the promoter region of *bcl*-2 and *bcl*-X were also identified in the promoter region of BAG-1 (Negrini

Fig. 8.7. Transactivation of BAG-1 promoter by p53 mutants derived from different tumors. BGP-luc was cotransfected with either CMV vector or the indicated human mutant *p53* expression vectors into HeLa cells. Cell extracts were then assayed for luciferase activity. Data represent the fold increase of luciferase activity for the *p53* mutants relative to the CMV control. Conditions were as in Fig. 8.5.


Luciferase activity (fold increase)
12
10
8
6
4
2
0
CMV
143A
175P
248W
273H
281G

et al., 1987; Grillot *et al.*, 1997; Fig. 8.1). Both WT1 and Ets proteins were shown to negatively regulate the *bcl*-2 promoter (Chen and Boxer, 1995; Heckman *et al.*, 1997). Therefore, the three anti-apoptotic genes, BAG-1, *bcl*-2 and *bcl*-X, may be regulated by the same transcription factors.

Computer analysis of the sequence of the BAG-1 promoter identified a 272 bp CpG island located from nt -472 to -200, indicating that BAG-1 expression may be regulated by methylation/demethylation. DNA methylation/demethylation is important for gene regulation and may play an important role in tumor progression. Activation of oncogenes by demethylation and inactivation of tumor suppressor genes by methylation has been suggested to be a significant event for different genes in all steps that lead to human cancer (Laird and Jaenisch, 1996; Baylin *et al.*, 1998). Since no rearrangement of the BAG-1 gene was found in human cancer cells (unpublished data), demethylation of the BAG-1 promoter could contribute to the enhanced expression of BAG-1 RNA in these cells. In addition, although BAG-1 RNA level was low in C33A cervical carcinoma cells (Yang *et al.*, 1998), promoter activity of the BAG-1 gene was high in this cell line (Fig. 8.3). The latter result indicates that all transcription factors necessary for initiating the transcription of BAG-1 RNA are present in C33A cells. Therefore, methylation of the endogenous BAG-1 promoter could be one of the reasons for the low transcription of BAG-1 RNA in C33A cells. It will be interesting to test these possibilities by examining the effect on luciferase activity of *in vitro* methylation of the BAG-1 promoter in the luciferase vector before its transfection into HeLa or C33A cells and *in vivo* demethylation by 5-azacytidine of the endogenous BAG-1 promoter DNA in normal human, HeLa or C33A cells.

It was reported that wild-type p53 can suppress the promoter activity of a number of cellular genes including c-*fos*, *hsp70*, *Rb* and *DP*1 (Ginsberg *et al.*, 1991; Shiio *et al.*, 1992; Agoff *et al.*, 1993; Gopalkrishnan *et al.*, 1998). Since *bcl*-2 transcription can also be downregulated by wild-type p53 (Miyashita *et al.*, 1994 a, b; Miyashita and Reed, 1995), enhanced expression of Bcl-2 in cancer

cells was at least partially due to the loss of p53-mediated *bcl*-2 suppression. Therefore, enhanced expression of BAG-1 in tumor cell lines (Takayama *et al.*, 1998; Yang *et al.*, 1998) may have resulted from loss of suppression by wild-type p53. Although my results showed that BAG-1 promoter activity relative to protein level was reduced by wild-type p53, no change in its activity was found when it was normalized using β-galactosidase as an internal control. The same observation was made for the c-*fos* promoter by others (Ryan and Vousden, 1998). Therefore, the suppression of gene promoters by p53 may have been caused by apoptosis, as suggested by Ryan and Vousden (1998). Alternatively, p53 may indirectly repress the promoter activity of some genes by modulating the binding of other transcription factors to the promoter. For example, p53 can suppress HIV-1 LTR activity by preventing Sp1 from binding to its binding sites in the LTR (Bargonetti *et al.*, 1997). Generally, wild-type p53 has been shown to inhibit viral or cellular promoters that do not have known p53-binding sites, while transactivating promoters, such as those of *WAF*1 and *bax*, which contain p53-binding sites. Considering all the data, the significance of wild-type p53 in transcription repression remains poorly understood.

Previous studies demonstrated that mutant p53 can confer a gain-of-function, for example inducing transformation, binding to Hsc70 or transactivation of a variety of cellular genes, including *hsp*70, *MDR*-1, *PCNA*, and c-*myc* (Hinds *et al.*, 1990; Chin *et al.*, 1992; Deb *et al.*, 1992; Tsutsumi-Ishii *et al.*, 1995 ; Frazier *et al.*, 1998). Since BAG-1 is an Hsp70-interacting protein (Takayama *et al.*,1997), this raised the possibility that BAG-1, like hsp70, may also be activated by mutant p53. In this report, I provided convincing evidence that gain of function p53 mutants, *p53*-143A, *p53*-175P, *p53*-248W, *p53*-273H, and *p53*-281G (Dittmer *et al.*, 1992), can transactivate the activity from a reporter gene driven from the BAG-1 promoter and transcription of BAG-1 RNA. Although the molecular mechanism for the transactivation of cellular genes by gain-of-function mutant p53 is uncertain, p53 mutants often modulate the transcription of genes such as BAG-1 that lack putative

p53-binding sites (Lanyi *et al.*, 1998) through an indirect mechanism. For *hsp*70, mutated p53 indirectly transactivated its promoter through a heat shock element (Tsutsumi-Ishii *et al.*, 1995). Sp1 has been suggested to mediate the transactivation of the HIV-1 LTR by mutant p53 (Subler *et al.*, 1994). Further experiments need to be performed to clarify how mutant p53 transactivates the BAG-1 promoter. However, my results do indicate that the enhanced expression of BAG-1 in human cancer cells may be due to the transactivation of the BAG-1 promoter by p53 mutants. Furthermore, recent studies indicate that mutant p53 can enhance the resistance of cells to apoptosis induced by anticancer drugs through a p53-independent mechanism (Li *et al.*, 1998; Blandino *et al.*, 1999). Combined with my results, this suggests that mutant p53 can cause drug resistance by increasing the level of anti-apoptotic protein BAG-1 through transactivating BAG-1 transcription. Therefore, understanding the transcriptional regulation of the BAG-1 promoter would be helpful to explain the molecular mechanism of not only BAG-1 overexpression in human cancers, but possibly also how *p53* gain-of-function mutants can lead to some human cancers and their resistance to chemotherapy.

SECTION VI CHAPTER 9

FUTURE DIRECTION

This thesis has described in detail the molecular cloning and characterization of human BAG-1. This study has provided information on how BAG-1 is involved in human cancers. However, to fully understand the molecular mechanism of BAG-1 function, several experiments could be done in the future:

1) The finding that BAG-1 is expressed as four protein isoforms is very important. Further, I and others in the laboratory found that the BAG-1 p50 isoform had not only a distinct subcellular localization, but also a different role in modulating vitamin D receptors, as compared with the other isoforms (Witcher and Yang, unpublished data). Therefore, it would be interesting to examine whether distinct BAG-1 isoforms also have different roles in other cellular process such as metastasis.

2) BAG-1 has previously been shown to be an anti-apoptotic protein. Moreover, in this study, BAG-1 was demonstrated to enhance 4-HPR-induced apoptosis in cervical cells. Further experiments examining the role of BAG-1-cellular protein interactions in apoptosis will help to explain this phenomenon.

3) Although BAG-1 has been shown to be overexpressed in various human cancers, there is no experimental data that show it is directly involved in tumorigenesis. Using soft-agar and nude mice assays, I have found that BAG-1 increased cell proliferation but not nude mouse tumorigenecity of C33A cells (unpublished data). It will be interesting to examine how BAG-1 is involved in multistage carcinogenesis via increasing cell proliferation. Comparing the cellular gene expression in BAG-1-overexpressing versus control cells, using cDNA microarray (Clontech), may finally solve the mystery.

4) In this study, tumor-derived p53 mutants were shown to transactivate the BAG-1 promoter. However, the DNA sequence that mediates this transcription activation has not been identified. Deletion and mutation analysis of BAG-1 promoter should finally identify the response elements for p53 mutants.

5) Although wild-type p53 was not found to have any effect on the BAG-1 promoter, it has been shown that p53 suppresses *bcl*-2 transcription by negatively regulate the 5'-UTR of the *bcl*-2 gene (Miyashita et al., 1994b). Therefore, the effect of wild-type p53 on BAG-1 transcription can be examined using exclusively the 5'-UTR of the BAG-1 gene.

6) Several putative transcription factor binding sequences, such as the WT1 binding site, which were shown to mediate the suppression of *bcl*-2 promoter by WT1 (Hewitt *et al.*, 1995), were identified in the BAG-1 promoter. Further examining the roles of these sequences in the regulation of BAG-1 transcription will be helpful in elucidating the molecular mechanism of BAG-1 overexpression in human cancers.

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