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ROLE OF APOPTOSIS IN MULTIDRUG RESISTANCE AND TUMORIGENESIS OF HUMAN CERVICAL CELLS: IMPLICATION OF BAG-1 AND OTHER APOPTOTIC PROTEINS

By

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ABSTRACT

Recent studies have indicated that inhibition of apoptosis may play an important role in both multistep carcinogenesis and multidrug resistance (MDR). Apoptosis is controlled through many cellular genes. The pattern of these apoptosis-regulating proteins varied in different cell types. The molecular mechanism of apoptosis in the multistep carcinogenesis and multidrug resistance of cervical cells is still poorly understood.

To examine the role of apoptosis in tumorigenesis and chemoresistance of human endocervical cells, a cisplatin-resistant endocervical cell line (HEN-16-2/CDDP) was established by treating an HPV16-immortalized human endocervical cell line previously established in this lab, HEN-16-2, with cisplatin. A phenotype of MDR was identified for HEN-16-2/CDDP by clonogenic survival efficiency assay using two structurally and functionally distinct anticancer drugs: cisplatin and paclitaxel.

The thresholds to undergo apoptosis of HEN-16-2/CDDP cells in response to various apoptotic stimuli was compared with that of its parental HEN-16-2 cells. HEN-16-2/CDDP cells were found to be significantly more resistant to cell death induced by several chemotherapeutic drugs, UV irradiation, anti-Fas antibody and heat shock. Moreover, the dysregulation of apoptosis in HEN-16-2/CDDP cells was found to confer tumorigenicity. Further characterization of HEN-16-2/CDDP cells indicated the following: 1) they displayed distinct morphologies in monolayer; 2) they had an increased rate of proliferation in medium containing physiological calcium levels; 3) they demonstrated anchorage-independent growth *in vitro*; 4) they expressed similar levels of pro-apoptotic genes, including p53, Bak, Bax and the anti-apoptotic gene Bcl-2,

compared to the drug-sensitive cell line, HEN-16-2; and 5) they expressed significantly higher levels of the anti-apoptotic gene Bcl-X_L as well as the p50 and p33 isoforms of BAG-1. Overexpression of BAG-1 in cervical carcinoma C33A cell line confers resistance to cisplatin, etoposide and doxorubicin, but not to actinomycin D and paclitaxel. BAG-1 also protects C33A cells from apoptosis induced by heat shock and UV irradiation.

The yeast two-hybrid system was established to screen BAG-1 interacting proteins from a human keratinocyte cDNA library. Eighteen positives were obtained from 2.5 $\times 10^6$ clones. Further analysis of the interacting clones identified four genes: *Hsp70*, *Hsp70*-2, *Hsc70* pseudogene and a putative novel *Hsp70Y*. Carboxyl-terminal amino acids of BAG-1 were found to be important in the mediation of the interactions.

Overexpression of Hsp70 or Hsp70-2 in C33A cells conferred the resistance to various apoptotic stimuli, including cisplatin, doxorubicin, etoposide, paclitaxel, actinomycin D, heat shock and UV irradiation.

In summary, this study provided the first *in vitro* evidence that inhibition of apoptosis conferred MDR and tumorigenesis in endocervical cells. Increased levels of Bcl-X_L and BAG-1 p50 and p33 isoforms were found to be associated with this phenotype. Hsp70s were identified as BAG-1-interacting proteins from a cDNA library using the yeast two-hybrid system, and further studies indicated that they may also contribute to the regulation of apoptosis.

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3-AT	3-amino-1,2,4-triazole
4-HPR	N-(4-hydroxyphenyl)retinamide
5-FU	5-fluorouracil
ABC	ATP-binding cassette
AD	transcriptional activation domain
Apaf-1	apoptotic proteinase activating factor-1
ATRA	all-trans retinoic acid
BD	DNA-binding domain
bp	base pairs
BSA	bovine serum albumin
CAD	caspase-activated Dnase
cdk	cyclin-dependent kinase
cDNA	complementary DNA
cisplatin	cis-diamminedichloroplatinum (II), CDDP
CSC	cigarette smoke condensate
DFF	DNA fragmentation factor
DISC	death-inducing signaling complex
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ETM	efflux transport machinery

FADD	Fas-associated death domain
FasL	Fas ligand
FasR	Fas receptor
FCS	fetal calf serum
FLIPs	FLICE (caspase-8)-inhibitory proteins
G1	first gap phase (of cell cycle)
G2	second gap phase (of cell cycle)
GST	glutathione S-transferase
HEN	human endocervical cells
HPV	human papillomavirus
HSILs	high-grade squamous intraepithelial lesions
IC_{50}	50% cell viability inhibiting concentration
ICAD	inhibitor of caspase activated DNase
IL-3	interleukin-3
IPTG	isopropyl-β-D-thiogalactoside
ITM	influx transport machinery
kb	kilobase
KDa	kilodalton
KGM	keratinocyte growth medium
LCR	long control regions
LSILs	low-grade squamous intraepithelial lesions
M	mitosis (of cell cycle)

mAb	monoclonal antibody
MDR	multidrug resistance
mRNA	messenger RNA
MTs	metallothioneins
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PARP	poly(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
Pgp	P-glycoprotein
PKCδ	protein kinase C δ
PMSF	phenylmethylsulfonyl fluoride
pRB	retinoblastoma protein
RA	retinoic acid
RNA	ribonucleic acid
Rpm	revolutions per minute
S	synthesis phase (of cell cycle)
SAPK	stress-activated protein kinase
SD	synthetic dropout
SDS	sodium dodecyl sulfate

Siah	SINA-homologous
SINA	Drosophila seven in absentia
SV40	Simian virus 40
TEMED	tetra-methylethylenediamine
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor-α
Topo II	topoisomerase II
TRADD	tumor necrosis factor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
UV	ultraviolet

CHAPTER 1

INTRODUCTION

1.1. Apoptosis, carcinogenesis and cancer therapy

1.1.1. General information on apoptosis

The term, apoptosis, is derived from the Greek word used to describe the shedding of leaves from a tree during autumn. Apoptosis, as seen for example in the developing embryo and during tissue turnover in the adult, is a highly regulated cell suicide process.

Developmentally regulated cell death, which has been studied in both invertebrates and vertebrates, has been referred to as programmed cell death (PCD) since the middle of the 19th century (Vogt, 1842). With the finding that regulated forms of cell death also occur in adult multicellular organisms, the term PCD was adopted to describe all forms of cell death that are mediated by an intracellular program. However, not until 1972 did a report formalize the existence of a form of cell death called apoptosis by describing its distinct morphological characteristics (Kerr *et al.*, 1972).

1.1.2. Morphological characteristics of apoptosis

The earliest recognized morphologic apoptotic changes are: compaction and segregation of the nuclear chromatin; formation of sharply delineated, uniform, fine granular masses that become marginated against the nuclear envelope; condensation of the nucleus and cytoplasm; and the loss of surface cellular protuberances called microvilli (see review Kerr *et al.*, 1994; Liepins and Bustamante, 1994). Progression of the

condensation is accompanied by convolution of the nuclear and cell outlines. This is followed by the breaking up of the nucleus into discrete fragments that are surrounded by double-layered envelopes and by budding or blebbing of the cell as a whole to produce plasma membrane-bound vesicles called apoptotic bodies. The extent of the nuclear and cellular budding varies with cell type. The cytoplasmic organelles within newly formed apoptotic bodies remain well preserved.

Apoptotic bodies arising in tissues are quickly phagocytosed by neighbouring cells or macrophages and degraded within their lysosomes. There is no inflammation associated with the invasion of specialized phagocytes into the tissue, such as occurs with necrosis, and various types of resident cells, including epithelial cells, participate in the removal of apoptotic bodies.

Similar morphologic events occur *in vitro*. However, most apoptotic cultured cells are in the floating population and apoptotic bodies formed in cell culture mostly escape phagocytosis and eventually degenerate.

In addition to apoptosis, cell death can also occur by necrosis and senescence. Necrosis is believed to occur in response to more intense cell injury, resulting in a loss of osmotic balance. The distinction between apoptosis and necrosis is obvious by electron microscopy. Condensation of nuclear chromatin occurs in the early stages of necrosis, but the chromatin is not radically redistributed, as it is in apoptosis, and edges of the chromatin clumps tend to be irregular and poorly defined. The cytoplasm of the necrotic cell becomes grossly swollen, and plasma and organelle membranes progressively disintegrate. Most important, necrosis, unlike apoptosis, is not under biological control.

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.

1.1.3. Biochemical mechanism of apoptosis

Along with the obvious morphological changes, distinct biochemical alterations are also associated with apoptosis. The most prominent is the random cleavage of the genome at intranucleosomal sites, which is detected in agarose gel electrophoresis as a DNA "ladder" composed of fragments in multiples of 180-200 base pairs (bp) (Wyllie, 1987). However, this type of DNA fragmentation does not occur in some experimental systems (Ucker *et al.*, 1992; Oberhammer *et al.*, 1993; Schulze-Osthoff *et al.*, 1994; Sakahira *et al.*, 1998; Janicke *et al.*, 1998).

Apoptosis triggered by various stimuli has in common the ability to induce activation of a family of cysteine proteases called caspases, such as caspase-3, which cleave a variety of specific protein substrates (for review, see Cryns and Yuan, 1997; Nuñez *et al.*, 1998). Caspases implement cell death and "act"as the execution arm for apoptosis (Alnemri *et al.*, 1996; Nuñez *et al.*, 1998). Caspases are crucial components of cell death pathways. They are normally present in the cell as zymogens that require proteolysis for activation of enzymatic activity. The mammalian caspases have been divided into upstream initiator caspases and downstream effector caspases, based on their sites of action in the proteolytic caspase cascade. Binding of initiator caspase precursors to activator molecules appears to promote procaspase oligomerization and autoactivation

by enzymatic cleavage of the procaspase into fragments. Enzymatic activation of initiator caspases leads to proteolytic activation of downstream effector caspases and then cleavage of a number of vital proteins, including poly(ADP-ribose) polymerase (PARP), gesolin, MEKK-1, and lamin (for review see Cryns and Yuan, 1997). PARP cleavage is observed in most forms of apoptosis (Kaufmann 1989; Kaufmann *et al.*, 1993), and is often used as a marker of apoptosis.

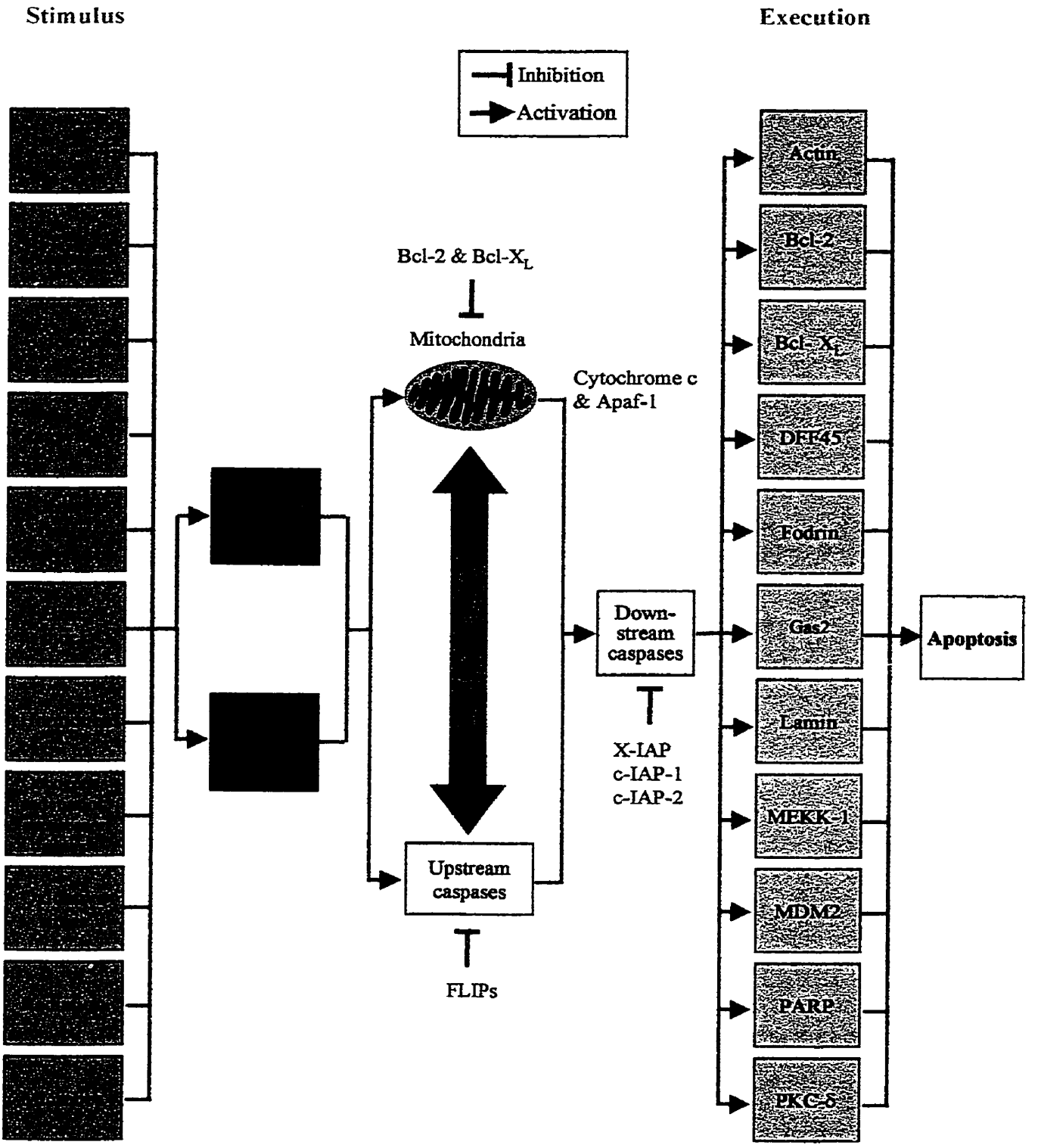
Among the downstream caspases identified, caspase-3 stands out because it is often activated by various cell death signals and cleaves many important cellular proteins, including PARP. It has been recently demonstrated that caspase-3 activates the endonuclease called caspase-activated DNase (CAD), which is responsible for the fragmentation of DNA, by specifically cleaving and inactivating the inhibitor of CAD (ICAD/DFF45) (Liu *et al.*, 1997; Enari *et al.*, 1998; Sakahira *et al.*, 1998).

1.1.4. Regulation of apoptosis

The regulation of apoptosis is summarized in Figure 1.1. Apoptosis can be triggered by a wide variety of stimuli, including chemotherapeutic drugs, ultraviolet light irradiation (UV), heat shock, cytokines, oxidative stress, growth factor deprivation, viral infections, genetic abnormalities, as well as normal differentiation and development. Moreover, the initiation of apoptosis involves biochemical changes that might be unique to each apoptotic stimulus (Ucker, 1997).

The p53 tumor suppressor gene, the "guardian of the genome", has been clearly linked to apoptosis induced by various stimuli (Levine, 1997). Two alternative cellular

Figure 1.1. Apoptosis pathways (adapted from Reed, 1998b). Apoptosis can be triggered by a wide variety of stimuli (examples on the left), all of which have in common the ability to eventually induce activation of downstream caspases that cleave a variety of specific protein substrates, leading to apoptosis. The p53 tumor suppressor gene plays an important role in apoptosis, although p53-independent pathways leading to apoptosis also exist. The complexity of the p53 response may depend on the cellular context. At least two pathways leading to activation of downstream effector caspases have been identified: a mitochondria-dependent pathway clearly governed by the Bcl-2 family of proteins and a parallel pathway involving activation of upstream caspases, such as those involved in Fas signaling. Extensive cross-talk probably exists between these two pathways (bidirectional arrow). Several members of a family of apoptosis-suppressing proteins called inhibitor of apoptosis proteins (IAPs) have been shown to bind directly to the active forms of the downstream effector caspases, but not the upstream initiator caspases. Some of the IAP family can bind to and inhibit effector caspases. FLIP/FLAME family of proteins bind to the inactive zymogens of certain upstream caspases and prevent their activation under some circumstances.


Stimulus
Execution
Inhibition
Activation
Bcl-2 & Bcl-X$_L$
Mitochondria
Cytochrome c
& Apaf-1
Down-
stream
caspases
X-IAP
c-IAP-1
c-IAP-2
Upstream
caspases
FLIPs
Actin
Bcl-2
Bcl-X$_L$
DFF45
Fodrin
Gas2
Lamin
MEKK-1
MDM2
PARP
PKC-δ
Apoptosis

responses occur as a result of p53 induction: growth arrest in the G1 phase of the cell cycle or apoptosis. p53 was recognized as a regulator of apoptosis following the observation that transfection or activation of wild-type p53 in tumor cells can result in rapid apoptotic cell death (Yonish-Rouach *et al.*, 1991; Levy *et al.*, 1993). Studies demonstrated that cell death induced by serum deprivation in Myc-overexpressing cells or in interleukin-3 (IL-3) -dependent thymocytes required functional p53 to induce apoptosis (Wagner *et al.*, 1994; Hermeking and Eick, 1994; Canman *et al.*, 1995). DNA strand breaks induce rapid p53 upregulation, but exactly how remains unknown. The upregulation of p53 is mostly post-transcriptional, involving both an increase in translation and a prolonged protein half-life (Dragovich *et al.*, 1998).

p53 is a sequence-specific DNA-binding protein, and known targets of p53 include genes associated with growth control, cell cycle checkpoints and DNA repair (e.g., WAF1/CIP1, WIP1, MDM2, EGFR, PCNA, cyclin D1, cyclin G, TGF-α, 14-3-3 σ, and GADD45), and apoptosis (Bax, Bcl-X_L, FasL, IGF-BP3, PAG608 and DR5) (reviewed by Amundson *et al.*, 1998). Activation of p53 results in a cascade of downstream events, depending on the cellular environment. Although most studies have focused on the involvement of p53 in regulating apoptosis, p53-independent apoptosis pathways were found to exist (Strasser *et al.*, 1994).

At least two apoptotic pathways leading to the execution of apoptosis have been identified (Figure 1.1): a mitochondria-dependent pathway that is clearly governed by Bcl-2 family proteins and a parallel pathway involving activation of upstream caspase-8, such as those involved in Fas and TNF receptor signaling.

Some cytokines, such as FasL and TNF-α, can bind to their receptors on the plasma membrane, causing trimerization of their receptors and thereby activation of an initiator caspase such as caspase-8 through interaction of the receptor with death adaptor proteins such as FADD or TRADD (Baker and Reddy, 1998; Nuñez *et al.*, 1998). In addition, other apoptotic stimuli, such as the anticancer therapeutic agent using cis-diamminedichloroplatinum (II) (cisplatin, CDDP), can cause mitochondria dysfunction. Mitochodrial dysfunction includes a reduction in the mitochondrial membrane potential ($\Delta\psi m$), production of reactive oxygen species (ROS), opening of the permeability transition pore (PTP), and the release of the intermembrane space protein, cytochrome c (see review Gross *et al.*, 1999). In response to cytochrome c binding, the apoptotic proteinase activating factor-1 (Apaf-1) can form a complex with and then activate initiator caspase-9 (Li *et al.*, 1997; Zou *et al.*, 1997). Cross-talk probably exists between these two pathways within the cell. For example, Bid, a pro-apoptotic Bcl-2 family protein, is cleaved into two fragments by caspase-8 in response to signaling by Fas or TNF receptor. The C-terminal fragment of Bid then binds to mitochondria, thus initiates the mitochondria-dependent pathway to apoptosis (Li *et al.*, 1998; Luo *et al.*, 1998).

Several members of a family of apoptosis-suppressing proteins called inhibitor of apoptosis proteins (IAPs) have been shown to bind directly to the active forms of the downstream effector caspases, such as caspase-3, and to potently inhibit their enzymatic activities (Deveraux *et al.*, 1997; Roy *et al.*, 1997). FLIP family proteins bind to the proforms of certain upstream caspases, such as procaspase-8 and procaspase-10, and prevent their activation under some circumstances (Imler *et al.*, 1997; Srinivasula *et al.*,

1997).

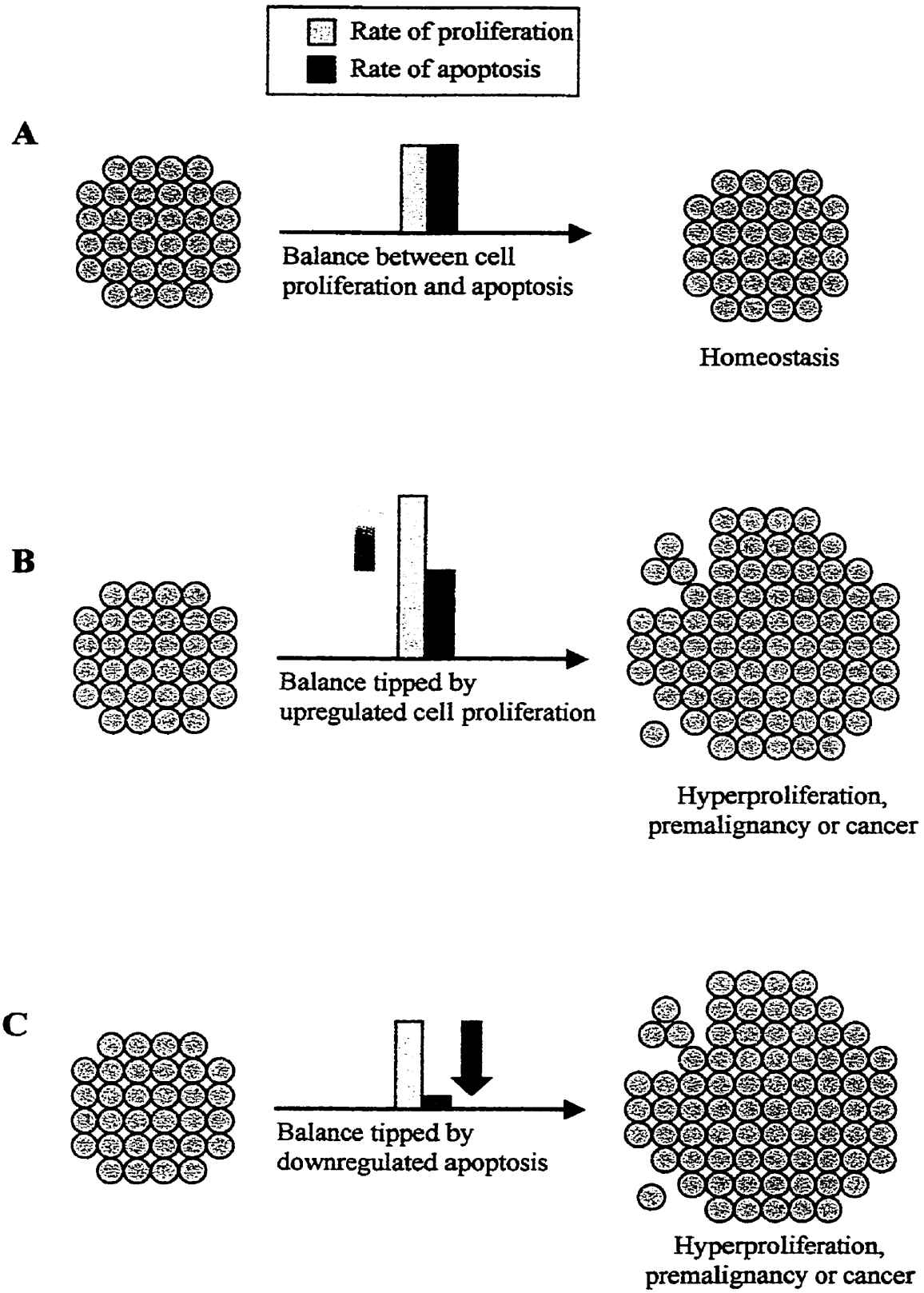
Different pathways involved in regulating cell proliferation and apoptosis may have different significance in various cells or within the same cell at different stages of development or differentiation. Extensive cross-talk probably also exists between proliferation and apoptosis within the cell. For example, Akt, a growth factor-dependent serine/threonine kinase, will phosphorylate Bad when the growth factor binds to the receptor. Bad is a Bcl-2 family protein and executes its pro-apoptotic function by binding to anti-apoptotic Bcl-2 and Bcl-X_L. Phosphorylated Bad is sequestered by cytosolic 14-3-3 protein, thus releasing and increasing the levels of free Bcl-2 and Bcl-X_L. Therefore, this growth factor-initiated signal pathway interferes with the Bcl-2- and Bcl-X_L-dependent regulation of the cellular apoptotic threshold through Bad phosphorylation (Zha *et al.*, 1996; Gajewski and Thompson, 1996).

1.1.5. Apoptosis in carcinogenesis

1.1.5.1. Cellular basis

Normal tissue has carefully balanced proliferation and apoptosis. Rates of proliferation are paired with rates of apoptosis so that cell numbers remain constant and tissue homeostasis is maintained (Figure 1.2A). However, given the critical role of apoptosis, it is not surprising that dysregulation of apoptosis occurs frequently during pathological disturbances. Neoplasia is a good example in which apoptosis is dysregulated. Carcinogenesis is characterized by the abnormal accumulation of cells. This accumulation of cells is generally accepted to be the result of enhanced cellular

Figure 1.2. The effect of relative rates of proliferation and apoptosis on homeostasis and carcinogenesis (adapted from Thompson, 1995). The rates of cell proliferation and apoptosis are indicated by the yellow and blue bars, respectively. In mature organisms, cell number is controlled by the net effects of cell proliferation and apoptosis, which are normally balanced and lead to homeostasis (A). In the absence of compensatory changes in the rates, increased cell proliferation (B), or decreased apoptosis (C), can result in cell accumulation, as seen in hyperproliferation, premalignancy and cancer.


Rate of proliferation
Rate of apoptosis
A
Balance between cell proliferation and apoptosis
Homeostasis
B
Balance tipped by upregulated cell proliferation
Hyperproliferation, premalignancy or cancer
C
Balance tipped by downregulated apoptosis
Hyperproliferation, premalignancy or cancer

proliferation (Figure 1.2B). However, recent mounting evidence suggests that the downregulation of apoptosis is also of critical importance resulting in an abnormal accumulation of cells during the initiation, promotion and transformation of some neoplasms (Figure 1.2C) (Reed, 1994; Reed *et al.*, 1995; Thompson, 1995).

In addition, apoptosis is important for metastasis. Apoptosis induced in the absence of native or the presence of foreign environment-specific factors in foreign sites may serve to prevent the survival of cancer cells that migrate from their native tissues (Williams *et al.*, 1990; Neiman *et al.*, 1991; Raff *et al.*, 1993; Cyster *et al.*, 1994; Frisch and Francis, 1994; Boudreau *et al.*, 1995). However, metastatic tumor cells have circumvented this homeostatic mechanism and can survive at sites distinct from the tissue in which they arose. For instance, epithelial cells that detach from the extracellular matrix in the process of metastasis rely on inhibition of apoptosis for survival in the absence of integrin-mediated signaling (Rabinovitz and Mercurio, 1996). Inactivation of the apoptosis pathway is thus a central event in the development of cancers.

1.1.5.2. Molecular basis

Recent advances are beginning to shed some light on the molecular basis for the role of apoptosis in carcinogenesis. Mutations or dysregulated expression of apoptosis-related genes, often in synergy with other genetic lesions that result in a high rate of proliferation, can be shown to result in tumor development. The relationship between the acquisition of these genetic lesions and the development of cancer is complex and highly tissue-specific.

Several types of genes that can be critical in the regulation of apoptosis have been defined. Bcl-2 production at abnormally high levels or in aberrant patterns is found in approximately half of all human cancers, suggesting that deregulated expression of this proto-oncogene represents one of the most common events associated with human malignancy (Reed, 1995a). Recently, it has been demonstrated that *Bcl-2* is only one member of a gene family that can control the cellular apoptotic threshold (Boise *et al.*, 1993; Kozopas *et al.*, 1993; Lin *et al.*, 1993; Oltvai *et al.*, 1993).

The wild-type p53 gene product influences several essential processes that prevent potentially oncogenic mutant cell propagation. It appears to have a direct role in DNA repair and also plays a role in regulating DNA repair enzymes, arresting cellular growth and inducing apoptosis following exposure to genotoxic stress. Loss of p53 function is strongly associated with the development and progression of many tumor types (Hollstein *et al.*, 1994). Moreover, studies in transgenic mice have confirmed the notion that the tumor suppressor role of p53 *in vivo* is closely linked to its ability to induce apoptosis (Symonds *et al.*, 1994; Donehower *et al.*, 1995).

A number of viral oncoproteins have been shown to play roles in regulating apoptosis. Examples are the E1B of adenovirus and E6 of human papillomavirus (HPV). E1B and E6 disable the p53 pathway in apoptosis, canceling the pRB-mediated cell death response to E1A or E7, respectively (Rao *et al.*, 1992; Debbas and White, 1993; White *et al.*, 1994).

IAPs can block downstream effector caspase and therefore inhibit apoptosis. An example is survivin, one member of the IAP family. Survivin is not detectable in adult

differentiated tissue; however, it is expressed in most cancers tested, including lung, colon, breast, prostate, pancreatic cancers, high grade lymphomas, neuroblastomas and gastric carcinoma (Ambrosini *et al.*, 1997; Adida *et al.*, 1998; Kawasaki *et al.*, 1998; Lu *et al.*, 1998). Overexpression of FLIPs (the inhibitors of upstream initiator caspases) is also detected in some human cancers (Irmler *et al.*, 1997).

1.1.6. Apoptosis in cancer chemo- and radiotherapies

Cancer chemo- and radiotherapies kill targeted malignant cells by causing irreversible cellular damage. The mechanism of this action was previously thought to be due to cell necrosis. However, recent studies have indicated that induction of apoptosis is the primary cytotoxic mechanism of action of most radio- and chemotherapeutic agents (Kerr *et al.*, 1994). Immunohistochemical assays specific for apoptotic cell death have revealed that cell death by apoptosis, not necrosis, often follows radiotherapy or chemotherapy (Eastman, 1990; Hickman, 1992). Consistent with this notion, clinical data has suggested that there are prognostic links between treatment outcome and distinct molecular genetic alterations that are known to regulate apoptosis (Fung and Fisher, 1995). Overexpression of *Bcl*-2 or related genes can result in a multidrug resistance (MDR) phenotype *in vitro* (Reed, 1995b; Thompson, 1995).

1.2. Drug resistance of cancer cells

1.2.1. General information

The response of tumors to chemotherapy varies. Failure to respond is frequent and

is due usually to the emergence of drug resistance. Drug resistance is one of the major obstacles hindering the success of conventional antineoplastic agents. In Canada, drug resistance is the principal explanation for the high mortality rate (approximately 65 %) from many cancers found in men and/or women (NCIC, Canadian Cancer Statistics, 1999). The significance of this is exemplified by the fact that cancer causes a greater loss of potential years of life than any other disease, including heart disease.

Patterns of drug resistance are divided into two groups: intrinsic and acquired. Intrinsic drug resistance is seen when previously untreated cancers are resistant to chemotherapeutic agents and is observed in patients with malignant melanomas, non-small cell lung cancers, pancreatic cancers, renal cancers, and colon cancers. Tumors with acquired drug-resistance, including breast carcinomas, small cell lung cancers, acute leukemias, ovarian carcinomas, and cervical carcinomas, are responsive to initial treatments, but often become refractory to further therapy. Relapse of tumors, particularly during or shortly after the completion of therapy, generally heralds the emergence of tumor cells that are resistant to the antineoplastic agents used initially and often to other drugs to which the patient was never previously exposed.

The phenomenon of clinical drug resistance has prompted studies to identify the mechanisms involved. Using *in vitro* (tissue culture) and *in vivo* (animal and xenograft) models, a number of physical and biochemical mechanisms of drug resistance have been identified (Table 1.1). Physiological resistance to chemotherapy implies the host-drug-tumor interactions or anatomic drug barriers. Some physiological and biochemical mechanisms can overlap. Traditionally, the term "drug resistance" in basic science refers

to biochemical resistance that is seen in the targeted tumor cells, as opposed to changes such as the detoxification of drugs by the non-malignant liver.

Table 1.1. General mechanisms contributing to drug resistance

Physiological Mechanisms
Anatomic drug barriers
Host-drug-tumor interactions
Biochemical Mechanisms
Decreased intracellular drug accumulation
Decreased influx
Increased efflux
Altered intracellular drug metabolism
Increased inactivation
Decreased pro-drug activation
Increased drug detoxification
Altered drug targets
Changes in molecules involved in repair of cellular damage
Cellular oncogene and tumor suppressor genes in drug resistance
Apoptosis regulation in drug resistance

1.2.2. Biochemical mechanisms

To understand the complexity of the biochemical mechanisms of drug resistance, it is essential to realize that cell killing by each cytotoxic drug is a complex process (Figure 1.3). While some drugs can enter the cell by passive diffusion through the plasma membrane lipid bilayer, other agents require the presence of special membrane carriers and/or pores for entry. Some drugs are inactive in the form in which they enter the cell

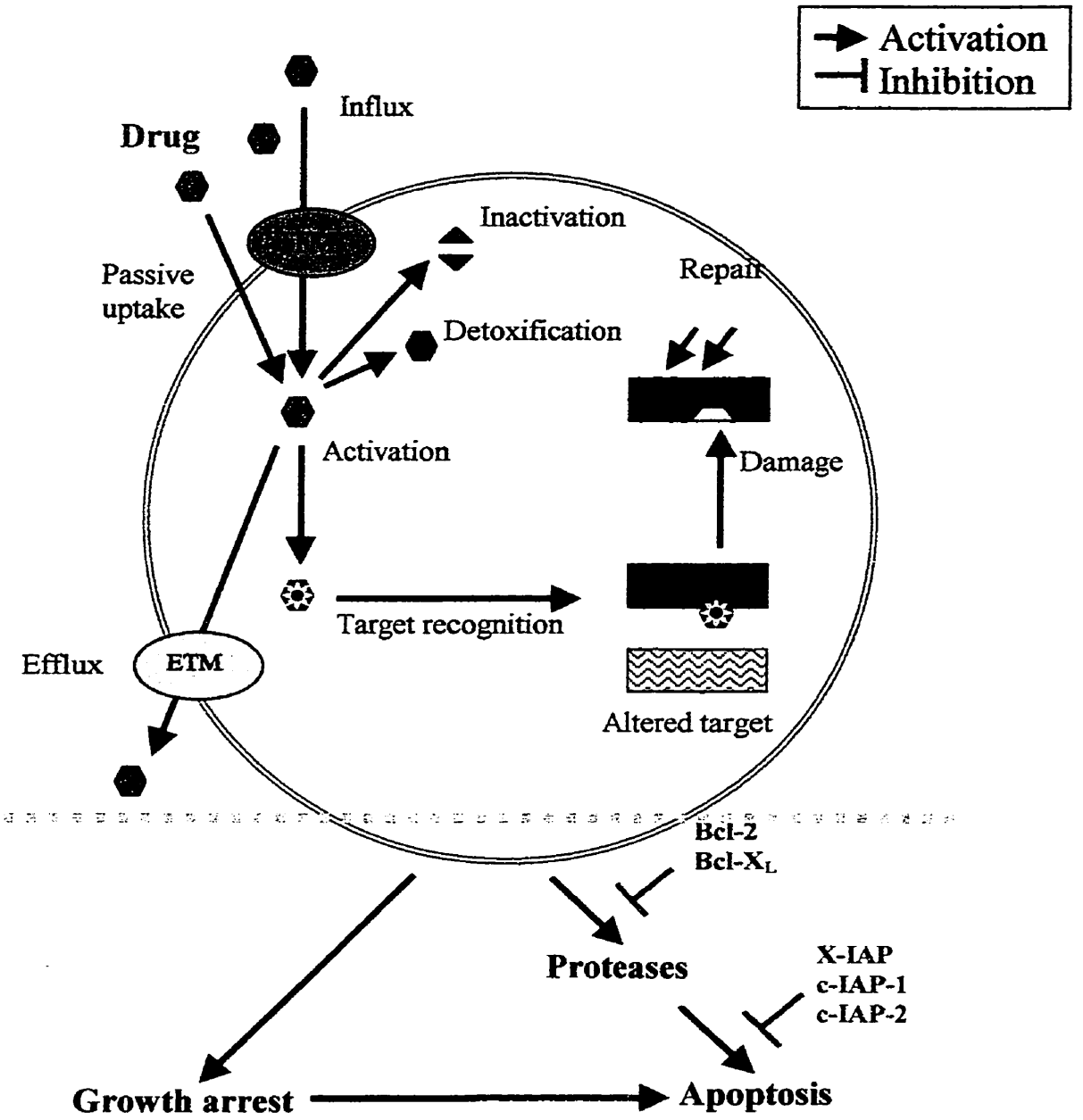


Figure 1.3. Drug resistance pathways (adapted from Roninson, 1997). The potential biochemical changes are indicated as black arrows. The dashed line separates two types of MDR mechanisms. Above the dashed line, are the mechanisms that limit the extent of drug-induced cellular damage; below the dashed line, are the mechanisms that alter the cellular response to the damage. ITM, influx transport machinery. ETM, efflux transport machinery.

and need to be activated through chemical modification by appropriate cellular enzymes. An active drug inside the cell needs to reach and bind to its specific target, such as DNA or microtubules, and interfere with its normal function. These events could trigger cell growth arrest or apoptosis.

The alteration of any step between drug uptake and cellular damage response genes could result in drug resistance. Generally, the mechanisms of drug resistance can be described as those which limit the extent of drug-induced damage or alter the cellular response, and can be categorized into the following six areas.

1.2.2.1. Altered intracellular drug influx or efflux

Decreased intracellular accumulation of cytotoxic agents due to decreased influx or increased efflux is one of the most common mechanisms of drug resistance. This may result from decreased drug influx due to a defective carrier-mediated transport system. Decreased influx via a reduction in high affinity folate-binding protein transport as well as via a reduced folate carrier has been identified in methotrexate resistant cells (Hill *et al.*, 1979; Sirotnak *et al.*, 1981; Antony *et al.*, 1985; Dixon *et al.*, 1994). Similarly, a deficient membrane influx transport system has been identified in cells resistant to nitrogen mustard (Goldenberg *et al.*, 1970).

Enhanced drug efflux may also lower intracellular steady state levels of drugs. Classical MDR is frequently associated with overexpression of P-glycoprotein (Pgp), a transmembrane transport protein capable of expelling and maintaining tolerable intracellular levels of certain cytotoxic drugs (Juliano and Ling, 1976; Endicott and Ling,

1989). Pgp belongs to the ATP binding cassette (ABC) family of transporter molecules, directly binds cytotoxic compounds and expels them from the cell through an energy-dependent efflux mechanism (Hyde *et al.*, 1990; Beck and Qian, 1992; Germann *et al.*, 1993; Breuninger *et al.*, 1995; Bosch and Croop, 1996). Transducing the cDNA of *mdr-1* (the gene encoding Pgp) into tissue culture cells resulted in an MDR phenotype, even before stable cell lines were selected (Gros *et al.*, 1986; Croop *et al.*, 1987). In addition, the incorporation of purified Pgp into liposomal membranes has demonstrated that Pgp is able to hydrolyze ATP and transport drugs (Saeki *et al.*, 1992; Thierry *et al.*, 1992; Sharom *et al.*, 1993; Naito and Tsuruo, 1995; Shapiro and Ling, 1995; Dong *et al.*, 1996; Eytan *et al.*, 1997). MDR-related protein (MRP) family members, MRP1, MRP2, MRP3, MRP4, MRP5, and cMOAT, are other members of the ABC family of transporters (Cole *et al.*, 1992; Krishnamachary and Center, 1993; Zaman *et al.*, 1994; Lautier *et al.*, 1996; Kool *et al.*, 1997).

Lung-related protein (LRP) is also associated with an MDR phenotype (Scheper *et al.*, 1993). It has been identified as the major component of certain nucleoprotein particles (vaults), which possibly translocate cytotoxic drugs from nuclei to cytoplasmic vesicles, which in turn release their contents at the cell surface (Scheper *et al.*, 1993; Izquierdo *et al.*, 1996).

1.2.2.2. Altered intracellular drug metabolism

The cytotoxicity of many chemotherapeutic agents is determined by the enzymatic conversion of the drugs into their active metabolites. For example, 5-fluorouracil (5-FU)

is a pro-drug, and must be activated to its cytotoxic form by the targeted tumor. Resistance to such nucleic acid base and nucleoside drugs has been associated with decreased conversion of these analogues to their cytotoxic nucleoside and nucleotide derivatives by phosphorylases, kinases and the phosphoribosyltransferase salvage pathway (Drahovsky and Kreis, 1970).

Furthermore, cellular factors involved in detoxifying chemotherapeutic agents could impact on the cytotoxicity of drugs that have gained access to the intracellular compartment of tumor cells. A number of mechanisms may permit detoxification. For example, glutathione (GSH), is an important intracellular antioxidant. When a compound is conjugated with GSH, the compound becomes more hydrophilic, more readily excreted, and usually less toxic (O'Brien and Tew, 1996). A group of cytosolic enzymes termed GSH-S-transferases (GSTs) conjugate certain drugs with GSH. This detoxification mechanism may require vesicle-mediated transport of GSH-drug conjugates by a poorly understood ATP-dependent GS-X pump. Overexpression of GST-π leads to resistance to alkylating drugs and to platinum compounds through their conjugation with GSH (Ozols *et al.*, 1990). Further study indicates that many drug-resistant cell lines have increased expression or activity of GSH and/or related enzymes (O'Brien and Tew, 1996).

Metallothioneins (MTs) are low molecular weight intracellular proteins characterized by high cysteine content and affinity for binding heavy metals. These proteins are located mainly in parenchymal tissues such as the liver, gut and kidneys, where they play a role in the detoxification of cadmium, platinum and certain other heavy

metals and in the regulation of normal zinc and copper metabolism. Overexpression of MTs has been correlated with acquired resistance of cancers to cytotoxic alkylating agents and to cisplatin through an unknown mechanism (Mousseau *et al.*, 1993; Kelley *et al.*, 1988; Lohrer and Robson, 1989; Kaina *et al.*, 1990).

In addition, enhanced inactivation of pyrimidine and purine analogues by elevated deaminases and oxidases has been linked to resistance toward these agents (Steuart and Burke, 1971; Hunt and Hoffee, 1983).

1.2.2.3. Altered drug targets

The mechanisms of cytotoxicity of several antineoplastic drugs involve interactions between the drugs and essential intracellular enzymes that consequently alter or inhibit normal functions. Quantitative or qualitative changes in these enzyme targets of antineoplastic drugs can compromise drug efficacy.

Topoisomerase (Topo) II is a nuclear enzyme that modifies the topologic state of DNA to facilitate strand relaxation, controlled cleavage, and religation of the DNA helix during replication and repair. It also serves a role during chromosome segregation in mitosis. Topo II is an important target of several antineoplastics, including etoposide and doxorubicin, which bind to and inhibit Topo II enzymatic religation, thereby stabilizing the enzyme-DNA cleavage complex. Qualitative changes affecting Topo II activity in selected cell lines result in an MDR phenotype similar to that described for Pgp, with the characteristic exception of preserved sensitivity to microtubule-targeting agents, such as Taxol (Potmesil *et al.*, 1987). Alterations of several other enzymes, including the

methotrexate-targeted dihydrofolate reductase, are associated with drug-resistance (Haber *et al.*, 1981).

1.2.2.4. Changes in molecules involved in repair of cellular damage

Cells contain multiple complex systems involved in damage repair, especially in their membranes and DNA. Because such damage may occur as a direct or secondary consequence of cytotoxic drug action, altered repair mechanisms can influence drug sensitivity of tumor cells.

For instance, alkylating agents induce lethal DNA damage by forming covalent bonds with nucleophilic sites in DNA. The N7 and O6 atoms of guanine are probably the main targets for alkylation of DNA. Repair of DNA adducts represents one of the main mechanisms of cellular protection, and one important DNA repair enzyme is O6-methylguanine-DNA methyltransferase (MGMT), which removes alkyl adducts from the O6 atom of the guanine base (Gerson *et al.*, 1995). A striking correlation between MGMT activity and resistance to nitrosourea and cisplatin has been demonstrated both *in vitro* and in xenograft models (Pegg *et al.*, 1984; Gerson *et al.*, 1994).

Correlation between mutations in DNA mismatch repair genes, such as h*MLH*1, and resistance to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and cisplatin has been reported (Kat *et al.*, 1993). Introduction of wild-type h*MLH*1 gene-carrying chromosome 3 into hMLH1 mismatch repair-deficient HCT-116 human colon cancer cells restored mismatch repair efficiency and conferred increased sensitivity to MNNG (Koi *et al.*, 1994).

1.2.2.5. Cellular oncogenes and tumor suppressor genes in drug resistance

It has recently been appreciated that changes in the level or activity of cellular oncogene and tumor suppressor gene products may be responsible for resistance to a broad range of drugs. There are a number of such cellular genes whose upregulation, downregulation or mutation contributes to drug resistance.

For example, c-*fos* and c-*jun* proto-oncogene levels can be elevated in chemoresistant cells (Bhushan *et al.*, 1992; Yamazaki *et al.*, 1994; Moffat *et al.*, 1996). Also, a relationship between cisplatin resistance and overexpression of the c-*myc* gene has been observed in an erythroleukemia cell line (Sklar and Prochownik, 1991). Transfection of c-*myc* enhanced cisplatin resistance of NIH 3T3 cells, and antisense oligonucleotide to c-*myc* RNA enhanced cisplatin sensitivity of urinary bladder cancer cells (Niimi *et al.*, 1991; Mizutani *et al.*, 1994; Sanchez-Prieto *et al.*, 1995;). Serially transplanted tumors that survived treatment with cisplatin displayed elevation in c-*myc* expression, and their growth could be inhibited by c-*myc* antisense RNA (Walker *et al.*, 1996). In addition, blocking Ras oncogene product function by farnesyltransferase inhibitors caused increased radiosensitivity and chemosensitivity (Bernhard *et al.*, 1996; Danesi *et al.*, 1996). Furthermore, studies have correlated *Her-2/neu* oncogene expression with intrinsic MDR of human non-small cell lung cancer cells (Tsai *et al.*, 1993), and transfection of *Her-2/neu* conferred chemoresistance on these cells (Tsai *et al.*, 1995). Blocking *Her-2/neu* receptor function by emodin and tyrphostin, inhibitors of tyrosine kinase activity, sensitized *Her-2/neu*-overexpressing lung cancer cells to *in vitro* killing by cisplatin, doxorubicin, or etoposide (Tsai *et al.*, 1996; Zhang and Hung, 1996).

One of the roles of p53 is to regulate the cell cycle. Functional p53 is required for G1 cell cycle arrest in response to DNA damage. The loss or mutation of p53 can affect drug sensitivity or resistance (Ruley, 1996; Velculescu and El-Deiry, 1996; Gallagher *et al.*, 1997; Coukos and Rubin, 1998). pRB is another cell cycle regulator/tumor suppressor gene that is involved in drug resistance (Wang *et al.*, 1998; Yamamoto *et al.*, 1998; Yoo *et al.*, 1998). Studies have shown that cells null for the $p21^{WAF1/CIP1}$ cell cycle regulator display defective repair of *in vitro* damaged DNA and are more sensitive to the cytotoxic effects of a variety of cytotoxic drugs, as well as to UV (McDonald *et al.*, 1996; Waldman *et al.*, 1996). Recently, $p27^{KIP1}$ has been also demonstrated to play a role in drug resistance in some neoplasia (for review, see Lloyd *et al.*, 1999).

1.2.2.6.. Apoptosis regulation in drug resistance

In addition to the role of apoptotic inhibition in oncogenesis (section 1.1.5), it is becoming clear that the same process is involved in the drug resistance of many cancers. This is because essentially all chemotherapeutic drugs available to date, as well as radiation, ultimately act on tumor cells through apoptosis. The Bcl-2 family proteins have been implicated not only in the pathogenesis of cancer, but also in resistance to chemotherapy. Anti-apoptotic Bcl-2 expression correlates with poor response to chemotherapy and shorter survival for patients with some types of lymphomas, acute myelogenous leukemias, and prostate cancers (Lotem *et al.*, 1993; Campos *et al.*, 1993; Reed, 1995a; 1995b; 1998). Conversely, reductions in Bcl-2 achieved by antisense methods sensitize cells to multiple chemotherapeutic drugs (Webb *et al.*, 1997).

Therefore, the ability of Bcl-2 to block cell death induced by all types of anticancer drugs indicates that Bcl-2 represents a novel MDR protein. Overexpression of Bcl-2 protein prevents drug-induced apoptosis. Antineoplastic agents interfere with DNA synthesis, or interfere with microtubule formation in tumor cells that contain high levels of Bcl-2, but the cells remain viable for protracted periods of time, resulting in enhanced clonogenic survival. Bcl-X_L, another anti-apoptotic Bcl-2 family member, can also confer high-level resistance to chemotherapeutic agents (Minn *et al.*, 1995; Taylor *et al.*, 1999).

Downregulation of pro-apoptotic Bcl-2 family proteins can result in drug resistance. Consistent with this notion, overexpression of Bax protein rendered tumor cells more sensitive to many anticancer drugs (Bargou *et al.*, 1996). Ablating Bax expression reduced drug-induced apoptosis (Perez *et al.*, 1997).

The Bcl-2 family members are also linked to drug sensitivity and resistance through their regulation by p53. Numerous studies have linked p53 to apoptosis in cases when DNA is damaged by anticancer drugs (see section 1.1.4). Inactivation of p53 correlates with enhanced resistance to anticancer drugs and a poorer prognosis in most human malignancies (Harris and Hollstein, 1993; Lowe *et al.*, 1994). In a controlled experiment using genetically defined tumors in immunocompromised mice, mutation of p53 was associated both with resistance to chemotherapy and with tumor relapse (Lowe *et al.*, 1994).

1.3. Molecular mechanism of multistage cervical cell carcinogenesis

1.3.1. The role of HPVs in cervical oncogenesis

Cervical cancer was recognized as a sexually transmitted disease more than a century ago and since then numerous infectious agents have been suggested to play a causative role (reviewed by zur Hausen and de Villiers, 1994; Alani and Münger, 1998). A subset of the more than 90 papillomaviruses (HPVs) was detected in more than 90% of cases of cervical carcinoma, providing compelling evidence for HPV infection as a causative factor (zur Hausen, 1991a; Bosch *et al.*, 1995; Alani and Münger, 1998).

HPVs are small DNA viruses that are found in many vertebrate species and contain approximately 8 kb double-stranded circular genomes, which are generally divided into three distinct regions: early (E), late (L), and long control regions (LCR) (Figure 1.4). The early region open reading frames (ORFs) are designated E1, E2, E4, E5, E6 and E7, and encode proteins required for viral DNA replication, viral RNA transcription, viral and cellular gene regulation, and oncogenesis. The late ORFs, L1 and L2, encode viral capsid proteins. The LCR, also called the noncoding region or upstream regulatory region, is found between the 3' end of the late region and 5' end of the early region. It contains DNA elements that regulate HPV RNA transcription and DNA replication by interacting with viral and cellular transcription and replication factors (Hoppe-Seyler and Butz, 1994).

The anogenital HPVs are generally categorized into two groups based on their ability to induce viral-associated cancers: low-risk (not associated with cancer) and high-risk (cancer-associated) (de Villiers, 1989). Generally, low-risk HPVs, such as HPV6 and 11, are associated with benign genital condylomas and oral and other papillomas; while high-risk HPVs, such as HPV16 and 18, are increasingly associated with normal

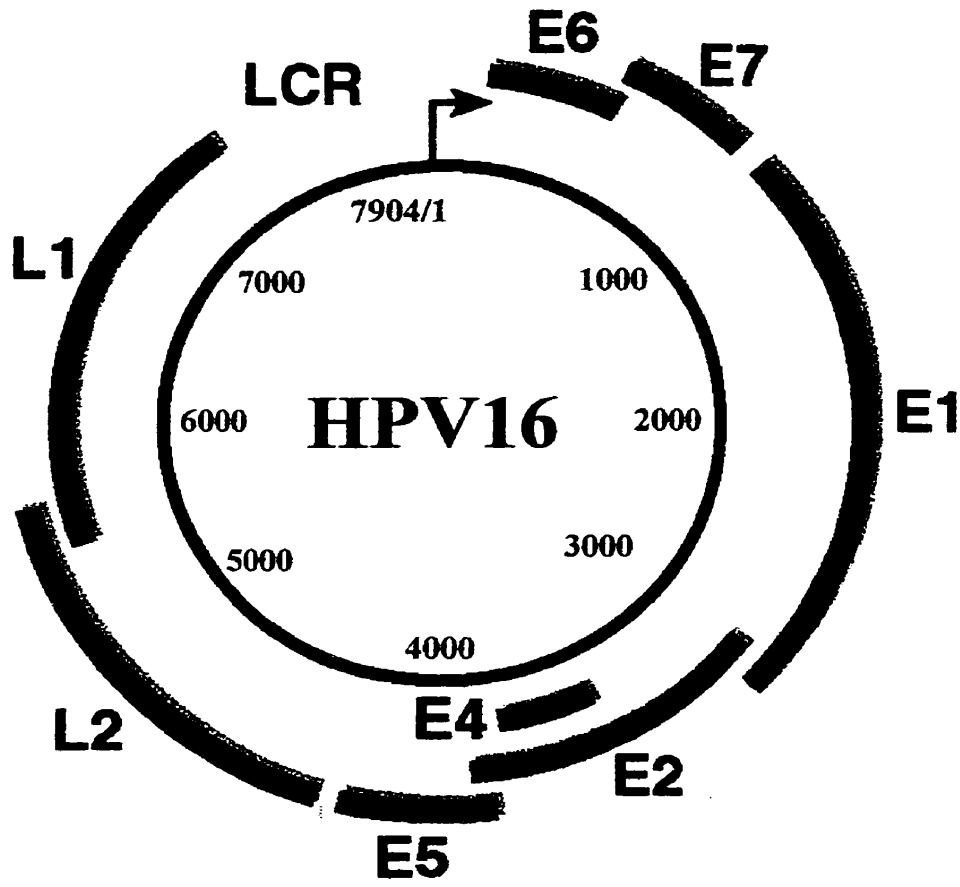


Figure 1.4. Genomic organization of HPV16 (adapted from Alani and Münger, 1998). All papillomaviruses contain a double-stranded circular DNA genome of approximately 8 kb. Transcription occurs from only one strand of DNA. Nucleotide positions are indicated in the circle.

epithelia, low-grade squamous intraepthelial lessions (LSILs), high-grade squamous intraepthelial lessions (HSILs), and invasive carcinomas (zur Hausen, 1991a).

Although infection with high-risk HPV types is relatively common, few infected women eventually develop cervical carcinoma. In addition, the interval between primary HPV infection and cervical cancer is usually several decades, suggesting that HPV infection alone is insufficient to generate the fully malignant phenotype (zur Hausen, 1994; Ponten *et al.*, 1995). Therefore, other events are also required for the development of cervical cancer.

In vitro studies demonstrated that transfection of high-risk HPVs, such as HPV16 and 18, can initiate immortalization of ectocervical and endocervical cells (Pirisi *et al.*, 1987; Woodworth *et al.*, 1988; Tsutsumi *et al.*, 1992; Pecoraro *et al.*, 1989; Woodworth *et al.*, 1989). Furthermore, cotransfection of HPV16 or 18 with an oncogene, such as c-*myc*, v-*fos* or H-*ras*, transformed rodent cells and primary human fibroblasts and keratinocytes *in vitro* (DiPaolo *et al.*, 1989; Durst *et al.*, 1990; Pei *et al.*, 1993), suggesting that HPVs can cooperate with oncogenes in carcinogenesis.

1.3.2. Functional consequences of high-risk HPV E6 and E7 oncogenes

Two genes of the high risk HPVs, namely *E6* and *E7*, can immortalize and transform cells by cooperating with other oncogenes (Bedell *et al.*, 1989; Phelps *et al.*, 1988; Storey *et al.*, 1988; Storey and Banks, 1993). The E6 and E7 viral oncoproteins of high-risk HPVs have also been shown to be selectively maintained in most virally induced tumors (Schneider-Gädicke and Schwarz, 1986; Pater and Pater, 1988; Hawley-

Nelson *et al.*, 1989; Woodworth *et al.*, 1989; van den Brule *et al.*, 1991; zur Hausen, 1991b).

One property of E6 and E7 that may contribute to oncogenic genetic changes is their fortuitous modulation of the cellular response genes to DNA damage (Figure 1.5). Normally, DNA damage results in the accumulation of wild-type p53 protein (section 1.1.4), which increases $p21^{waf1/cip1}$, which in turn inhibits the activation of cyclin-cdk complexes that phosphorylate retinoblastoma tumor suppressor protein (pRB). Consequently E2F transcription factors therefore remain associated with pRB and are unable to activate transcription of genes required for progression from G1 into S phase. Consequently, cells are arrested in G1, and this G1 arrest is thought to allow repair of damaged DNA.

The immortalization and transformation activities of high-risk HPV E6 and E7 correlate, at least in part, with their inactivation of p53 and pRB (Dyson *et al.*, 1989; Heck *et al.*, 1992; Mansur *et al.*, 1993; Münger *et al.*, 1989; Nakagawa *et al.*, 1995; Werness *et al.*, 1990). The E6 oncoprotein of the high-risk HPVs 16 and 18 binds p53 and promotes its ubiquitin-mediated degradation (Werness *et al.*, 1990; Scheffner *et al.*, 1990; 1994). Similarly, the E7 protein has been shown to bind pRB and modify pRB function (Dyson *et al.*, 1989; Dyson *et al.*, 1992; Davies *et al.*, 1993). The cyclins, cdk and other cellular targets that regulate normal cellular function may also be dysregulated by viral E6 and E7 oncoproteins. Moreover, apoptotic cell death is another important response to DNA damage that may also be influenced by HPV oncoprotein expression. In cervical tumors that are not associated with HPV infection, p53 and pRB may be

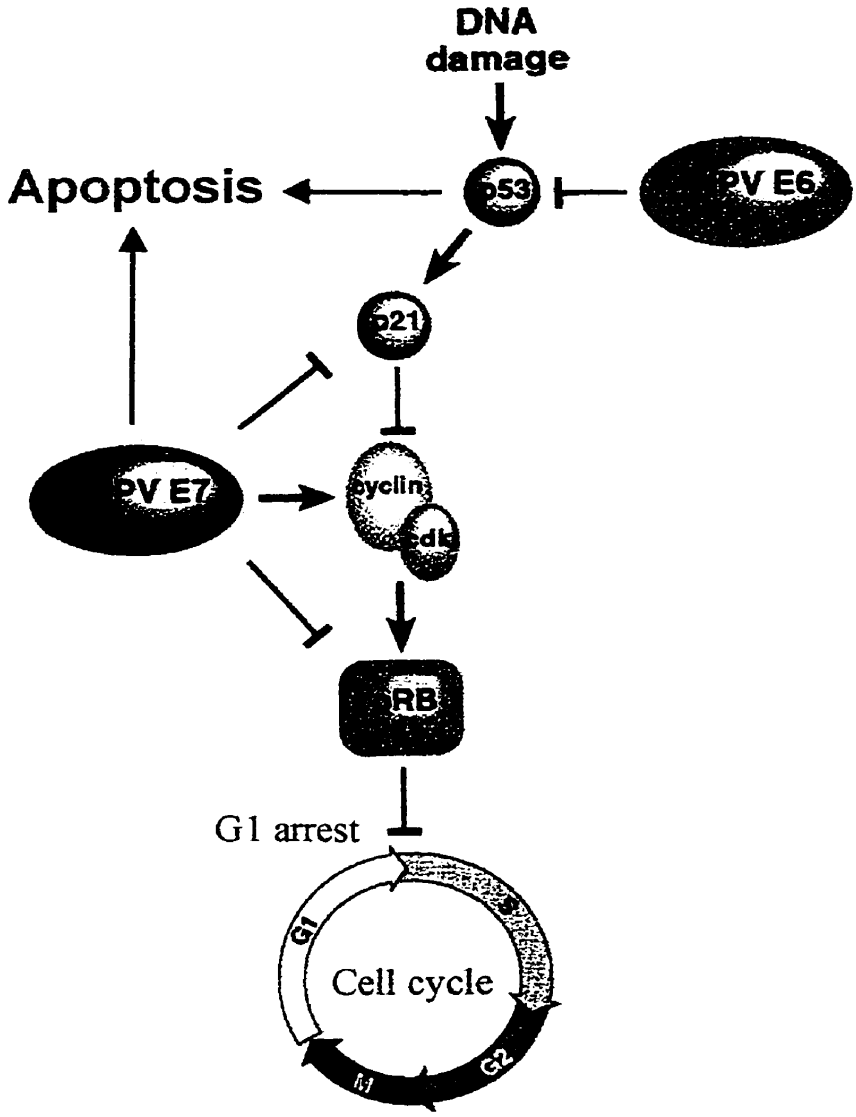


Figure 1.5. Effects of HPV oncoproteins on the cell cycle and apoptosis pathway (adapted from Alani and Münger, 1998).

inactivated by mutations, such as deletions, splice site changes and codon substitutions, rather than by binding to a viral protein (Crook *et al.*, 1992).

1.3.3. *In vitro* cervical cell model of *in vivo* multistage carcinogenesis

The initiation of cervical cancers is mostly caused by HPV infection (section 1.3.1). After initiation, the cells may be exposed to some tumor promoting reagents and experience further genetic alterations, such as mutation and activation or inactivation of oncogenes and tumor suppressor genes (section 1.3.2). A single cell acquires a growth advantage and/or inhibition of apoptosis to form a clonal malignant tumor (section 1.1.5.1). Both physical and chemical factors could contribute to the further progression of cervical cancers after initiation.

Carcinogenesis, or the process of cancer development, in most instances involves a long period of latency. During this time, endogenous and/or exogenous carcinogenic agents act on individual cells and cause genetic alterations, mostly involving oncogenes and tumor suppressor genes. Most cancers are clonal in origin. Cancer development involves several successive rounds of gene mutations, and tumor progression is usually a multistage progression.

Similar to the preceeding description of cervical carcinogenesis *in vivo*, the multistage nature of cancer also can be observed in the carcinogenesis of human cervical cells *in vitro*. Figure 1.6 is a schematic representation of *in vivo* and *in vitro* cervical carcinogenesis. In this scheme, HPVs initiate carcinogenesis *in vivo* after infecting endocervix-derived metaplastic cells at the transformation zone, where almost all cervical

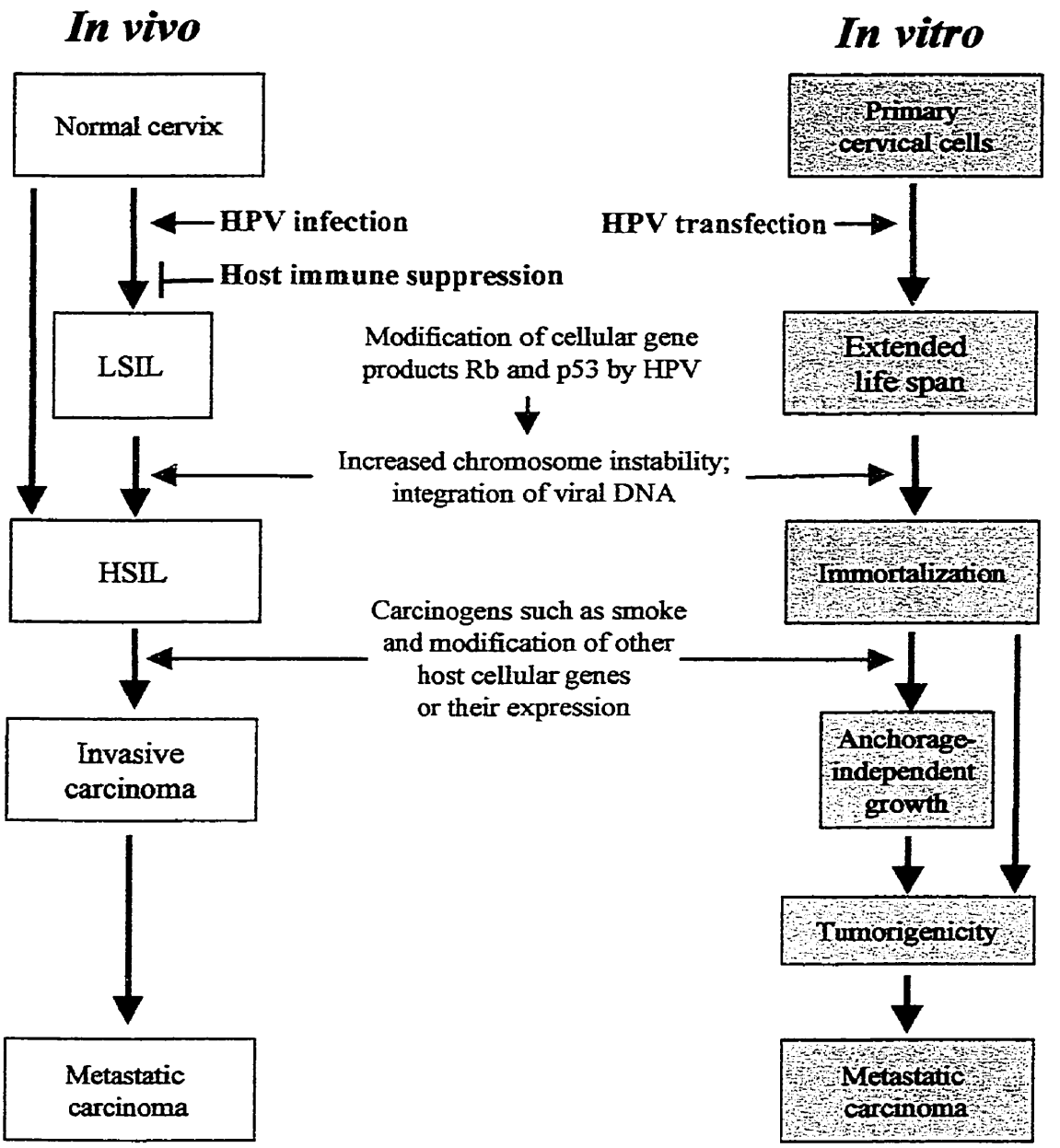


Figure 1.6. *In vivo* and *in vitro* cervical multstep carcinogenesis.

neoplasia is formed (Vousden, 1989; Sun *et al.*, 1997). Alternatively, normal metaplastic endocervical cells *in vitro* are HPV-transfected, immortalized, and become transformed (Tsutsumi *et al.*, 1992; Sun *et al.*, 1992; 1997; Yang *et al.*, 1996a).

High-risk and low-risk HPVs are found in low-grade squamous intraepithelial lesions (LSILs) *in vivo* and both types also extended the cell life span in *in vitro* cell culture. Further modifications of cellular genes resulted in mainly high-risk HPV-containing high-grade squamous intraepithelial lesions (HSILs) *in vivo* or isolated clones of immortalized cells *in vitro*. However, these events are insufficient to transform cervical cells *in vivo* or *in vitro* and produce invasive cervical carcinoma. Other factors, such as smoking, or cigarette smoke condensate (CSC) are required to efficiently induce further changes in other cellular genes and transform some of the high-risk HPV-containing HSILs, or -immortalized cells to form invasive tumors (Yang *et al.*, 1996a; Nakao *et al.*, 1996).

1.4. BAG-1 and its associated proteins

BAG-1 is a protein with multiple isoforms: p50, p46, p33 and p29. Each isoform is initiated from an alternate translational start site (Yang *et al.*, 1998a; Zapata *et al.*, 1998). The BAG-1 p50 isoform is distributed predominantly in the nucleus, while other isoforms are located mainly in the cytoplasm or membranes (Yang *et al.*, 1998a), suggesting that BAG-1 is a multifunctional protein.

BAG-1 was initially identified as Bcl-2 binding proteins (Takayama *et al.*, 1995). Bcl-2 is a key inhibitor of apoptosis (see section 1.1.4), and BAG-1 can enhance the

ability of Bcl-2 to inhibit apoptosis induced by staurosporine and anti-Fas antibody in Jurkat T cells (Takayama *et al.,* 1995), or by NGF withdrawal in neuronal PC12 cells (Schulz *et al.,* 1997). BAG-1 can interact with Raf-1, which can bind to Bcl-2 and cooperate in the suppression of apoptosis, and activates its protein kinase *in vitro* and *in vivo* (Wang *et al.,* 1996). In addition, BAG-1 was found to bind hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF) receptors, and enhance their ability to inhibit apoptosis (Bardelli *et al.,* 1996). Further, overexpression of BAG-1 inhibits the apoptosis induced by staurosporine in 3T3 fibroblasts and C33A cervical carcinoma cells (Takayama *et al.,* 1995; Yang *et al.,* 1999a), heat shock in GM701 immortalized fibroblasts (Takayama *et al.,* 1997), and IL-3 withdrawal in IL-3-dependent B cell line Ba/F3 (Clevenger *et al.,* 1997). Thus, BAG-1 was identified as a Bcl-2-dependent and -independent anti-apoptotic molecule.

BAG-1 is capable of interacting with various other cellular proteins (Figure 1.7). BAG-1 can to form complexes with a number of steroid hormone receptors, such as estrogen receptor (ER), androgen receptor (AR), and glucocorticoid receptor (GR) and modulates their function (Zeiner and Gehring, 1995; Froesch *et al.,* 1998; Kullmann *et al.,* 1998). Recently, BAG-1 was demonstrated to interact with Siah-1A, which can inhibit p53-dependent cell-cycle arrest, and to inhibit Siah-1A function (Matsuzawa *et al.,* 1998). In late 1997, BAG-1 was demonstrated to interact with heat shock protein 70 chaperones (Hsp70s) and modulate their chaperone activity (Takayama *et al.,* 1997, Zeiner *et al.,* 1997, Höhfeld and Jentsch, 1997). I also independently identified Hsp70 and Hsp70-2 as BAG-1-binding proteins using the yeast two-hybrid system during that

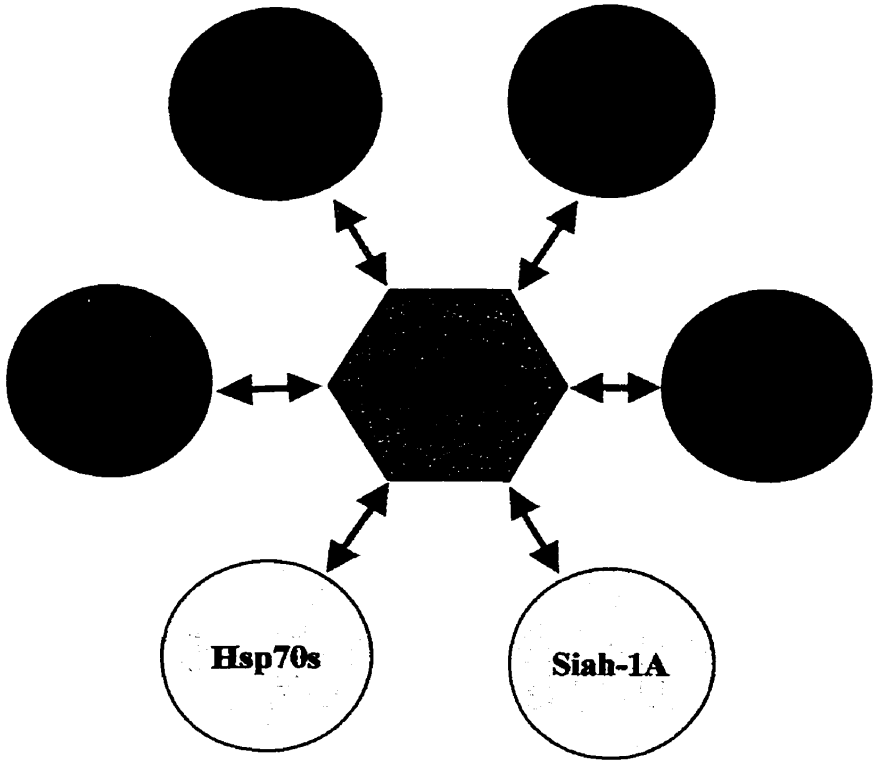


Figure 1.7. BAG-1-interacting proteins. Proteins in blue color were demonstrated to interact with BAG-1 before 1997. Proteins in yellow were demonstrated to interact with BAG-1 since 1997, when screening for BAG-1-interacting proteins using the yeast two-hybrid system began.

time. The Hsp70 family comprises molecular chaperones that play a key role in the folding, translocation and degradation of proteins in eukaryotic cells through their capacity to bind and stabilize non-native protein conformations (Hartl, 1996; Rüdiger *et al.*, 1997; Bakau and Horwich, 1998). Hsp70 is also known to regulate apoptosis (Gabai *et al.*, 1995; Mosser *et al.*, 1997; Jäättelä *et al.*, 1998) and may thus play a role in tumorigenicity and drug resistance (Kaur and Ralhan 1995, Ralhan *et al.*, 1995; Jäättelä 1995; Vargas-Roig *et al.*, 1998).

BAG-1 was demonstrated to increase pulmonary metastases in mice (Takaoka *et al.*, 1997). Furthermore, BAG-1 is present at much higher levels in cervical tumors and breast tumors than in surrounding normal tissue (Yang *et al.*, 1999a, 1999b). BAG-1 has also been shown to be expressed at higher levels in lung, breast and cervical tumor cell lines than their non-tumor counterparts (Takayama *et al.*, 1998; Yang *et al.*, 1998b; 1999a; 1999b; Zapata *et al.*, 1998). Moreover, the increased expression of BAG-1 correlates with enhanced resistance of cervical carcinoma cells to apoptosis induced by cisplatin (Yang *et al.*, 1998b). All these observations suggest that BAG-1 may have an important role in carcinogenesis and drug resistance through inhibition of apoptosis.

1.5. Objective of this study

From the foregoing review of the literature, it can be concluded that apoptosis plays an important role both in multistep carcinogenesis and in cancer chemotherapy resistance. Apoptosis is controlled through cellular genes including pro-apoptotic genes (e.g., *p53, Bax, Bak*) and anti-apoptotic genes (e.g., *Bcl-2, Bcl-*X_L, and *BAG-1*). Also, alterations in

these apoptosis-regulating genes have been implicated to have an important role in carcinogenesis and cancer drug resistance. The expression patterns of these apoptosis-regulating proteins vary in different cell types. Therefore, my hypothesis is that deregulation of apoptosis plays a role in the process of multistep carcinogenesis and MDR of human endocervical cancer.

The molecular mechanism of apoptosis in the multistep carcinogenesis process and MDR is still poorly understood. An understanding of the role of apoptosis in multistep carcinogenesis and MDR of endocervical cancer is important in cancer research. The objective of this study is to further characterize the role of apoptosis in carcinogenesis and MDR of human cervical cells.

For these purposes, firstly, HPV 16-immortalized endocervical cells were treated with cisplatin and a multidrug-resistant endocervical cell line was established. Then, the response to various apoptotic stimuli, cellular morphology, growth characteristics, tumorigenicity, and cellular apoptosis-regulating gene expression of the immortalized and multidrug-resistant cells were analyzed.

Secondly, since BAG-1 was found to be overexpressed in our multidrug resistant cells, BAG-1 was then stably transfected into the low BAG-1-expressing cervical carcinoma cell line, C33A, to determine whether overexpression of BAG-1 can recapitulate the drug-resistance.

Thirdly, the yeast two-hybrid system was established and employed to screen a complementary DNA (cDNA) library. Hsp70 and Hsp70-2 were isolated as BAG-1–binding proteins. Furthermore, mutation analyses of the functional domain of BAG-1

which is required for interaction with Hsp70 and Hsp70-2 were conducted *in vitro* and *in vivo*. The effect of BAG-1 on Hsp70 chaperones-mediated protein refolding activity *in vivo* was also assayed. Additionally, Hsp70 and Hsp70-2 were stably transfected into C33A cells and the role of their interaction with BAG-1 in drug resistance was further characterized.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Keratinocyte growth medium (KGM) and Dulbecco's modified Eagle medium (DMEM) were purchased from GIBCO-BRL and ICN, respectively. GIBCO-BRL was the supplier for the fetal calf serum (FCS) and trypsin-ethylenediamine tetraacetic acid (EDTA). Penicillin-streptomycin was obtained from ICN. HEN-16-2 and HEN-16-2T cells were established by Dr. K. Tsutsumi and Dr. X. Yang in the laboratory, respectively (Tsutsumi *et al.*, 1992; Yang *et al.*, 1996a). HeLa and C33A cell lines in the laboratory stock were previously purchased from ATCC.

Taq DNA polymerase, restriction endonucleases and their respective 10× reaction buffers were obtained from GIBCO-BRL. The coupled transcription/translation TNT system was supplied by Promega.

GIBCO-BRL supplied the 1 kb and 100 bp DNA ladder markers. Low melting point agarose, agarose, acrylamide, N,N'-methylenebisacrylamide, urea and 10 mM dNTPs (dGTP, dATP, dTTP and dCTP) were all purchased from GIBCO-BRL. Baker Inc., Bio-Rad, and Carnation were the suppliers of 2-mercaptoethanol, N,N,N',N'-tetra-methylethylenediamine (TEMED) and skim milk powder respectively.

Cisplatin, actinomycin D, doxorubicin, etoposide, 5-FU, staurosporine, sanguinarine, paclitaxel (taxol), N-(4-hydroxyphenyl)retinamide (4-HPR or fenretinide) and all-trans retinoic acid (ATRA) were all purchased from Sigma Chemical Co..

Mouse anti-BAG-1 monoclonal antibody (mAb) was generated and prepared in our lab (Yang *et al.*, 1998a). Rabbit polyclonal antibodies for Bcl-X_L, Bax and mouse mAbs for Bak, Bcl-2, Hsp70 and caspase-3 were obtained from Santa Cruz Biotechnology. Other mouse mAbs used were: anti-p53 (DAKO), anti-actin (Sigma Chemical Co.) and anti-PARP (PharMingen International).

The yeast two-hybrid system kit was purchased from Clontech. Invitrogen and United States Biochemical Co. supplied the TA Cloning Kit version 3.0 and Sequence Version 2.0 Sequencing Kit, respectively. Kodak was the supplier of X-ray film.

Eight-well tissue chamber slides; 35 mm, 60-mm and 100-mm tissue culture plates; and culture tubes were obtained from NUNC. Eppendorf micro test tubes for PCR were obtained from Fisher.

2.2. Cell culture

HEN-16-2 and HEN-16-2/CDDP were cultured in serum-free KGM containing 1% penicillin/streptomycin. HeLa, HEN-16-2T, C33A, C33A-BAG-1, C33A-Hsp70 and C33A-Hsp70-2 were cultured in DMEM containing 10% FCS and 1% penicillin/streptomycin.

All cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. After three days or when the cells had reached approximately 80% confluence, the medium was aspirated from the plates and the cells were washed with phosphate-buffered saline (PBS). Then, 2 ml of trypsin-EDTA was added into each plate, which was placed in the incubator for 10 minutes. For cells cultured in KGM, 8 ml of PBS containing 10%

FCS was added to the trypsinized cells to quench the activity of trypsin. The cells were then suspended and centrifuged at 1,000 rpm for 10 minutes. The cell pellets were resuspended with KGM and passaged at a dilution of 1:10, 1:4, or 1:2 into plates. For cells cultured in DMEM containing 10% FCS, 8 ml of this medium was added directly to the trypsinized cells, which were further passaged at 1:10, 1:4, or 1:3 into fresh plates.

2.3. Establishment of endocervical MDR HEN-16-2/CDDP cell line

As shown in Figure 2.1, the multidrug-resistant human endocervical cell line HEN-16-2/CDDP was established from the HPV-immortalized human endocervical cell line HEN-16-2. HEN-16-2 cells which were normally maintained in KGM were first adapted to grow in DMEM. At each passage, they were incubated for 24 hours in DMEM containing 5 μM CDDP. The CDDP-containing medium was then replaced with fresh DMEM for additional incubation for 3 days. This treatment was repeated for approximately 50 passages, and then the cells were cultured in KGM (Figure 2.1).

2.4. Clonogenic survival assays

Clonogenic assays were performed, as previously described (Vasey *et al.*, 1996). Briefly, 10^3 cells were seeded into 60-mm diameter tissue culture plates and allowed to attach overnight. Cells were then incubated with each drug for 24 h, washed twice with sterile PBS at 37°C, and incubated in drug-free KGM for 10-14 days. The cells were were stained with 2% crystal violet in methanol and colonies of 50 or more cells were scored. The surviving fraction was calculated as the ratio of the colony-forming

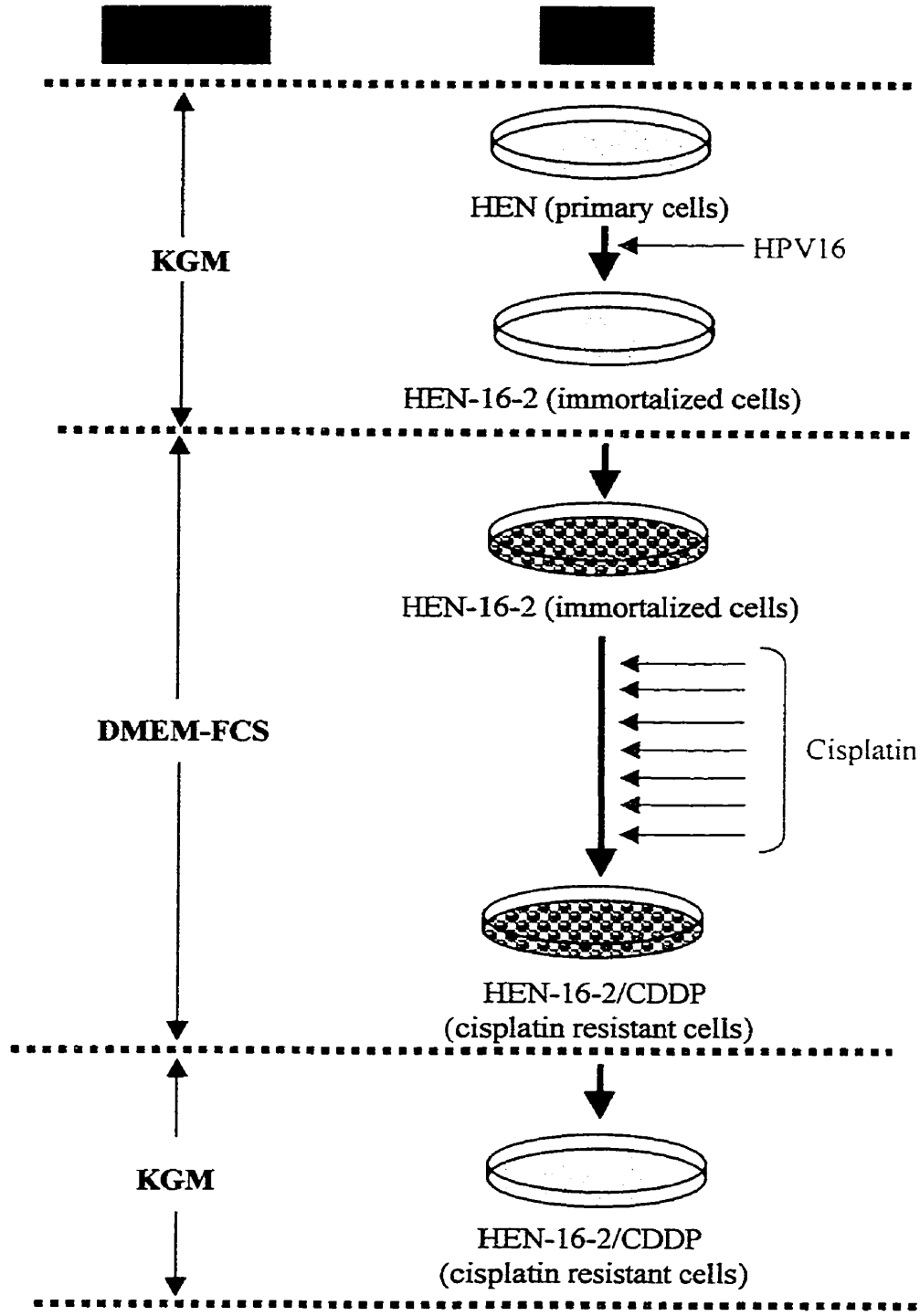


Figure 2.1. Schematic representation of method used to establish HEN-16-2/CDDP cells. The dotted lines indicate the initiation and termination of serum adaption.

efficiency of drug-treated and untreated cells. The drug concentration resulting in 50% cell viablility inhibition (IC_{50}) was determined from each clonogenic survival curve (Nagane *et al.*, 1997).

2.5. Measurement of growth rate and saturation density

Exponentially growing cells were trypsinized, centrifuged, and resuspended in 2 ml medium. Cell numbers were counted with a hemocytometer, and approximately 2×10^4 cells were dispensed into 60-mm plates for each cell line. Cell numbers were counted from each of three plates every two days for 8 days.

The saturation density of each cell line was measured by the same procedure 5 days after the cells reached confluence.

2.6. Light microscopy and scanning electron microscopy

For light microscopic analysis of morphology, cells were cultured in 8-well tissue culture chamber slides. The chamber frame was released from the slides, before the cells were studied under light microscopy. Cell morphology was documented with Kodak Tmax 400 film.

For SEM analysis, approximately 50,000 cells were seeded on 24-well tissue-culture plates containing acid-cleaned coverslips (Thermanox, no. 5414; Lux Scientific Corp.). Cells were allowed to attach to the coverslips at 37 °C in a humidified incubator containing 5% CO_2 overnight. Cells were fixed with Karnovsky fixative 2.5% glutaraldehyde (J. B. EM Services) in 0.1 M sodium cacodylate buffer. Cells were

dehydrated in a 25%, 50%, 75% and 100% graded ethanol series followed by Freon-113 substitution. All samples were dried simultaneously, sputter-coated with gold and examined in a Hitachi S-570 SEM, as previously described (Liepins and Younghusband, 1985).

2.7. Soft agar or anchorage-independent growth assays

The 0.7% agarose underlying gel was prepared by mixing equal volumes of 2× DMEM containing 20% FCS and low melting point agarose melted in sterilized water, dispensing 2.5 ml into 60-mm plates, and leaving the plates at room temperature until the gel solidified. Subseqently, HEN-16-2 and HEN-16-2/CDDP and positive control HEN-16-2T and HeLa cells were trypsinized, resuspended in 2× DMEM, and counted using a hemocytometer. The 0.35% agarose overlaying gel was prepared by mixing approximately 10^5 cervical cells in 2.5 ml of 2× DMEM with 2.5 ml of 0.7% low melting point agarose in sterilized water, pouring the mixture into the 60-mm plates containing the 0.7% underlying gel. The plates were placed at 4 °C for 5 minutes, and then incubated at 37 °C. One week later, 1 ml DMEM was carefully added onto the surface of the soft agar to replenish nutrients. Colony formation was monitored every two days for 2-4 weeks. Triplicate assays were performed for each cell line. Representative photographs were taken for documentation.

2.8. *In vivo* tumorigenicity assays

HEN-16-2 and HEN-16-2/CDDP cells were trypsinized and resuspended in PBS.

PBS (0.1 ml) containing 10^7 cells was injected into each of six 2-3 month old female immunocompremised nude mice, and tumor incidence was monitored weekly for 4-8 weeks. HeLa cells were similarly treated as a positive control. The average diameter of each tumor was measured when the mouse was sacrificed after 8 weeks.

The tumors were excised and fixed in 10% paraformaldehyde. The fixed tumors were embedded in paraffin, sectioned with a microtome, and then stained with hematoxylin and eosin (Sun *et al.*, 1992). Stained sections were examined and photographed with Kodak Tmax 400 film for documentation by light microscopy.

2.9. Western blotting

For protein extraction, cells were washed twice with ice-cold PBS, and 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% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 200 μg/ml phenylmethylsulfonyl fluoride (PMSF) and 20 μg/ml aprotinin] for 30 minutes and centrifuged at 4°C for 10 minutes, after which the supernatants were stored at -70°C. Protein concentration was determined using the DC Lowry Protein Assay Kit (Bio-Rad) as instructed by the manufacturer.

Protein extracts were boiled in 2× SDS-polyacrylamide gel electrophoresis (PAGE) gel loading buffer [200 mM Tris-HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol] for 3 minutes and resolved in an SDS-PAGE gel. The running gel was prepared with 8-12% acrylamide, 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulphate, and 6 μl TEMED. The stacking gel was composed of 5% acrylamide, 125

mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulphate and 5 µl TEMED. Electrophoresis was at 20V in SDS-PAGE running buffer (25 mM Tris, 250 mM glycine) using a Protean II minigel apparatus (Bio-Rad).

The proteins were subsequently transferred to Hybond enhanced-chemiluminescence nitrocellulose membranes (Amersham Corp) under semidry conditions in Towbin transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) using a Trans-Blot SD transfer apparatus (Bio-Rad). Membranes were blocked by gentle shaking for 1 hour in TBST [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20] and 5% skim milk powder. Membranes were incubated with the primary antibody diluted in TBST containing 5% skim milk powder overnight at 4 °C, and then washed in TBST. Membranes were incubated with horseradish peroxidase-conjugated secondary antibody diluted in TBST with 5% skim milk powder for 1 hour at room temperature, and then washed in TBST. Signals were detected using the enhanced chemilumiscence (ECL) system (Amersham) and subsequently exposing the membranes to ECL film, as instructed by the manufacturer.

After primary signals were detected with the ECL system, they were removed from the membranes by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50 °C for 30 minutes with occasional agitation. Then, membranes were rinsed with TBST and reprobed with anti-actin mAb and the signals were detected as for the primary signal.

Signal intensities were quantified by densitometry of bands with Eagle Eye II Still Video system (Stratagene).

2.10. Apoptosis assays

Pilot experiments were conducted to determine the optimum dose of anticancer drugs, anti-Fas antibody and UV for apoptosis by morphological criteria, such as blebbing, using light microscopy and SEM, as described in section 2.6; and two biochemical characteristics, activation of caspase-3 and cleavage of PARP, using Western blotting, as described in section 2.9. Unless otherwise stated, apoptosis was evaluated using the trypan blue exclusion assay to assess the percentage cell survival/cell death. This was done by trypsinizing cells, incubating them with 0.4% trypan blue solution (Sigma), and scoring more than 200 cells using a hemocytometer.

Stock solutions were stored in aliquots at -20°C after preparation as follows: 1mM actinomycin D, 5 mM cisplatin, 10 mM doxorubicin, 10 mM 5-FU, 1 μg/ml sanguinarine were prepared in sterile distilled water; 100 mM etoposide and 1 mM 4-HPR in DMSO; 30 mM ATRA acid, 4 mM paclitaxel (taxol), 2 mM staurosporine in ethanol; and 500 μg/ml anti-human Fas antibody in PBS containing 50% glycerol.

Approximately 24 hours prior to all treatments, about 5×10^4 cells were seeded per well in 12-well plates. Immediately prior to treating cells, all stock solutions were thawed and diluted in medium. UV treatment was as described (Aragane *et al.*, 1998). Briefly, immediately before UV treatment, cells were washed twice with prewarmed PBS and exposed to UV-B (290-320 nm) with an emission peak of 312 nm through PBS. A UV dose of 50 mJ/cm^2 was used. For heat shock, cells were incubated at 45°C for 45 minutes, then returned to culture at 37°C for 2 days.

2.11. Stable transfection of C33A cells

The coding regions of Hsp70 and Hsp70-2 cDNA were subcloned into the expression plasmid pCR3.1 (Figure 2.2A).

C33A cells were then transfected using LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) with 5 μg cDNA-containing, or empty vector PCR3.1, according to the protocol recommended by the manufacturer. Briefly, approximately 5×10^5 cells were seeded per 10-mm culture dish. Cells were then exposed to transfection mixtures for 5 hours at 37 °C. The transfection mixtures were then replaced with fresh DMEM. Cells were passaged 48 hours after transfection into three 100-mm plates and selected in the presence of 800 μg/ml G418 for 5-7 days. Well separated colonies were trypsinized, transferred to 96-well plates, then subcultured into 24-well and finally into 6-well plates in the presence of G418 until enough cells were present for protein extraction.

2.12. The yeast two-hybrid system cDNA library screening for identification of BAG-1 interacting proteins

2.12.1. Strategy of cDNA library screening

The yeast two-hybrid system (Fields and Song, 1989; Chien *et al.*, 1991; Fields and Sternglanz, 1994; Figure 2.3) was used as a genetic system to isolate BAG-1-interacting proteins *in vivo*. It uses the restoration of transcriptional activation to assay the interaction between BAG-1 and novel proteins. It relies on the modular nature of many site-specific transcriptional activators, such as yeast GAL4, consisting of a DNA-binding domain (BD) and a transcriptional activation domain (AD) (Figure 2.3A) (Keegan *et al.*, 1986).

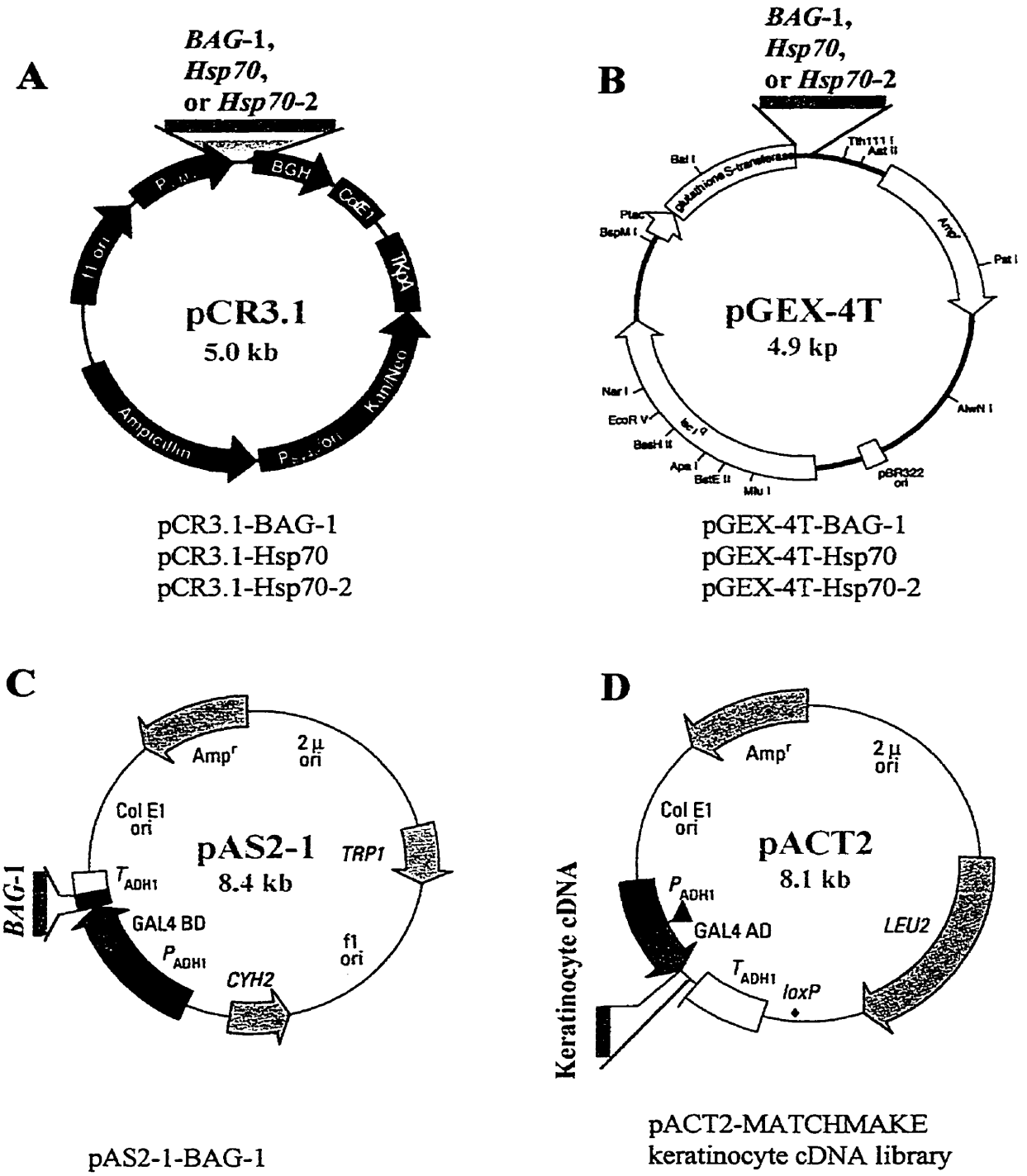


Figure 2.2. Construction of plasmids. A. pCR3.1. B. pGEX-4T. C. pAS2-1. D. pACT2. Parental vectors and their sizes are indicated in the circles.

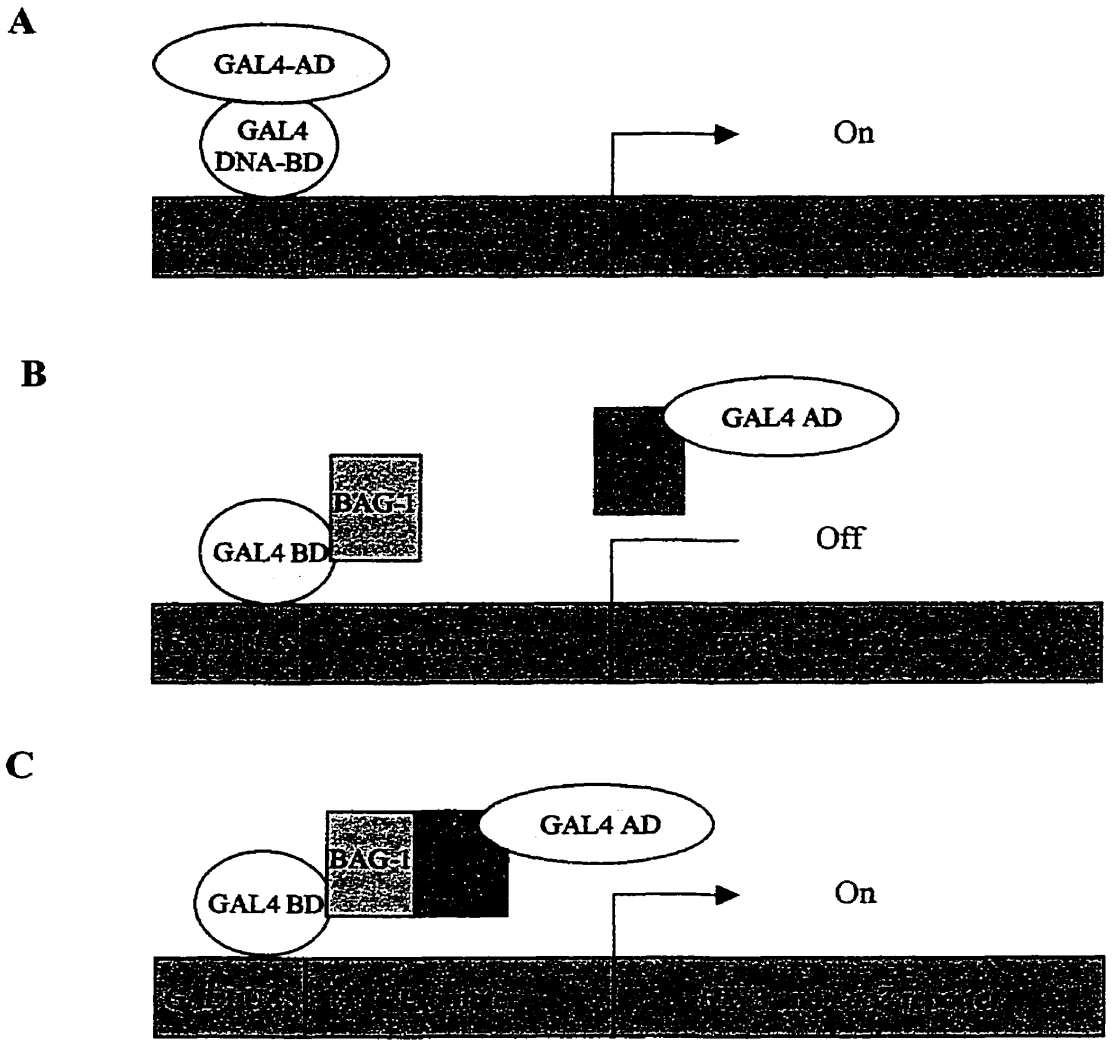


Figure 2.3. Mechanism of the method of the yeast two-hybrid system. A. Yeast GAL4 protein, the transcriptional activator required for the expression of genes encoding enzymes of galactose utilization, consists of two physically discrete modular domains. B. If a protein X from the library cannot interact with human BAG-1, the AD will not be brought to the DNA binding site to activate the reporter gene. C. If the protein Y, another protein from the library, can bind to human BAG-1, protein Y will bring the AD to the DNA binding site and thus activate reporter gene expression.

The BD serves to target the activator to the specific genes that will be expressed, and AD contacts other proteins of the transcriptional machinery to enable transcription to occur. The yeast two-hybrid system is based on the observation that the two domains of the activator need not be a single polypeptide and can be brought together by any two interacting fusion proteins, one of which contains the BD while the other has the AD.

The application of this system requires that two hybrid fusion plasmids be constructed for expressing BD and AD fusion proteins: a BD fused to the bait protein (in this case GAL4 BD-BAG-1, Figure 2.3B and C), and an AD fused to some proteins which may interact with the bait protein [in this case the human Keratinocyte MATCHMAKE cDNA Library (Clontech) proteins, such as AD-X and AD-Y (Figure 2.3B and C)]. The two hybrid plasmids are cotransformed into a yeast *S. cervisiae* host strain Y190 harboring the yeast *HIS*3 and the bacterial *lacZ* reporter genes, which contain an upstream GAL4 binding site. In Fig. 2.3, the interaction of BAG-1 with a novel library protein Y will activate the *HIS*3 and the *lacZ* reporter genes (Figure 2.3C), while library proteins not interacting with BAG-1, such as X, will not activate the reporter genes (Figure 2.3B).

2.12.2. Construct pSA2-1-BAG-1 plasmid

To construct a plasmid expressing GAL4 BD-BAG-1 p46 fusion protein, the BAG-1 isoform p46 cDNA fragment was amplified by PCR in a Hybraid Thermal Reactor (Bio/CAN). The forward primer was Ding1, and the reverse primer was B3-1 (Table 2.1). PCR of the BAG-1 p46 isoform was performed, as recommended by the

manufacturer (Stratagene), with minor modifications. 1 µl 50 ng/µl DNA and 1µl 10 pmol/µl of both primers, were mixed with 2 µl of 10× PCR buffer (Stratagene), 0.4 of µl 10 mM dNTP (GIBCO-BRL), 0.2 µl of 5 U/µl polymerase (Stratagene) and 14.4 µl water. PCR was performed as follows: 94°C for 3 minutes; 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; 94°C for 1 minute, 55°C for 1 minute, and 72°C for 8 minutes.

The GAL4 BD plasmid (pAS2-1-BAG-1) was then constructed by subcloning the PCR product into the vector pAS2-1 in-frame with GAL4 BD (Figure 2.2C).

2.12.3. Screening the human keratinocyte MATCHMAKE cDNA Library

The human keratinocyte MATCHMAKE cDNA Library of plasmids inserted into the yeast two-hybrid system AD vector pACT2 (Figure 2.2D) was purchased from Clontech. *S. cerivisiae* strain Y190 (MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, cyh^{r}2, LYS2::GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ), which was Trp$^-$, Leu$^-$ and His$^-$, was cotransformed with pAS2-1-BAG-1 and the MATCHMAKE cDNA Library, using the lithium acetate procedure as described by the manufacturer (Figure 2.4). The transformation mixture was then plated on 150-mm petri dishes containing synthetic dropout (SD) media lacking tryptophan, leucine, and histidine but including 25 mM 3-amino-1,2,4-triazole (3-AT), and incubated at 30 °C for 3-5 days. The transformants were screened for β-galactosidase activity using a filter lift assay, according to the protocol recommended by the manufacturer (Clontech). Briefly, colonies were transferred to Whatman #5 filters and

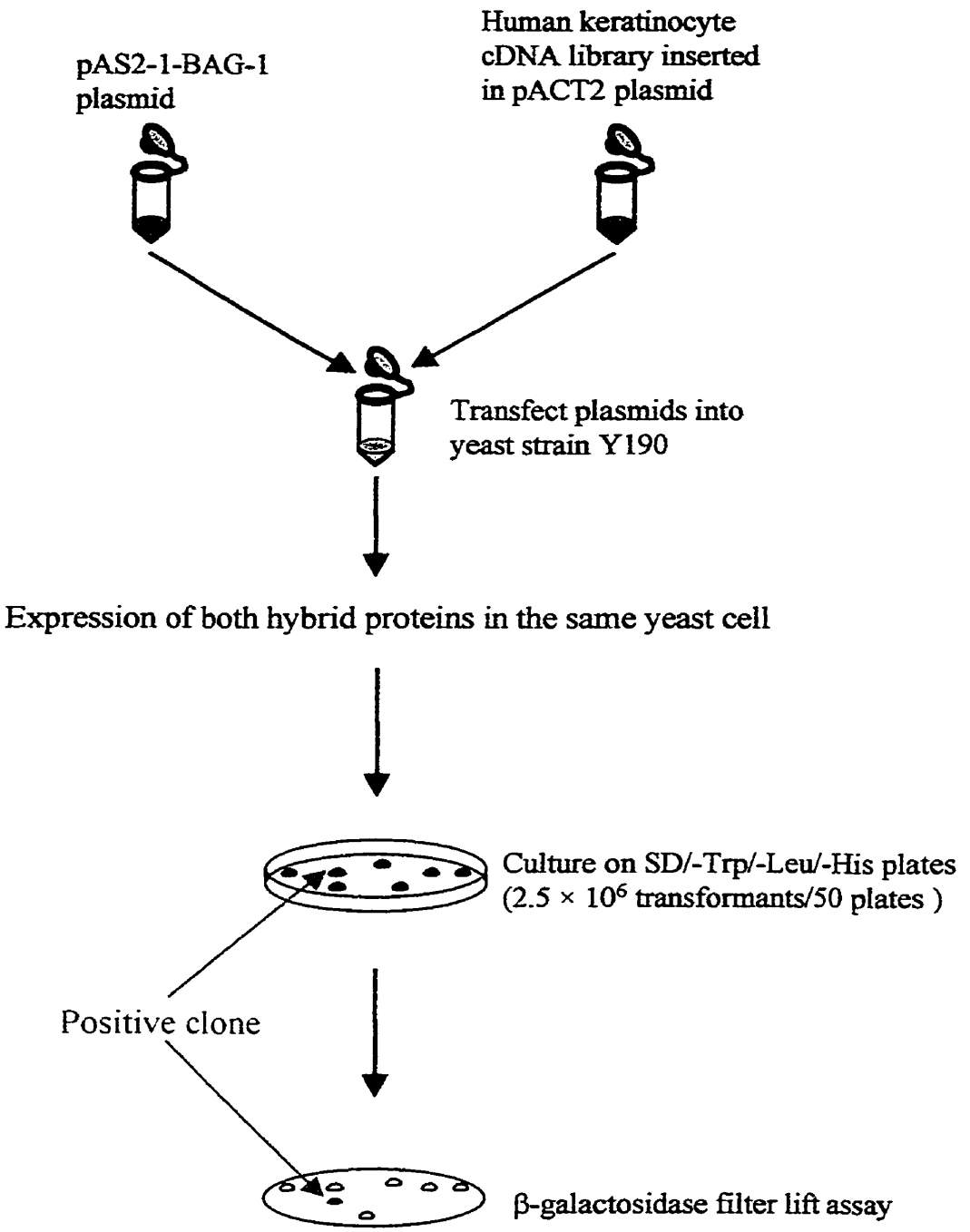


Figure 2.4. cDNA library screening for BAG-1-interacting protein positive clones.

cells were permeabilized by freezing for 10 seconds in liquid nitrogen, and thawing at room temperature. Filters were then overlaid onto another Whatman #5 filter saturated with Z buffer/X-gal solution [16.1 mg/ml $Na_2HPO_4.7\ H_2O$, 5.5 mg/ml $NaH_2PO_4.H_2O$, 0.75 mg/ml KCl, 0.246 mg/ml $MgCl_2.7\ H_2O$, 0.327 mg/ml X-gal, 0.3% (v/v) 2-mercaptoethanol], and incubated at room temperature for 30 minutes to overnight for color development. Approximately 2.5×10^6 yeast transformants were screened for BAG-1-interacting proteins.

2.12.4. Verifying postitive clones in yeast

As diagrammed in Figure 2.5, each of the initial His^+ and $LacZ^+$ yeast colonies was streaked out one to five times to segregate multiple pACT2-library plasmids within each single colony and β-gal filter lift assays were repeated on well-isolated colonies. The plasmids were then isolated from yeast, transfected into *E. coli*, and further amplified. These pACT2-library plasmids isolated from *E. coli* were then individually retransformed into yeast strain Y190 to test the specific interaction of the candidate library clones with pAS2-1-BAG-1, pAS2-1-LAM5, and pAS2-1 vector. Nonspecific interactions (those conferring His^+, $LacZ^+$ when paired with pAS2-1 or pAS2-1-LAM5) were considered false positives and eliminated, while clones specific for pAS2-1-BAG-1 bait fusion were retained.

2.12.5. DNA sequencing and analysis

Clones retained above were analyzed by DNA sequencing of both strands using a

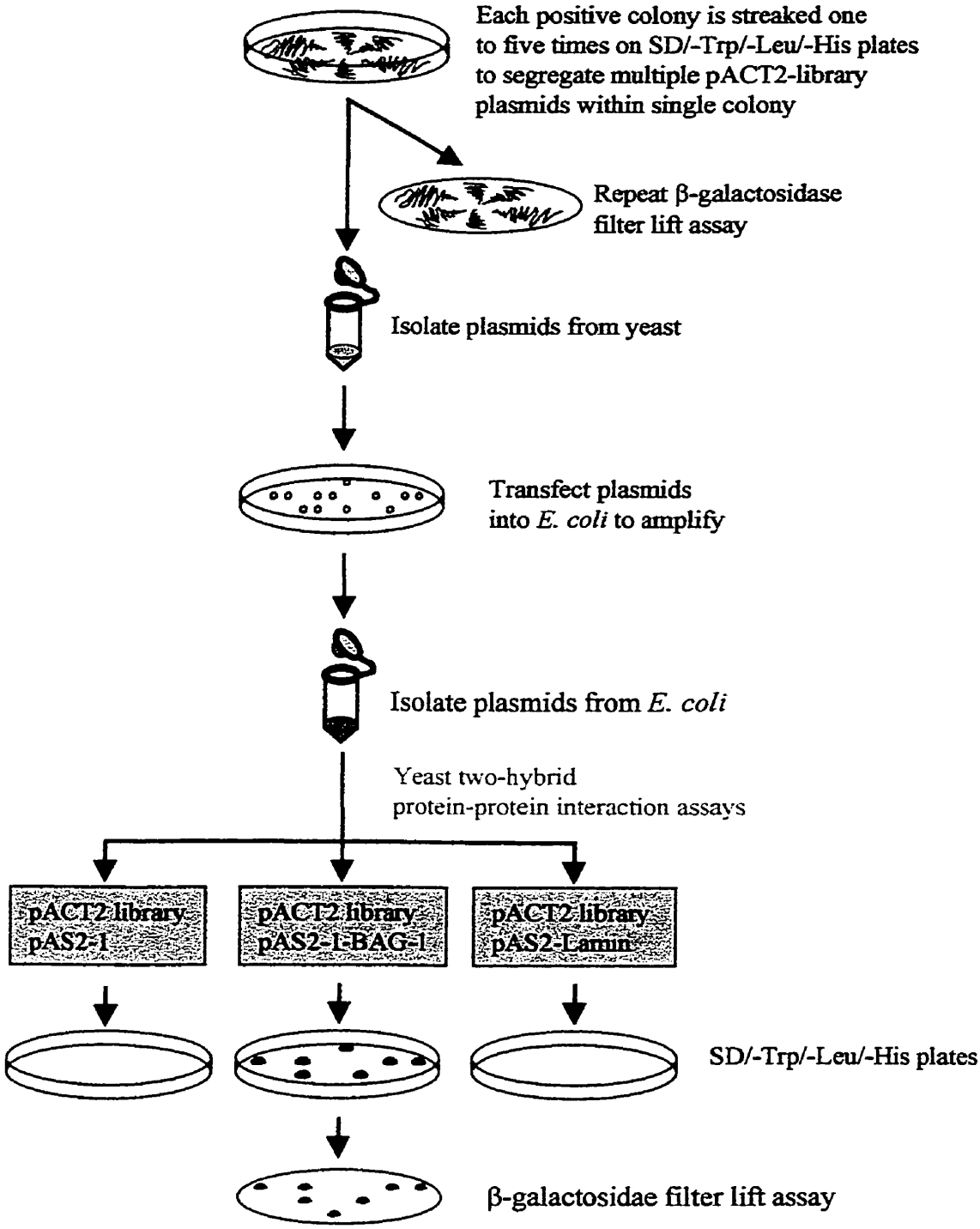


Figure 2.5. Method for verifying BAG-1-interacting protein positive clones.

Sequenase Version 2.0 kit (United States Biochemical Co.), according to the protocol recommended by the manufacturer. The sequences were then analyzed for sequence homology using the National Center for Biotechnology Information sequence databases through the Basic Alignment Search Tool (BLAST) program through the internet http://www.ncbi.nlm.nih.gov/BLAST/.

2.13. *In vitro* binding assay

2.13.1. *In vitro* transcription/translation

Hsp70, *Hsp70* ATPase domain, *Hsp70*-2, and *BAG*-1 cDNA were transcribed/translated *in vitro* in the presence of [^{35}S]-methionine (Amersham) using the TNT system (Promega), as described by the manufacturer.

2.13.2. GST-fusion protein production

To obtain GST-Hsp70 and GST-Hsp70-2, the coding regions of *Hsp70* and *Hsp70*-2 cDNA in pACT2 plasmids were subcloned in-frame into pGEX-4T-2 GST vector (Pharmacia, Biotech) (Figure 2.2B). The resulting plasmids were then transfected into the BL21 strain of *E. coli*. GST fusion proteins were purified according to the instructions of the GST gene fusion system manufacturer. Briefly, *E. coli* BL21 containing either parental pGEX-4T-2 plasmid or the plasmid with the inserted cDNA were grown at 30 °C with shaking until the A_{600} reaches 1.0. At this time, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM and the bacteria were cultured for an additional 5 hours, centrifuged, resuspended in PBS and sonicated in

eight 15 second bursts on ice. Triton X-100 was then added to a final concentration of 1% and the tubes were mixed gently for 30 minutes. Lysates were centrifuged at 12,000 ×g and supernatants were collected, combined with 400 µl of GSH sepharose beads and incubated at room temperature for 30 minutes with gentle agitation. After this, the unbound proteins were removed with three PBS washes. GST fusion proteins were eluted with 10 mM reduced GSH in 50 mM Tris-HCl, pH 8.0. GST fusion protein level and purity were determined by SDS-PAGE of 1 µl of protein samples and bovine serum albumin standards, and staining in Coommassie Brilliant Blue.

2.13.3. *In vitro* binding assays of BAG-1 and Hsp70s

GST protein interaction assays were performed, as previously described (Hanada *et al*, 1995). Briefly, 10 µg of GST fusion protein was incubated with 10 µl of GST-sepharose beads for one hour in 100 µl binding buffer (10 mM HEPES pH 7.2, 140 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA and 0.15% Nonidet P-40) to attach the proteins to the beads. Then, 5 µl of [^{35}S]-methionine *in vitro*-translated products were incubated with the GST fusion proteins for 2 hours at 4°C. The mixture was washed six times with binding buffer. The beads (in 20 µl binding buffer) were boiled in 20 µl 2× SDS gel loading buffer and 20 µl of centrifuge supernatants were subjected to SDS-PAGE. After electrophoresis, the gel was dried and X-ray film was exposed. GST and binding buffer were incubated with *in vitro* transcribed/translated proteins as negative controls.

2.14. Deletion mutation analysis of BAG-1 domains for binding Hsp70s *in vivo*

BAG-1 p50, p46, p33, and p29 isoforms, as well as a series of deletion mutations which were in-frame with the GAL4 BD of pAS2-1 were generated by PCR (Figure 2.6) using the the primers listed in Table 2.1.

The BAG-1 isoforms and mutations were then inserted into the pAS2-1 plasmid. These plasmids were then cotransformed with pACT2-Hsp70 or pACT2-Hsp70-2 into Y190, and the interactions were then determined by filter lift assays (see section 2.12.3).

Table 2.1. Primers used to generate BAG-1 isoforms and deletion mutations

Primer	Strand	Sequence	Amino acids	BD-BAG-1 plasmid
B5-0K	Sense	5'-CACCATGGCTCAGCGCGGG-3'	1-345	p50
B3-1	Antisense	5'-GCTCACTCCACATCGTCTTT-3'		
Ding1	Sense	5'-GATGAAGAAGAAAACCCGG-3'	72-345	p46
B3-1	Antisense	5'-GCTCACTCCACATCGTCTTT-3'		
B5-2K	Sense	5'-CACCATGGATCGGAGCCAGGAGGT-3'	116-345	p33
B3-1	Antisense	5'-GCTCACTCCACATCGTCTTT-3'		
B5-3K	Sense	5'-TCATCTCCTCCAAGATCTTCAT-3'	139-345	p29
B3-1	Antisense	5'-GCTCACTCCACATCGTCTTT-3'		
B5-4K	Sense	5'-CACCATGGAAACACCGTTGTCAG-3'	200-345	Δ 1
B3-1	Antisense	5'-GCTCACTCCACATCGTCTTT-3'		
B5-5K	Sense	5'-CACCATGGAGATCTTGGAGGAGA-3'	286-345	Δ 2
B3-1	Antisense	5'-GCTCACTCCACATCGTCTTT-3'		
B5-6K	Sense	5'-CACCATGGTTCAGGCATTCCTAG-3'	315-345	Δ 3
B3-1	Antisense	5'-GCTCACTCCACATCGTCTTT-3'		
B5-1K	Sense	5'-CACCATGGAGAAGAAAACCCGG-3'	72-320	Δ 4
B3-5	Antisense	5'-TCACTCGGCTAGGAATGCCTGAAC-3'		
B5-1K	Sense	5'-CACCATGGAGAAGAAAACCCGG-3'	72-291	Δ 5
B3-5K	Antisense	5'-TCATCTCCTCCAAGATCTTCAT-3'		
B5-1K	Sense	5'-CACCATGGAGAAGAAAACCCGG-3'	72-215	Δ 6
B3-4	Antisense	5'-TCACCCAATTAACATGACCCG-3'		
B5-1K	Sense	5'-CACCATGGAGAAGAAAACCCGG-3'	72-156	Δ 7
B3-3	Antisense	5'-TCAAAGGTCGTGCTTCTCATTGC-3'		
B5-1K	Sense	5'-CACCATGGAGAAGAAAACCCGG-3'	72-121	Δ 8
B3-2	Antisense	5'-TCATACCTCCTGGCTCCGATTCAT-3'		

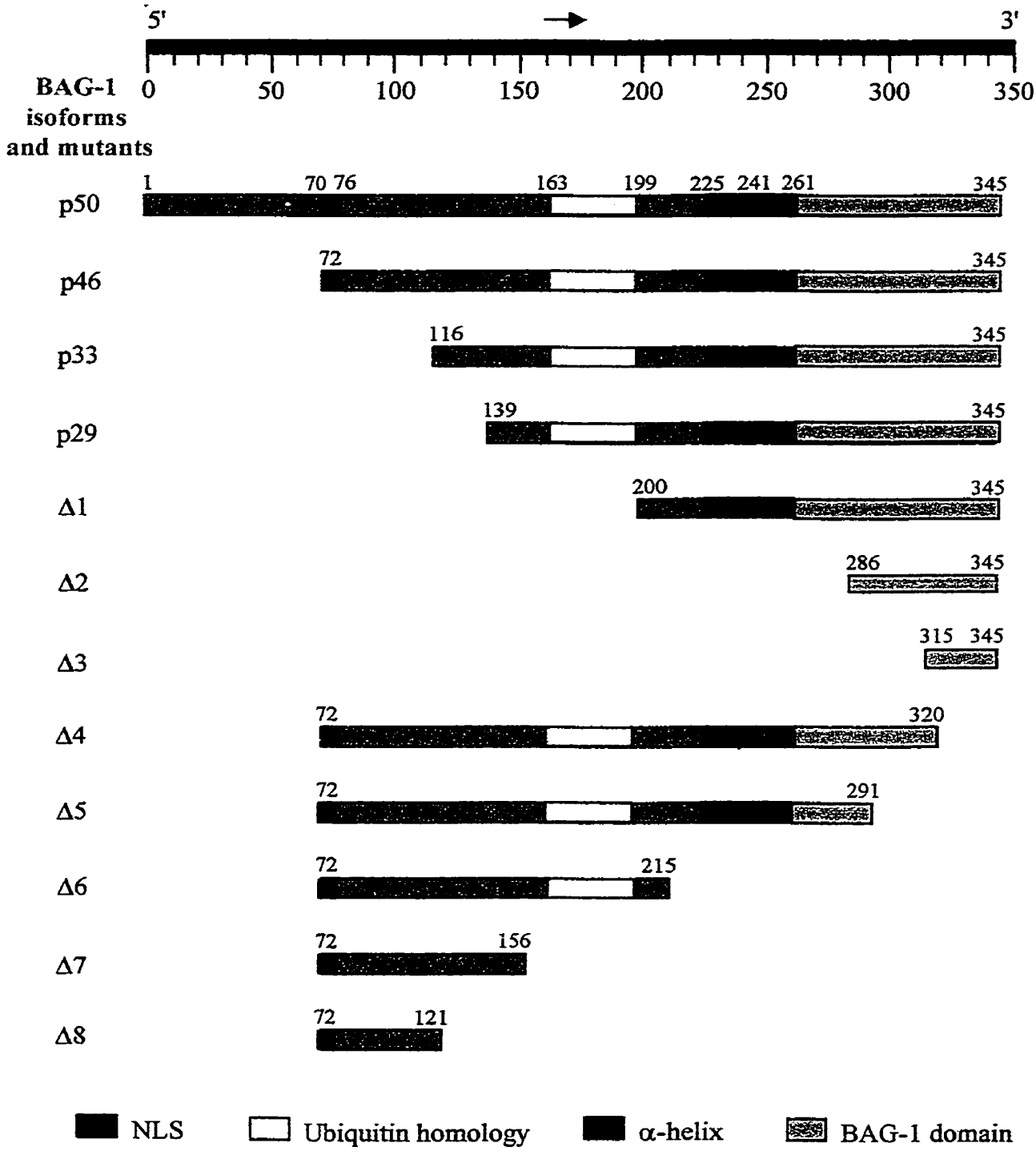


Figure 2.6. BAG-1 isoforms and deletion mutants. Numbers indicate the amino acids.

2.15. *In vivo* protein refolding assays

2.15.1. Transient transfection

Approximately 1×10^6 C33A-BAG-1 or C33A-NEO cells were seeded into 60-mm culture dishes, cultured 24 hours and transiently cotransfected with 1.0 μg of pSV-luciferase vector and 2.0 μg of pSV-β-galactosidase vector (Promega) using LipofectAMINE (Life Technologies, Inc.) for 5 hours at 37 °C. The transfection mixtures were then replaced with DMEM containing 10% FCS and 1% penicillin/streptomycin.

Cotransfected cells were cultured for 24 hours, trypsinized and 1×10^5 cells were aliquoted into each cell culture tube (Nunc) and cultured for a further 24 more hours.

2.15.2. Heat shock inactivation and refolding

For heat shock inactivation, transiently transfected cells were preincubated in a water bath at 37 °C for about 15 minutes and transferred within 3 seconds to 42 °C for 45 minutes.

For refolding experiments, the medium was replaced 30 minutes prior to heat shock with medium containing 20 μg/ml cycloheximide to inhibit new protein synthesis. After heat shock treatment, cells were incubated at 37 °C for 30 or 60 minutes to allow protein to refold. Luciferase or β-galactosidase activities before heat shock were taken as 100%.

2.15.3. Luciferase assays

Cells were chilled to 4 °C, washed with ice-cold PBS and lysed in 500 μl buffer A [25 mM H_3PO_4/Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 1% (v/v) Triton X-100, 15% (v/v)

glycerol, 1 mM EDTA] containing 0.5% (v/v) 2-mercaptoethanol. The lysates were stored at -70 °C.

After mixing 20 µl cell extract and 100 µl luciferase reagent (Biorad), luminescence was measured for 60 seconds in a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

2.15.4. β-galactosidase assays

β-galatosidase expression assays were performed on the same lysates, using 10 µl cell extract and 200 µl buffer Z (60 mM Na_2PO_4, 40 mM NaH_2PO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) and 40 µl 4 mg/ml ortho-nitrophenyl-n-D-galacto-pyranoside (ONPG). The reaction mixture was incubated at 37 °C for 50 minutes. The assays were terminated with 100 µl 0.5 M Na_2CO_3 and the absorbance was measured at 412 nm.

2.16. Statistical analysis

Statistical analysis was conducted using the Student's t-test. Differences with a value of $p < 0.05$ were considered to be significant.

CHAPTER 3

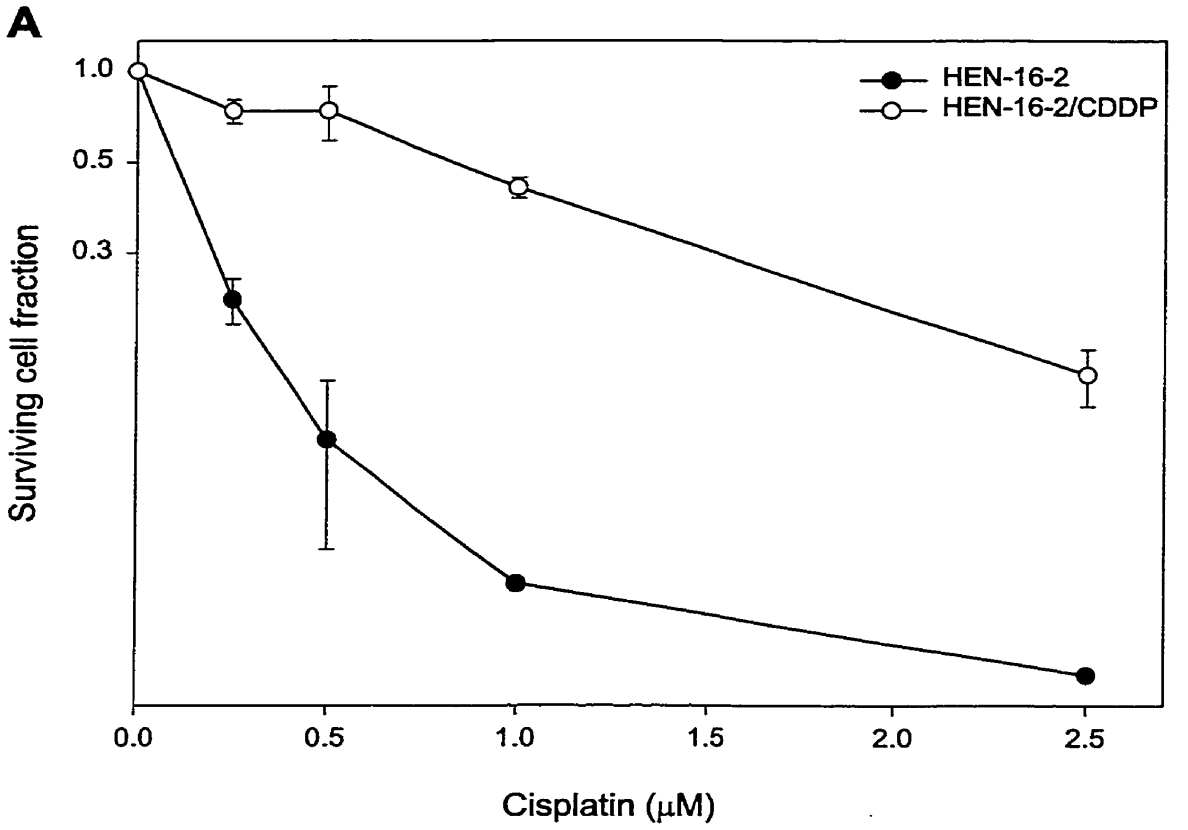
RESULTS

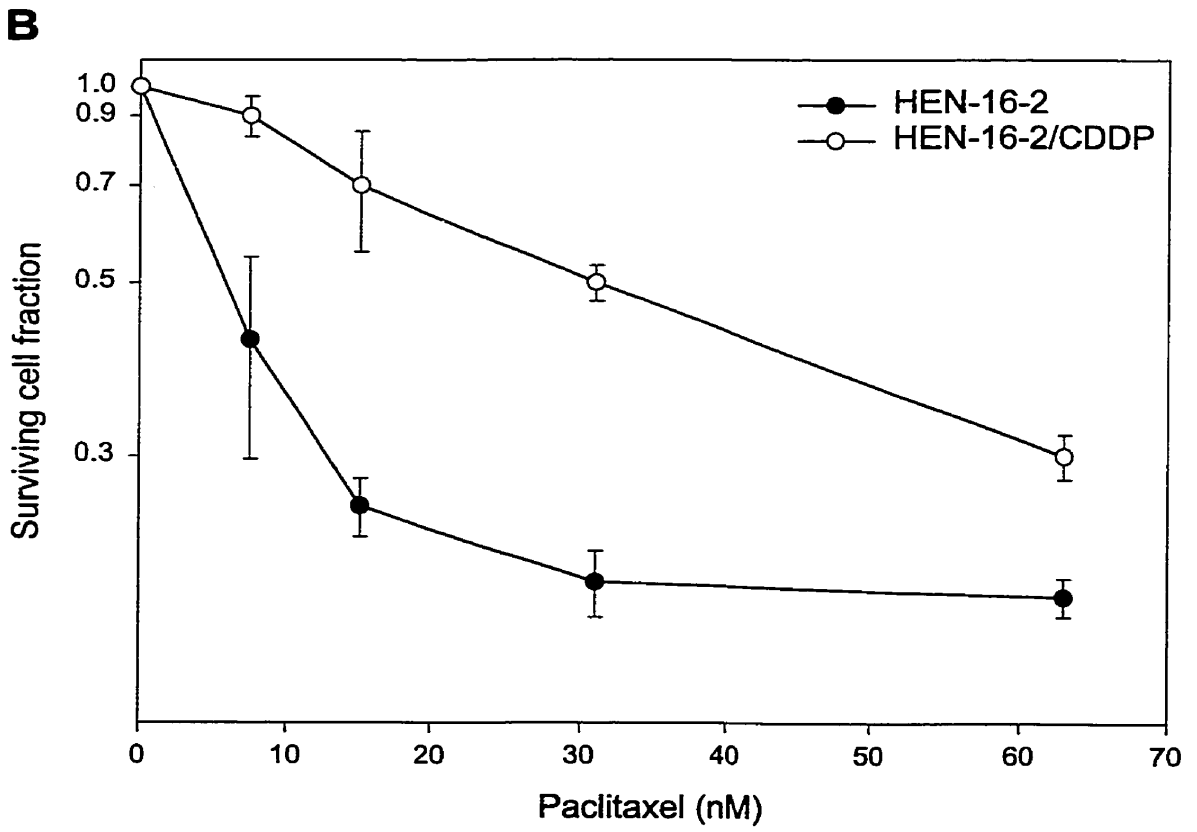
3.1. Establishment of MDR endocervical cell line HEN-16-2/CDDP

HPV16-immortalized endocervical cells, HEN-16-2, were treated with or without cisplatin and then subjected to clonogenic survival assays. Two structurally and functionally unrelated antineoplastic agents, cisplatin and paclitaxel, were used in these assays. Cisplatin is a platinum compound. Several mechanisms of anticancer action are proposed for cisplatin: intrastrand crosslinking of DNA, inhibiting DNA precursors, and unmasking antigenic sites on the cell membrane. Paclitaxel is a taxane alkaloid, which binds to microtubules and promotes the rate and extent of tubulin assembly into stable microtubules, thus preventing tubulin depolymerization and cell division.

Clonogenic survival curves showed that cisplatin-treated HEN-16-2 cells, and HEN-16-2/CDDP, were more resistant to cisplatin than their parental HEN-16-2 cells (Figure 3.1A), as well as to paclitaxel (Figure 3.1B). The resistance to cisplatin or paclitaxel is calculated as the concentration of drug resulting in 50% cell viablility inhibition (IC_{50}) and was 830 nM for cisplatin and 31 nM for paclitaxel, respectively. Compared to HEN-16-2, HEN-16-2/CDDP cells were found to be significantly more resistant to cisplatin (more than 8 fold) or paclitaxel (more than 5 fold), respectively. This indicates that HEN-16-2/CDDP cells have acquired resistance to structurally and functionally unrelated antineoplastic agents, cisplatin and paclitaxel, and thus have a phenotype of MDR.

Figure 3.1. Dose-dependent clonogenic survival of HEN-16-2 and HEN-16-2/CDDP after cisplatin and paclitaxel treatment. About 1,000 cells were seeded into 60-mm diameter tissue culture plates and allowed to attach overnight in KGM. Cells were then incubated with various concentrations of cisplatin (A) and paclitaxel (B) in KGM for 24 hours, washed twice with sterile PBS at 37°C and incubated in drug-free KGM for 10-14 days. Colonies were stained with 2% crystal violet in methanol and counted. Survival cell fraction was expressed as the ratio of the colony-forming cells of drug-treated and untreated control cells. Results represent the mean ± the standard deviation from three independent experiments.


A
1.0
0.5
0.3
Surviving cell fraction
HEN-16-2
HEN-16-2/CDDP
0.0
0.5
1.0
1.5
2.0
2.5
Cisplatin (μM)


B
1.0
0.9
0.7
0.5
0.3
Surviving cell fraction
HEN-16-2
HEN-16-2/CDDP
0
10
20
30
40
50
60
70
Paclitaxel (nM)

3.2. Response of multidrug resistant cells and its parental cells to various apoptotic stimuli

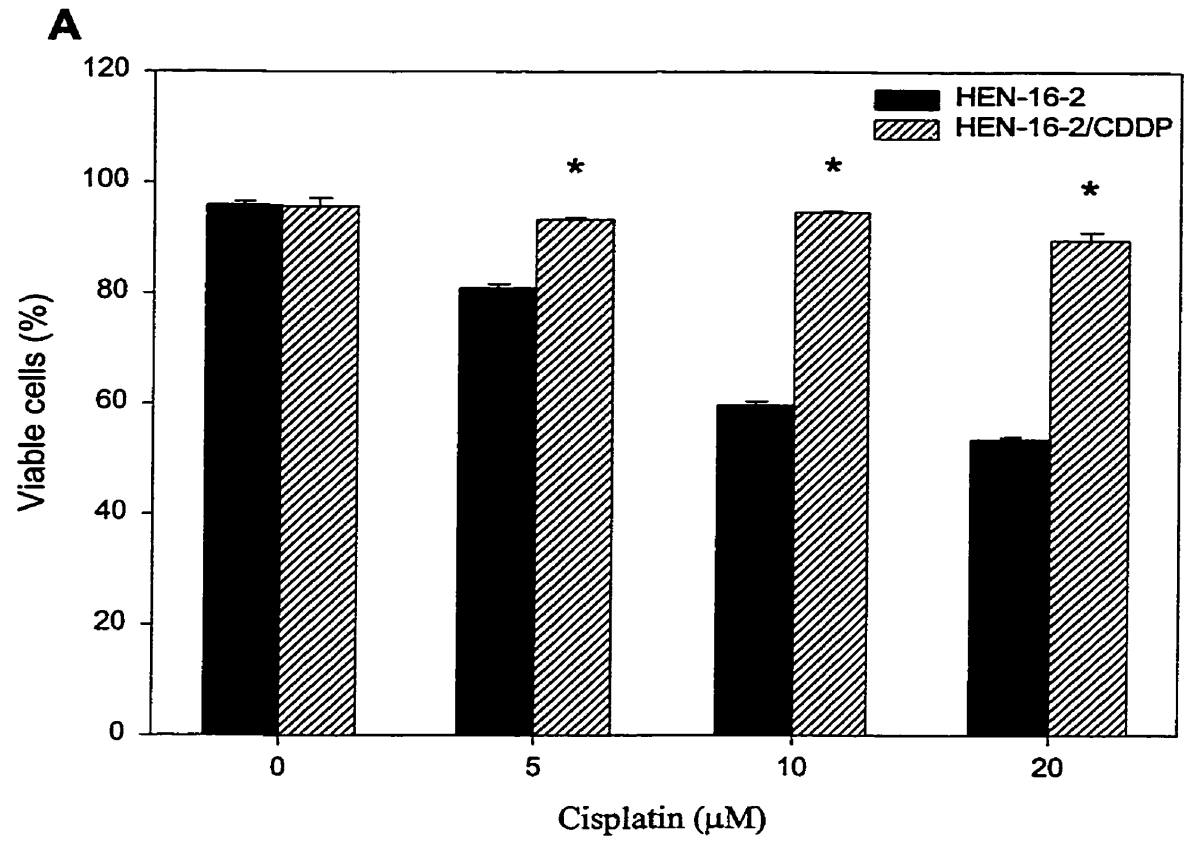
Abnormalities in apoptosis control can influence the sensitivity of cancer cells to chemotherapeutic drugs and other onslaughts (see section 1.2). Overexpression of anti-apoptotic proteins, such as Bcl-2, in some types of cells cause drug resistance. Bcl-2 does not interfere with the accumulation of drugs in tumor cells, obviate the initial damage induced by drugs, or alter the rate of cellular damage repair. It simply increases the threshold to apoptosis and prevents drug-induced cellular damage leading to apoptosis. Increasing the apoptosis threshold confers an MDR phenotype which can render cells more resistant to various apoptotic stimuli, including essentially all chemotherapeutic drugs (Reed, 1998b).

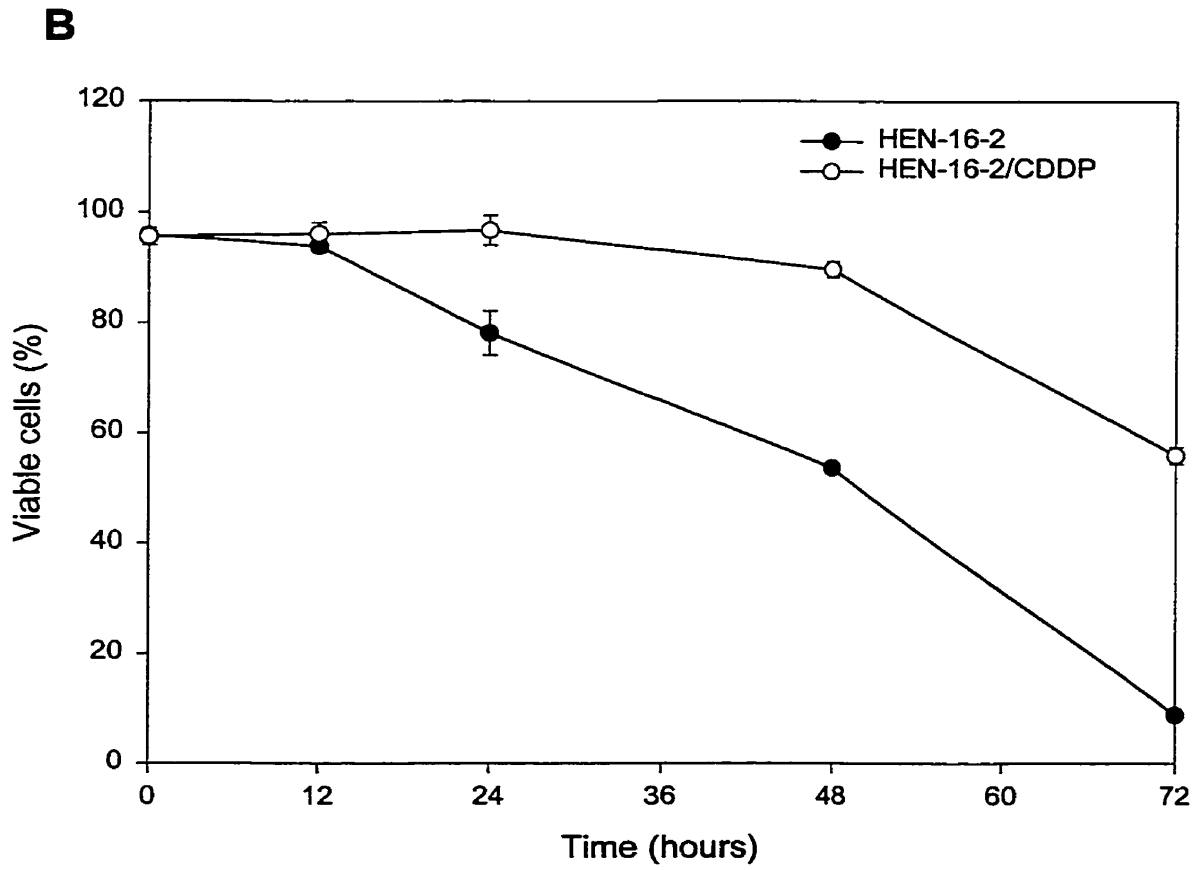
To determine whether the increased apoptotic threshold is the major mechanism of HEN-16-2/CDDP MDR, further studies were conducted on their apoptotic rate and that of the parental HEN-16-2 cells using various apoptotic stimuli that may induce apoptosis through different mechanisms, such as chemotherapeutic drugs, heat shock, UV light irradiation and anti-Fas antibody.

Compared with HEN-16-2, HEN-16-2/CDDP, displayed a significant inhibition of apoptosis induced by cisplatin (Figure 3.2 and 3.3), Sanguinarine (Figure 3.4), actinomycin D (Figure 3.5), doxorubicin (Figure 3.6), etoposide (Figure 3.7), paclitaxel (Figure 3.8), 5-FU (Figure 3.9A), staurosporine (Figure 3.9B), ATRA (Figure 3.10A) and 4-HPR (Figure 3.10B).

Furthermore, HEN-16-2/CDDP exhibited a higher survival rate than HEN-16-2,

Figure 3.2. Dose- and time-dependent induction of apoptosis in HEN-16-2 and HEN-16-2/CDDP cells by cisplatin. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean ± the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between HEN-16-2 cells and HEN-16-2/CDDP cells. A. Dose dependence. Cells were incubated with the indicated concentrations of cisplatin for 48 h. B. Time dependence. Cells were incubated with 20 μM cisplatin for the indicated periods.


A
120
100
80
60
40
20
0
Viable cells (%)
HEN-16-2
HEN-16-2/CDDP
*
*
*
0
5
10
20
Cisplatin (μM)


B
120
100
80
60
40
20
0
Viable cells (%)
HEN-16-2
HEN-16-2/CDDP
0
12
24
36
48
60
72
Time (hours)

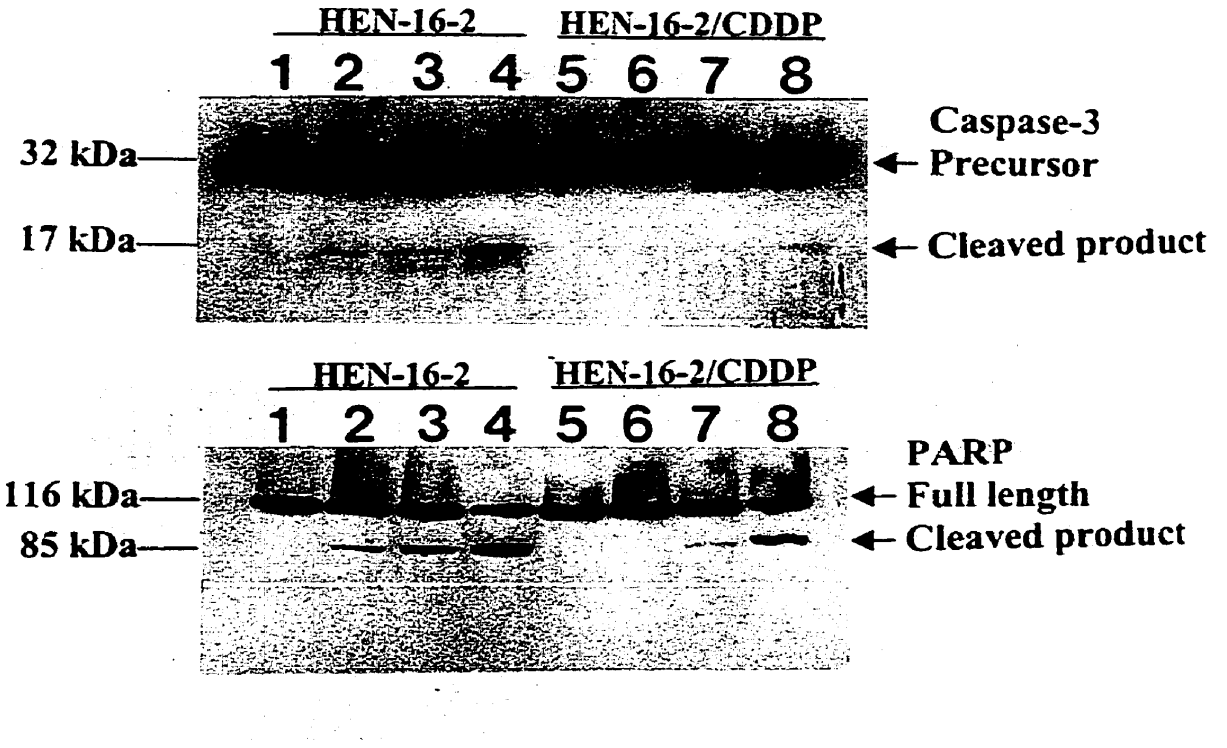


Figure 3.3. Representive result of caspase-3 activation and PARP cleavage in HEN-16-2 and HEN-16-2/CDDP cells. Lysates from cells treated in 0 μM (lanes 1 and 5) or 5 μM (lanes 2 and 6), or 10 μM (lanes 3 and 7), or 20 μM (lanes 4 and 8) cisplatin were immunoblotted with anti-caspase-3 or anti-PARP.

Figure 3.4. Representative result of morphological changes of cells undergoing apoptosis induced by sanguinarine. Apoptosis was induced by 1.5 μg/μl sanguinarine for 4 hours and examined by light microscopy (A and B) or scanning electronic microscopy (C and D). Panels E and F represent untreated cells examined by scanning electronic microscopy.

HEN-16-2

HRN-16-2/CDDP

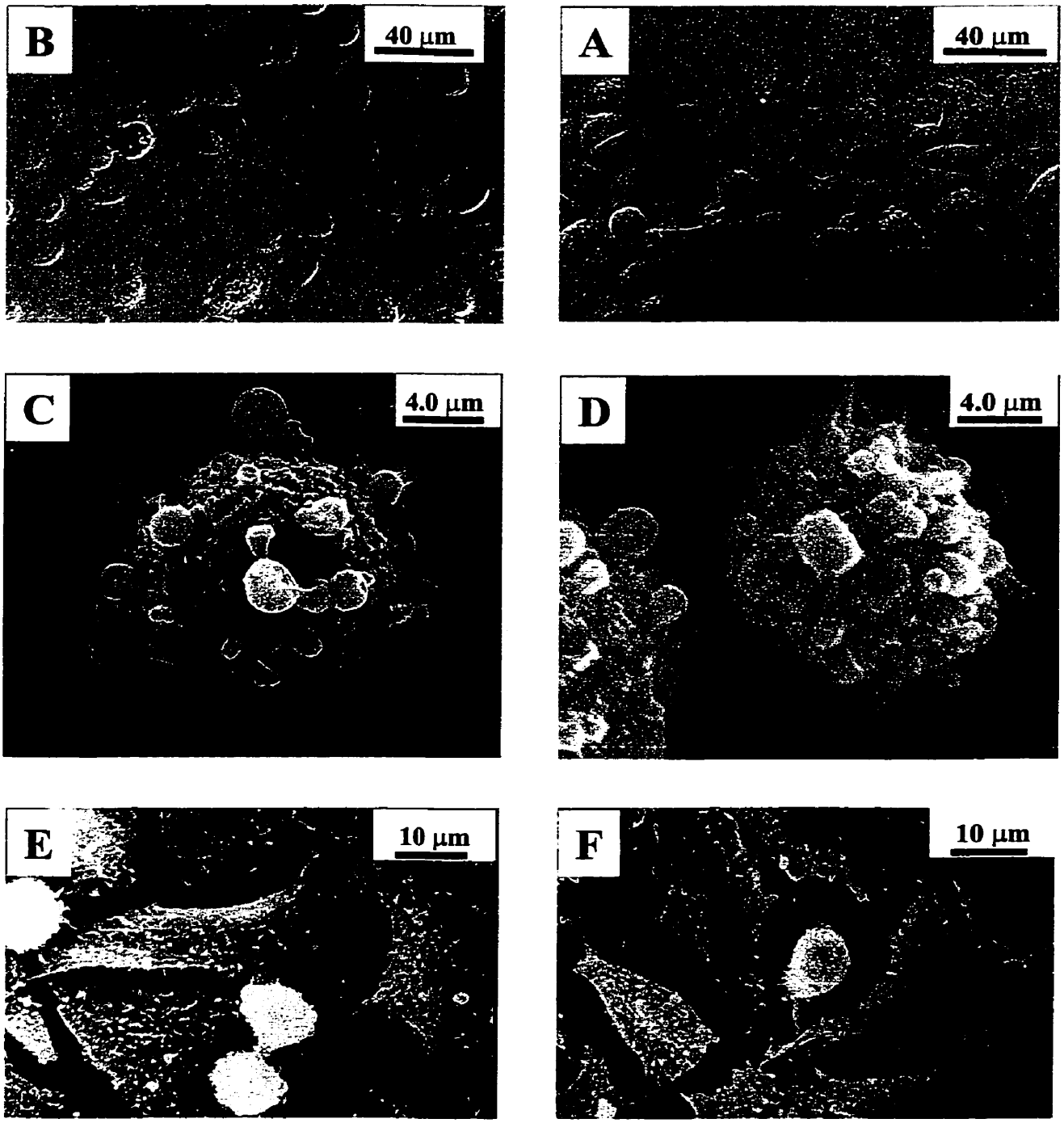
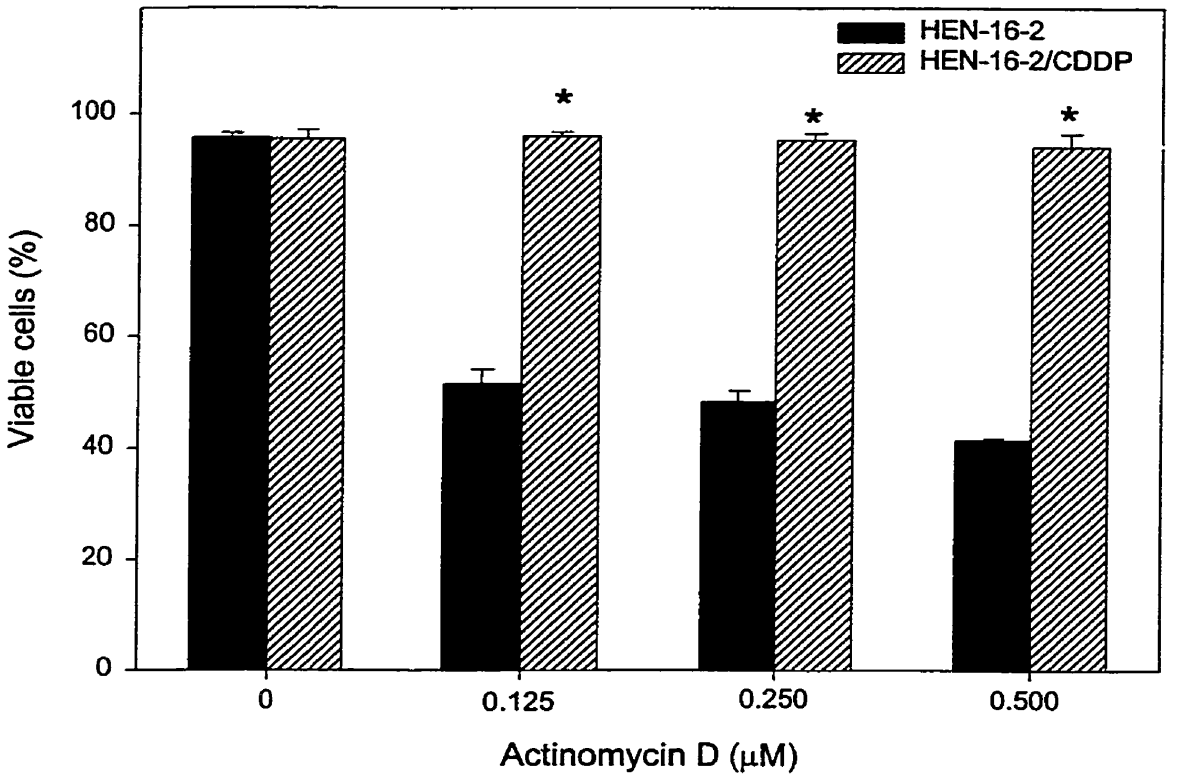


Figure 3.5. Dose- and time-dependent induction of apoptosis by actinomycin D in HEN-16-2 and HEN-16-2/CDDP cells. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean $\pm$ the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between HEN-16-2 cells and HEN-16-2/CDDP cells. A. dose-dependence. Cells were incubated with the indicated concentrations of actinomycin D for 48 h. B. Time-dependence. Cells were incubated with 1 μM actinomycin D for the indicated periods.

A


HEN-16-2
HEN-16-2/CDDP
Viable cells (%)
100
80
60
40
20
0
*
*
*
0
0.125
0.250
0.500
Actinomycin D (μM)

B

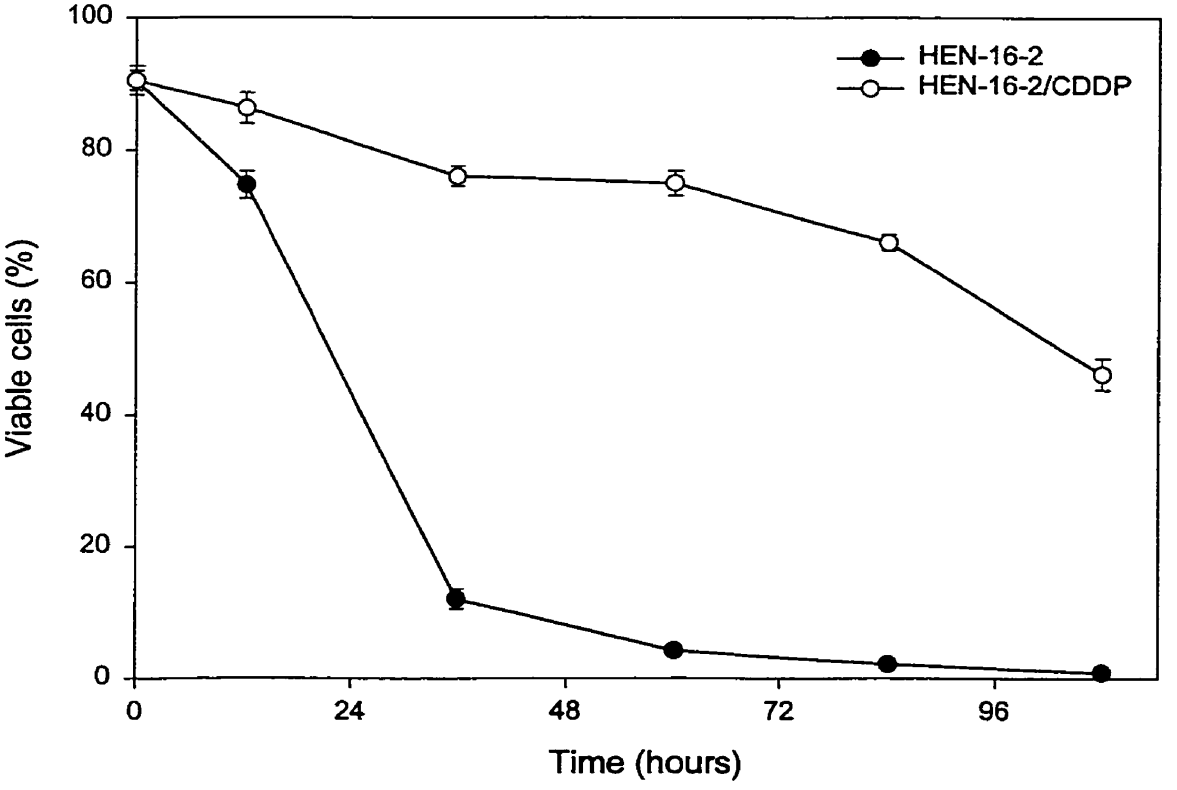
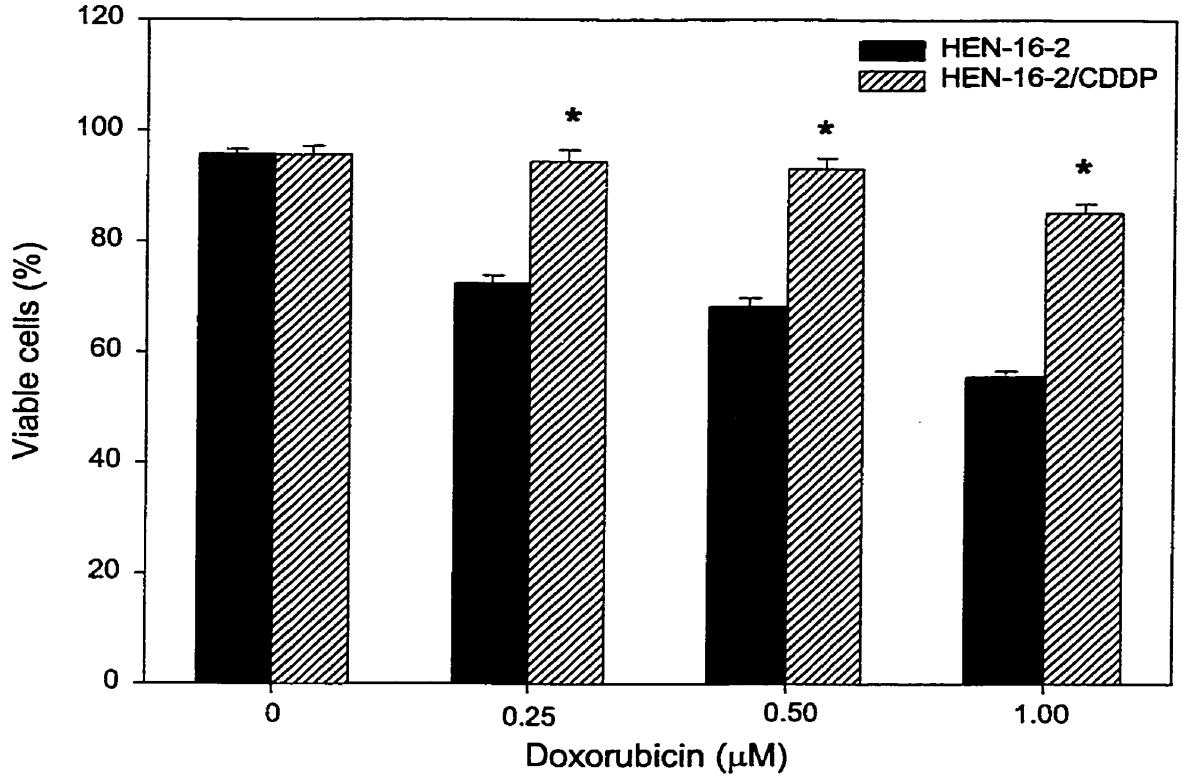

HEN-16-2
HEN-16-2/CDDP
Viable cells (%)
100
80
60
40
20
0
0
24
48
72
96
Time (hours)

Figure 3.6. Dose- and time-dependent induction of apoptosis by doxorubicin in HEN-16-2 and HEN-16-2/CDDP cells. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean $\pm$ the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between HEN-16-2 cells and HEN-16-2/CDDP cells. A. Dose dependence. Cells were incubated with the indicated concentrations of doxorubicin for 48 h. B. Time dependence. Cells were incubated with 1 μM doxorubicin for the indicated periods.

A


120
100
80
60
40
20
0
Viable cells (%)
HEN-16-2
HEN-16-2/CDDP
*
*
*
0
0.25
0.50
1.00
Doxorubicin (μM)

B

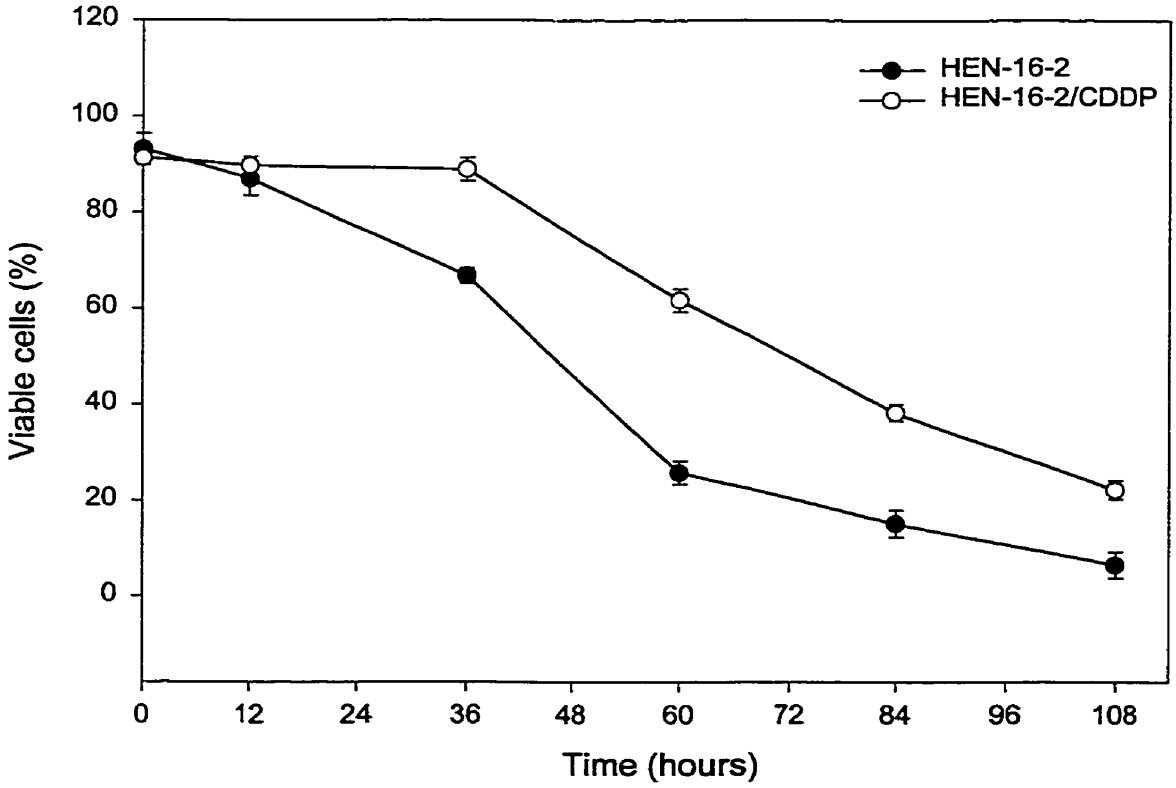
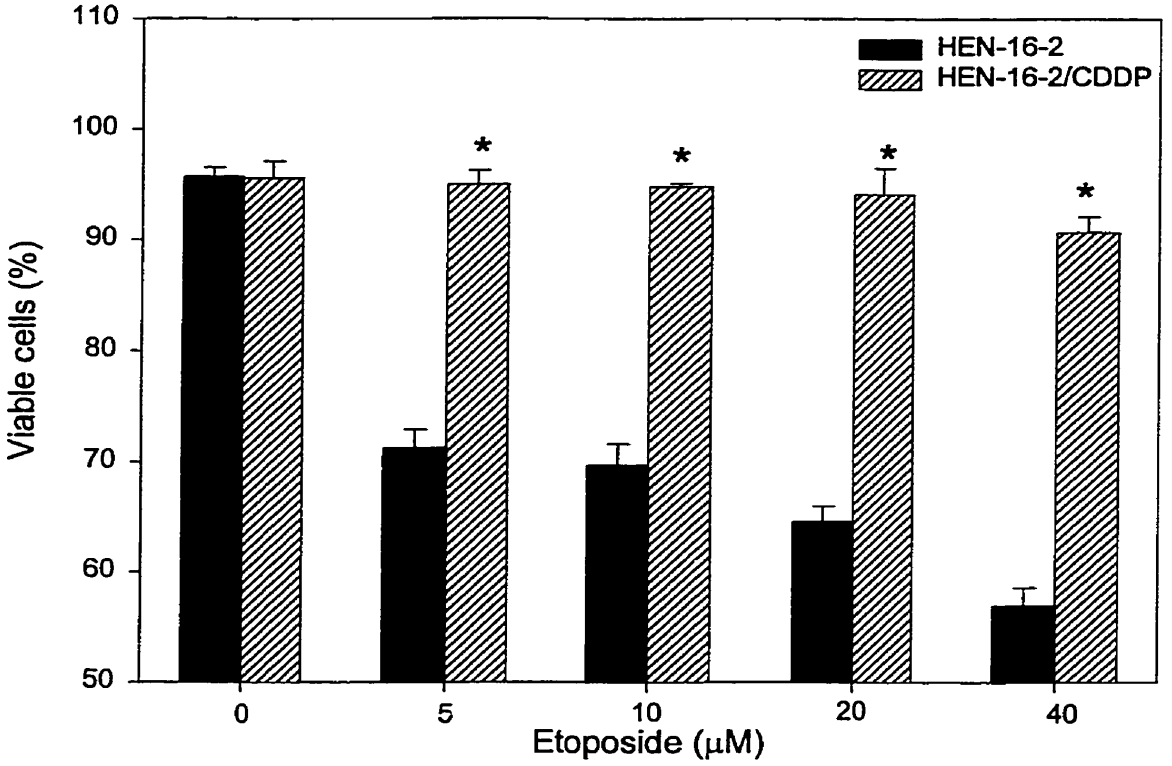

120
100
80
60
40
20
0
Viable cells (%)
HEN-16-2
HEN-16-2/CDDP
0
12
24
36
48
60
72
84
96
108
Time (hours)

Figure 3.7. Dose- and time-dependent induction of apoptosis by etoposide in HEN-16-2 and HEN-16-2/CDDP cells. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean $\pm$ the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between HEN-16-2 cells and HEN-16-2/CDDP cells. A. dose dependence. Cells were incubated with the indicated concentrations of etoposide for 48 h. B. time dependence. Cells were incubated with 40 μM etoposide for the indicated periods.

A


HEN-16-2
HEN-16-2/CDDP
Viable cells (%)
110
100
90
80
70
60
50
*
*
*
*
0
5
10
20
40
Etoposide (μM)

B

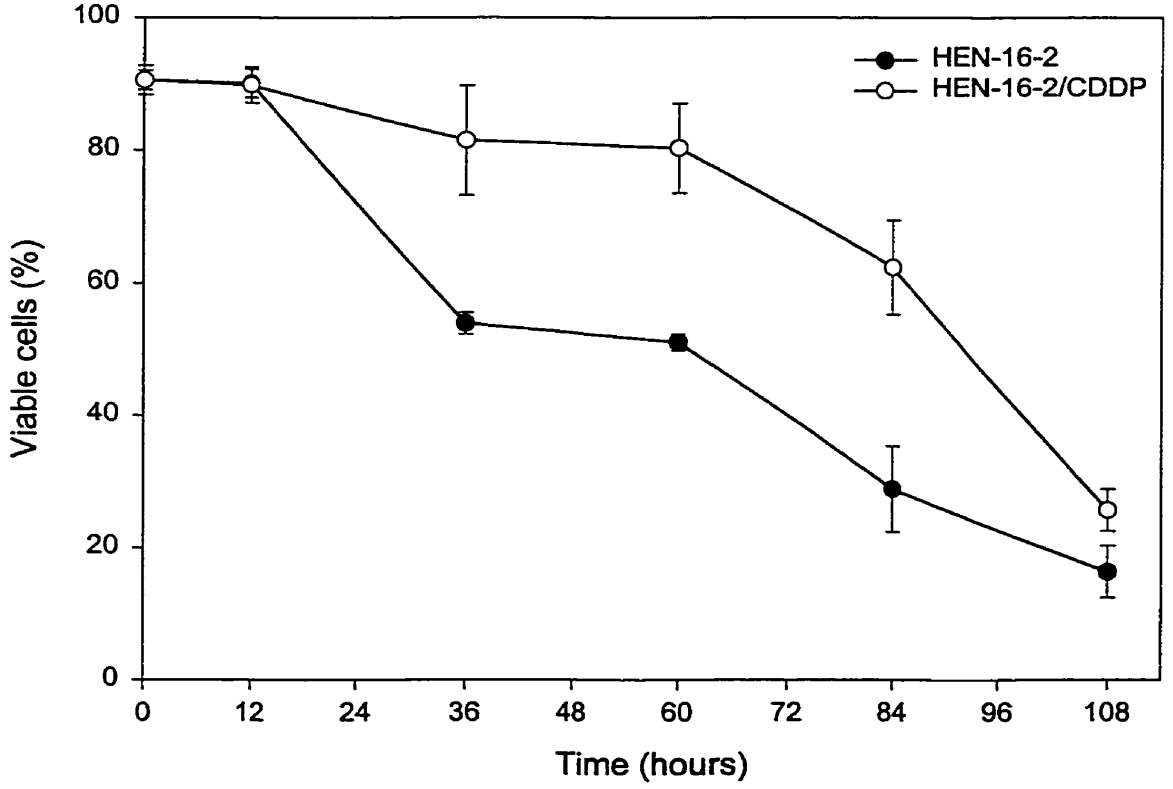
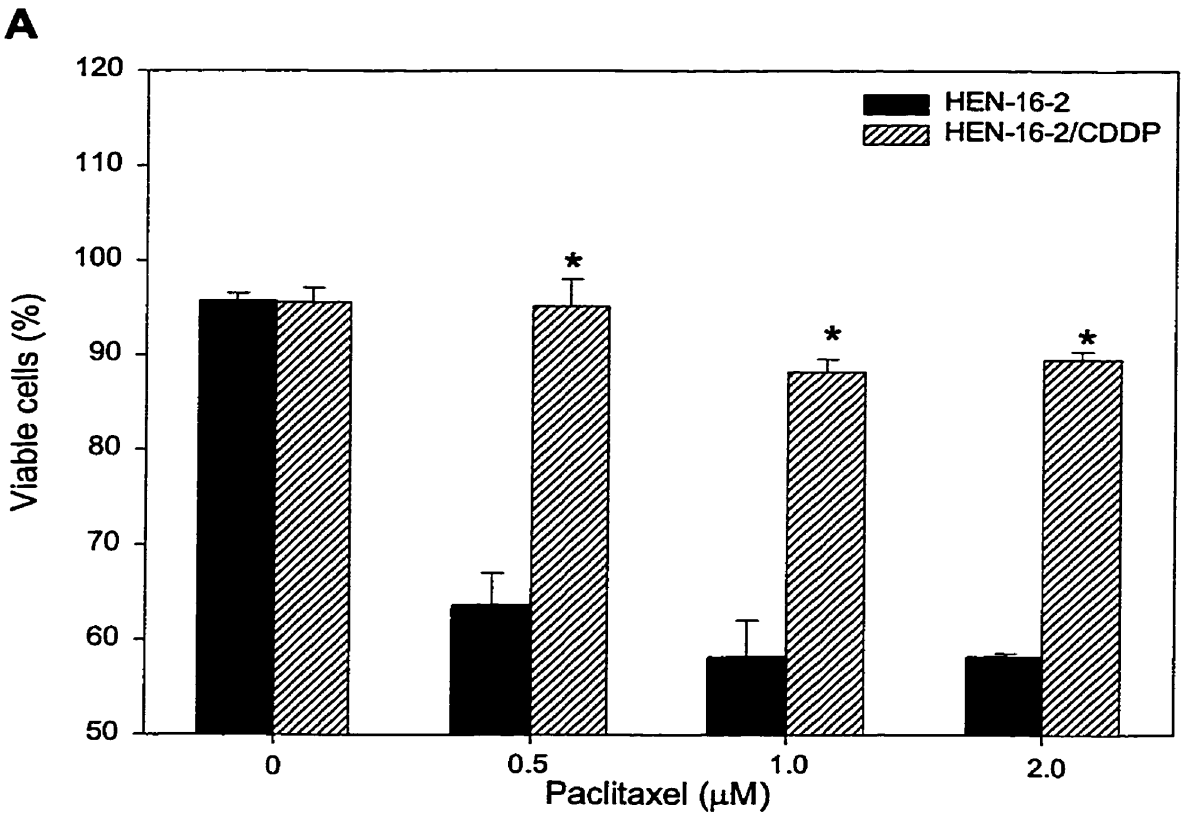

HEN-16-2
HEN-16-2/CDDP
Viable cells (%)
100
80
60
40
20
0
0
12
24
36
48
60
72
84
96
108
Time (hours)

Figure 3.8. Dose- and time-dependent induction of apoptosis by paclitaxel in HEN-16-2 and HEN-16-2/CDDP cells. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean $\pm$ the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between HEN-16-2 cells and HEN-16-2/CDDP cells. A. Dose dependence. Cells were incubated with the indicated concentrations of paclitaxel for 48 h. B. Time dependence. Cells were incubated with 5 μM paclitaxel for the indicated periods.


A
120
110
100
90
80
70
60
50
Viable cells (%)
HEN-16-2
HEN-16-2/CDDP
*
*
*
0
0.5
1.0
2.0
Paclitaxel (μM)

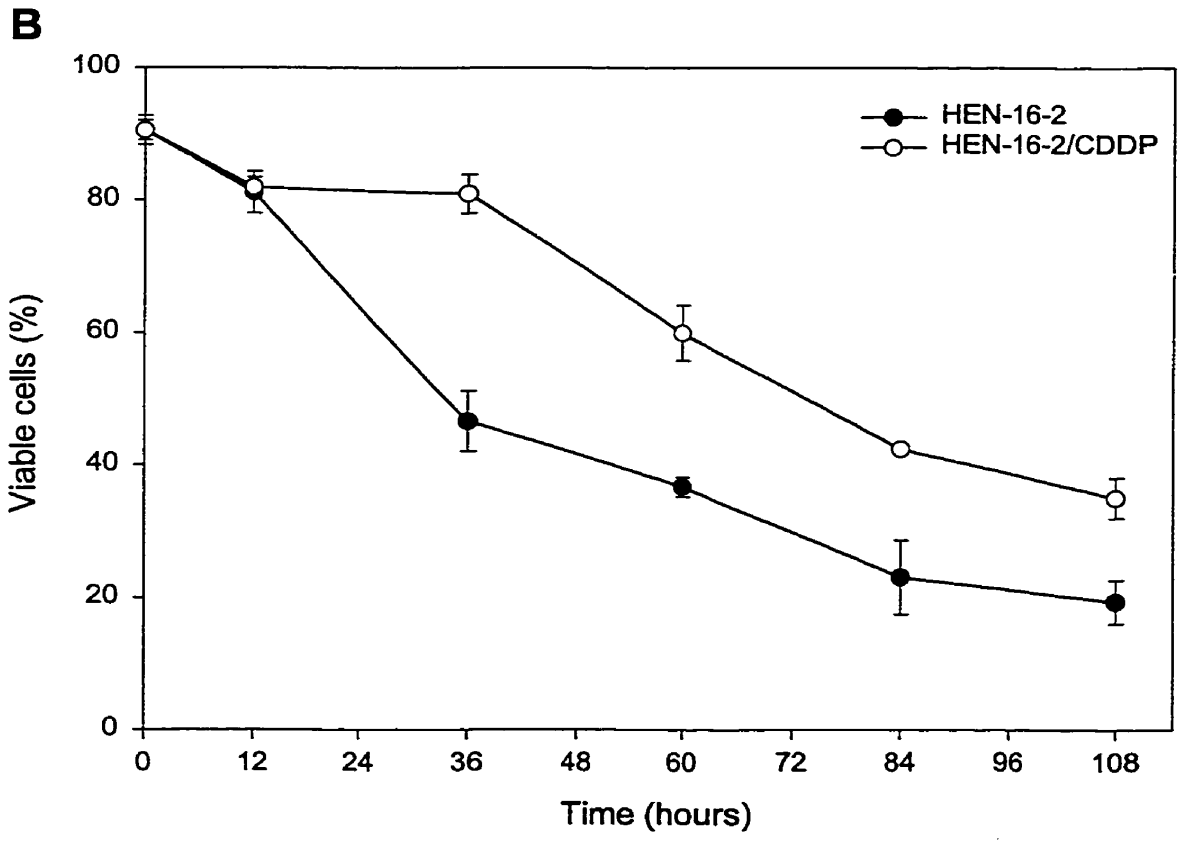
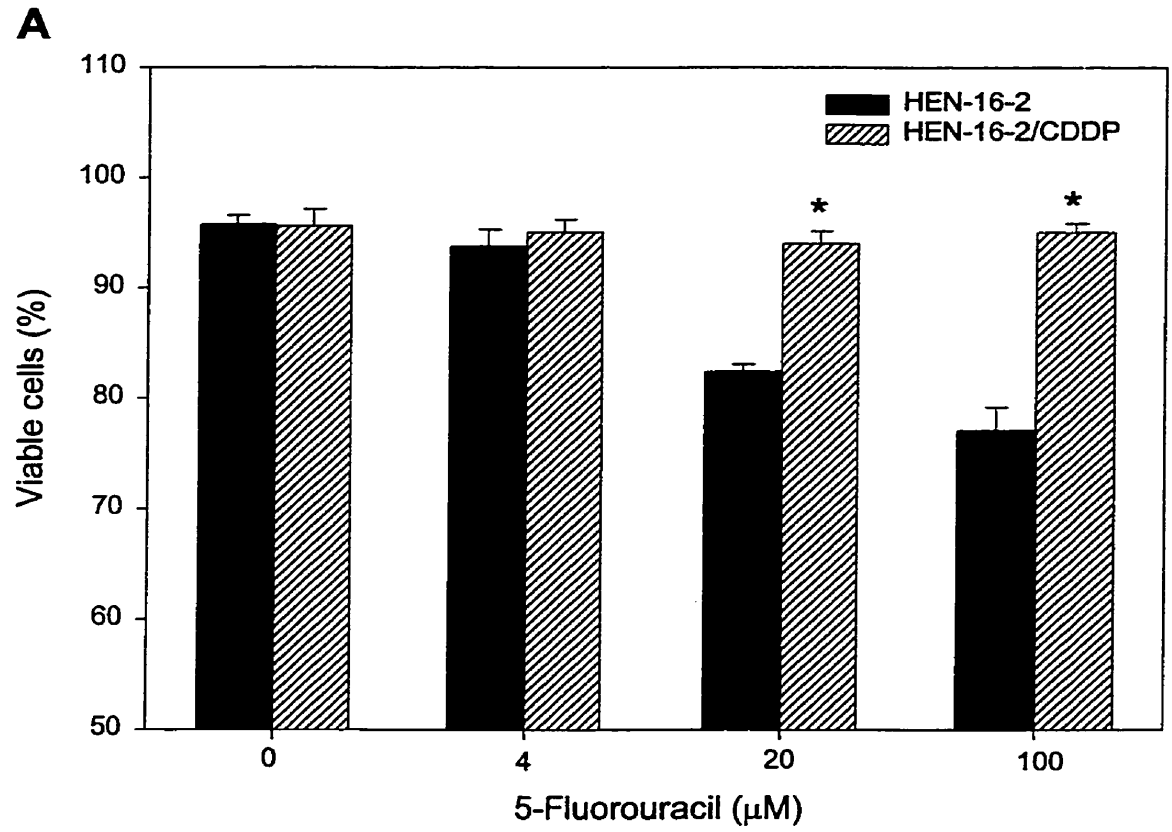

B
100
80
60
40
20
0
Viable cells (%)
HEN-16-2
HEN-16-2/CDDP
0
12
24
36
48
60
72
84
96
108
Time (hours)

Figure 3.9. Dose-dependent induction of apoptosis by 5-FU and staurosporine in HEN-16-2 and HEN-16-2/CDDP cells. Cells were incubated with the indicated concentrations of 5-FU or staurosporine for 48 h. A. 5-FU. B. Staurosporine. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean ± the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between HEN-16-2 cells and HEN-16-2/CDDP cells.


A
110
100
90
80
70
60
50
Viable cells (%)
HEN-16-2
HEN-16-2/CDDP
*
*
0
4
20
100
5-Fluorouracil (μM)

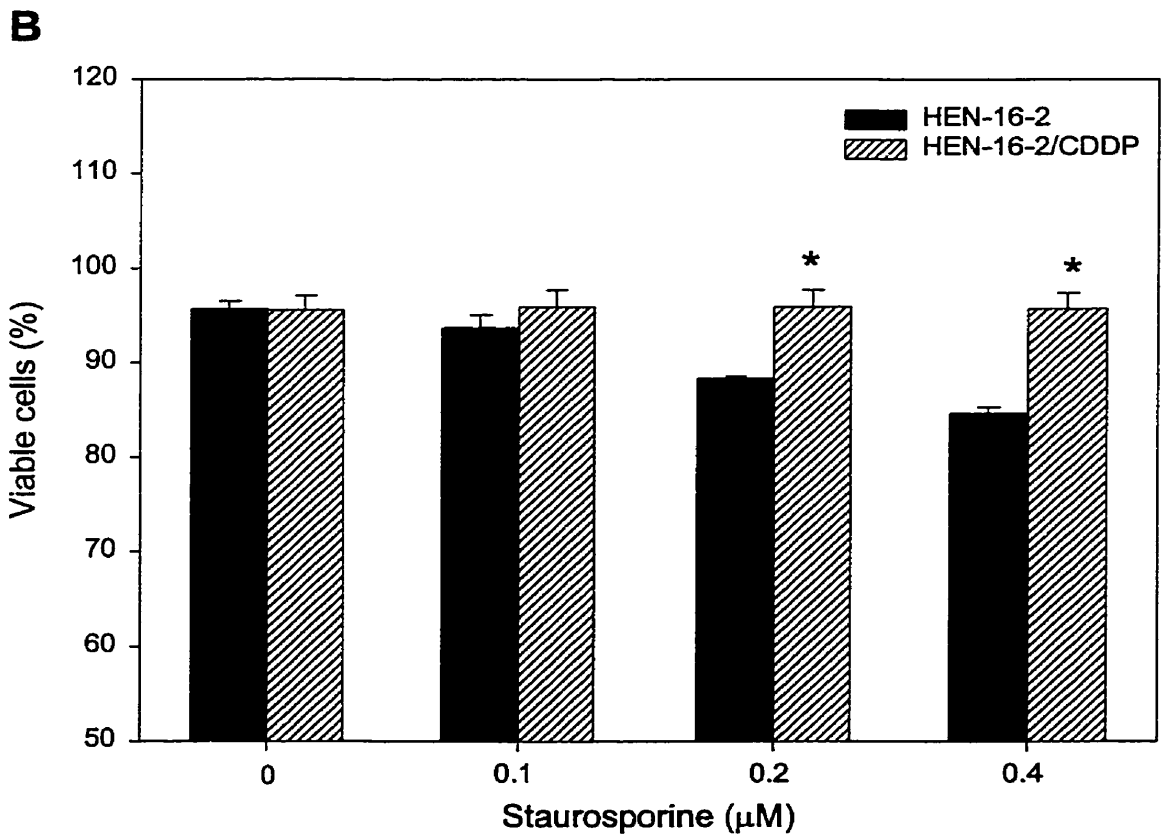
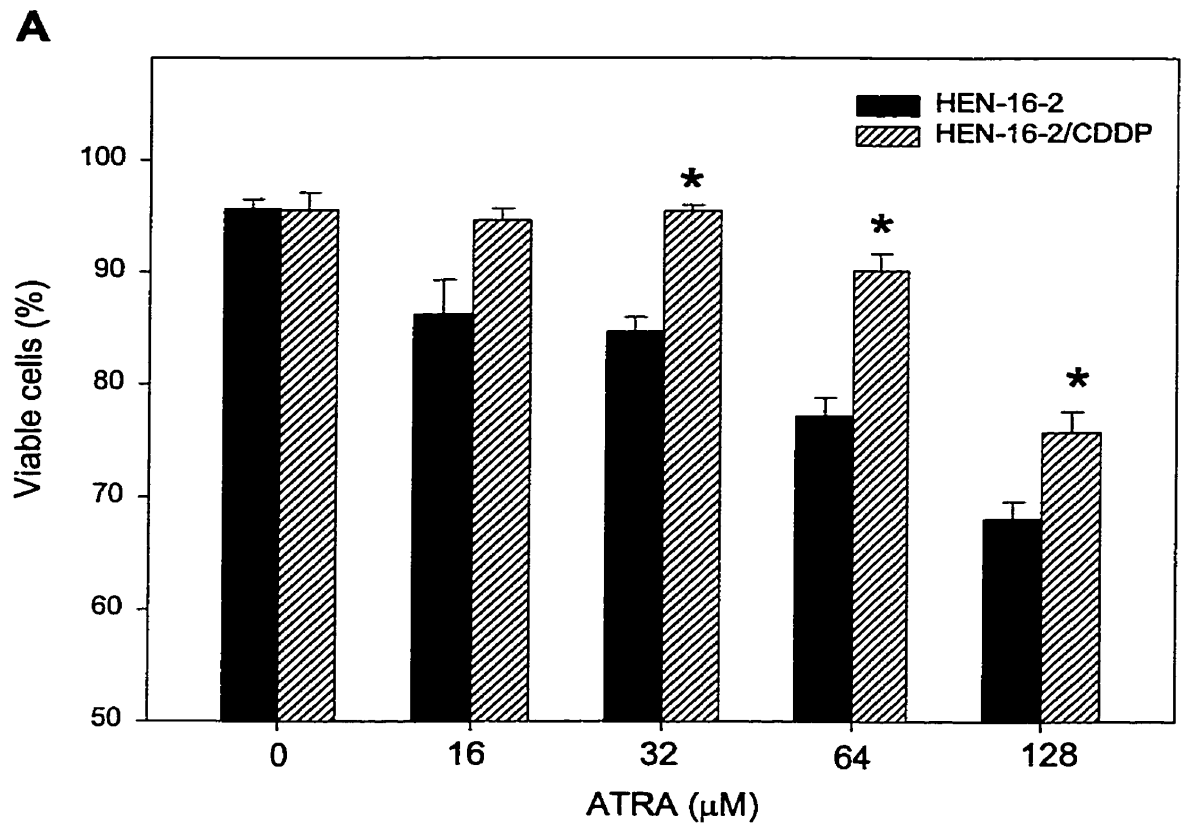
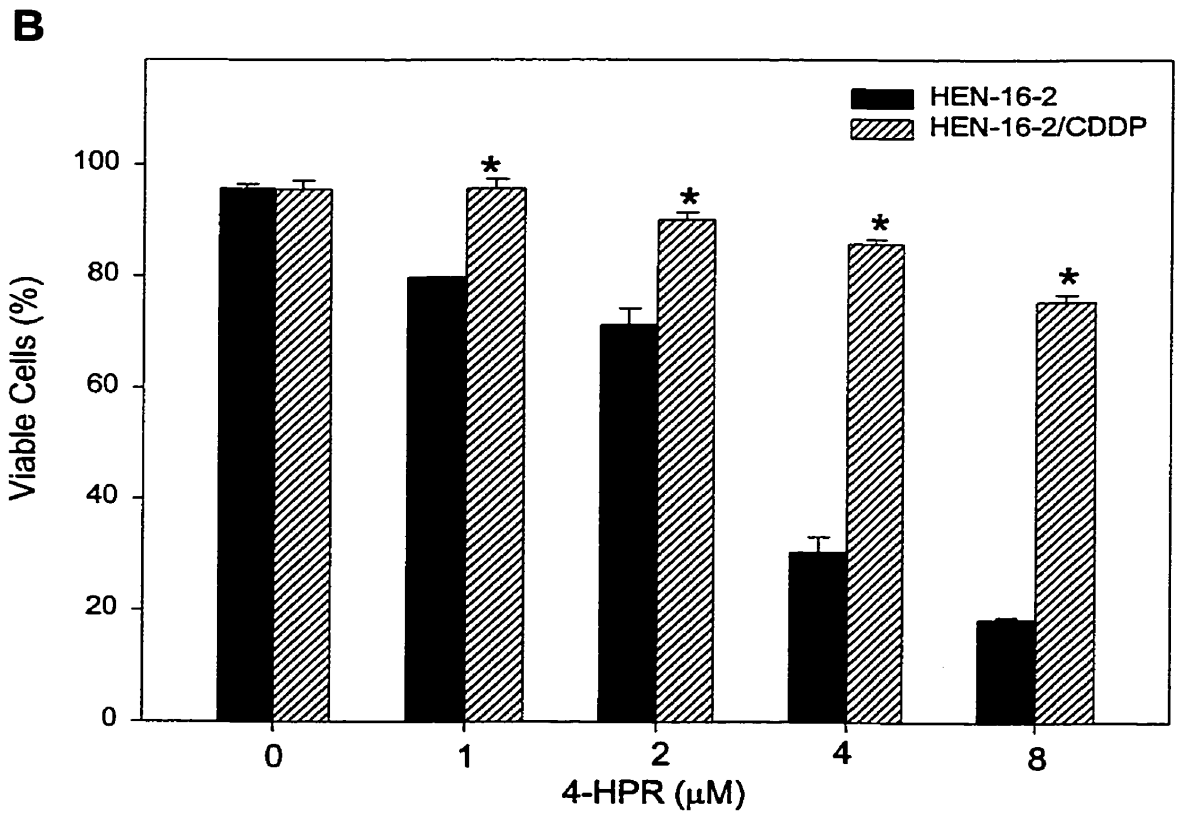

B
120
110
100
90
80
70
60
50
Viable cells (%)
HEN-16-2
HEN-16-2/CDDP
*
*
0
0.1
0.2
0.4
Staurosporine (μM)

Figure 3.10. Dose-dependent induction of apoptosis by ATRA and 4-HPR in HEN-16-2 and HEN-16-2/CDDP cells. Cells were incubated with the indicated concentrations of ATRA or 4-HPR for 48 h. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean ± the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between HEN-16-2 cells and HEN-16-2/CDDP cells. A. ATRA. B. 4-HPR.


A
HEN-16-2
HEN-16-2/CDDP
Viable cells (%)
100
90
80
70
60
50
0
16
32
64
128
ATRA (μM)
*
*
*


B
HEN-16-2
HEN-16-2/CDDP
Viable Cells (%)
100
80
60
40
20
0
0
1
2
4
8
4-HPR (μM)
*
*
*
*

when treated with 0.5 μg/ml anti-Fas antibody, 50 mJ/cm^2 UV irradiation or heat shock at 45 °C for 45 minutes (Figure 3.11).

3.3. Morphology of HEN-16-2/CDDP

The morphology of cultured cervical epithelial cells is an indication of the differentiation potential and oncogenicity of the normal or abnormal tissue from which the cells are derived (Turyk *et al.*, 1989; Vooijs, 1991). In KGM, a serum-free medium that contains 0.15 mM calcium, both types of the cell grew actively and formed keratinocyte-like cells (Figure 3.12A and B). However, when the cells were cultured in 10% serum plus high calcium-containing DMEM, HEN-16-2 immortalized cells were slow growing and flat, branched, heterogeneously sized and unevenly distributed (Figure 3.12C). In contrast, HEN-16-2/CDDP cell line cultures had higher nucleus/cytoplasm ratio, and showed morphology and distribution that were comparable to those of the cells cultured in serum-free KGM (Figure 3.12D).

3.4. Growth characteristics of HEN-16-2/CDDP

Table 3.1 summarizes the growth rate, the saturation density and anchorage-independent growth in soft agar of HEN-16-2/CDDP compared with its parental cell line HEN-16-2.

In serum-free KGM, the average growth rate decreased slightly in multidrug resistant HEN-16-2/CDDP cells (Figure 3.13 and Table 3.1). The doubling times of HEN-16-2 and HEN-16-2/CDDP in KGM were 37 hours and 44 hours, respectively. In

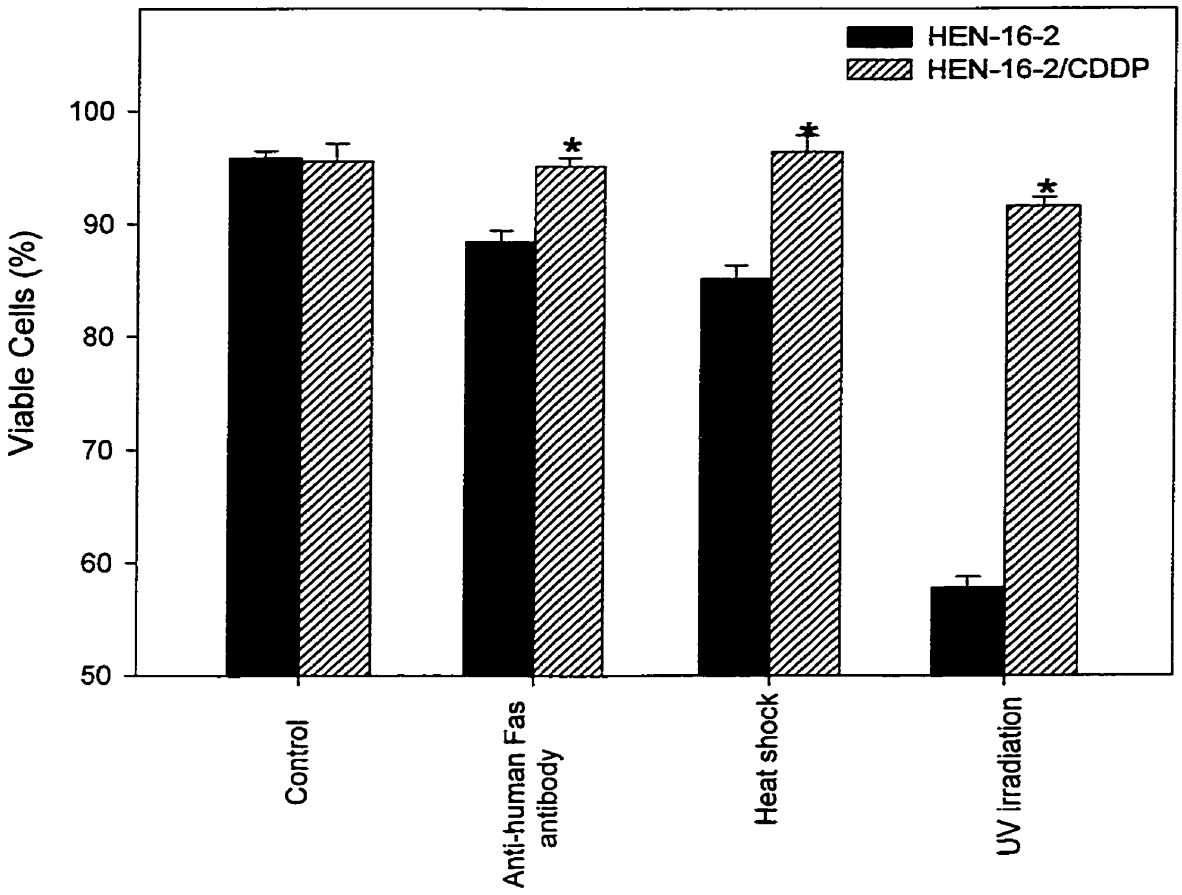


Figure 3.11. Induction of apoptosis by anti-Fas antibody, UV irradiation and heat shock in HEN-16-2/CDDP and HEN-16-2 cells. Cells were exposed to 0.5 μg/ml anti-Fas antibody for 24 h, heat shock at 45 °C for 45 minutes or 50 mJ/cm^2 UV irradiation. Cells subjected to heat shock and UV irradiation were then returned to normal culture for 48 hours. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean ± the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between HEN-16-2 cells and HEN-16-2/CDDP cells.

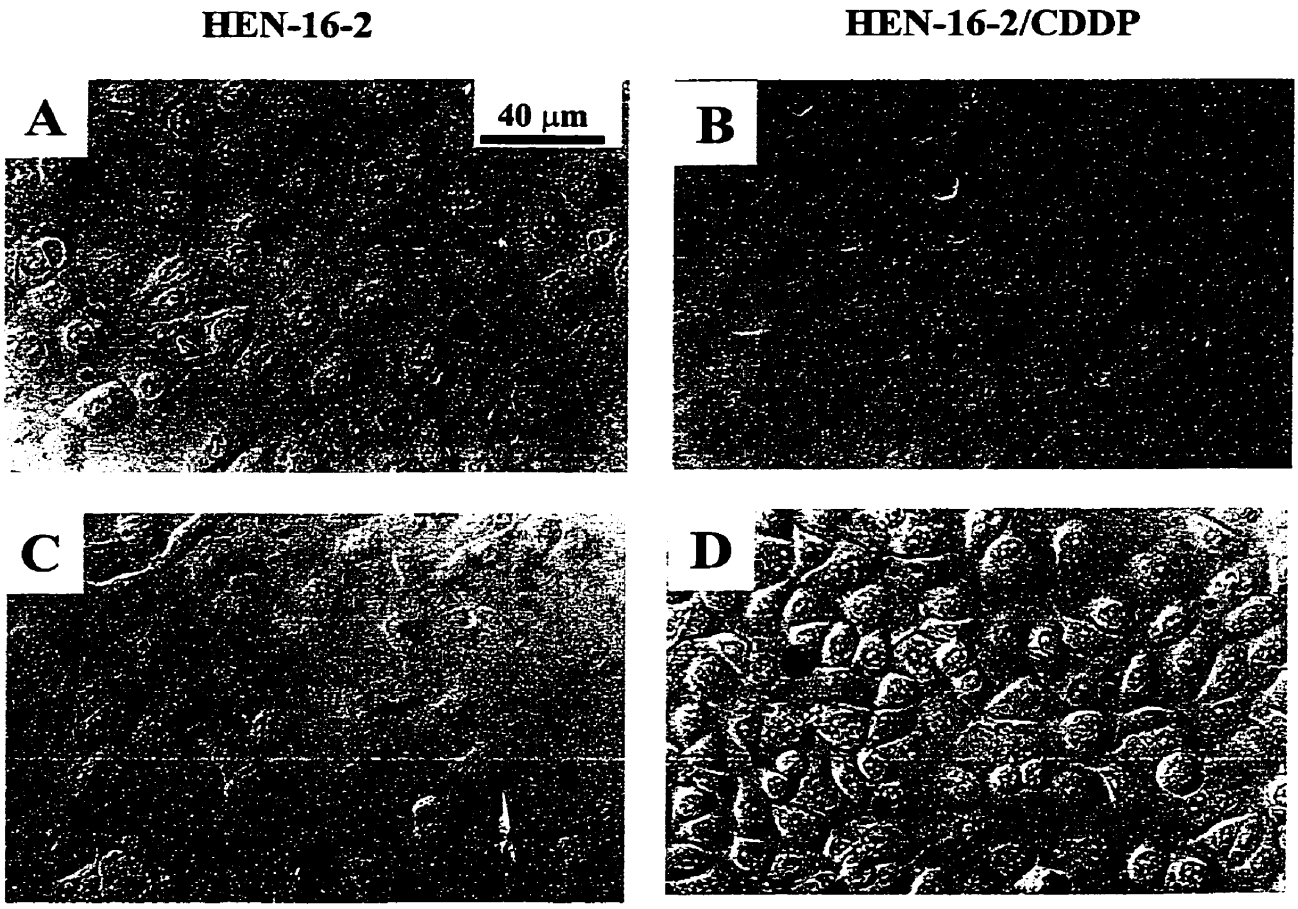
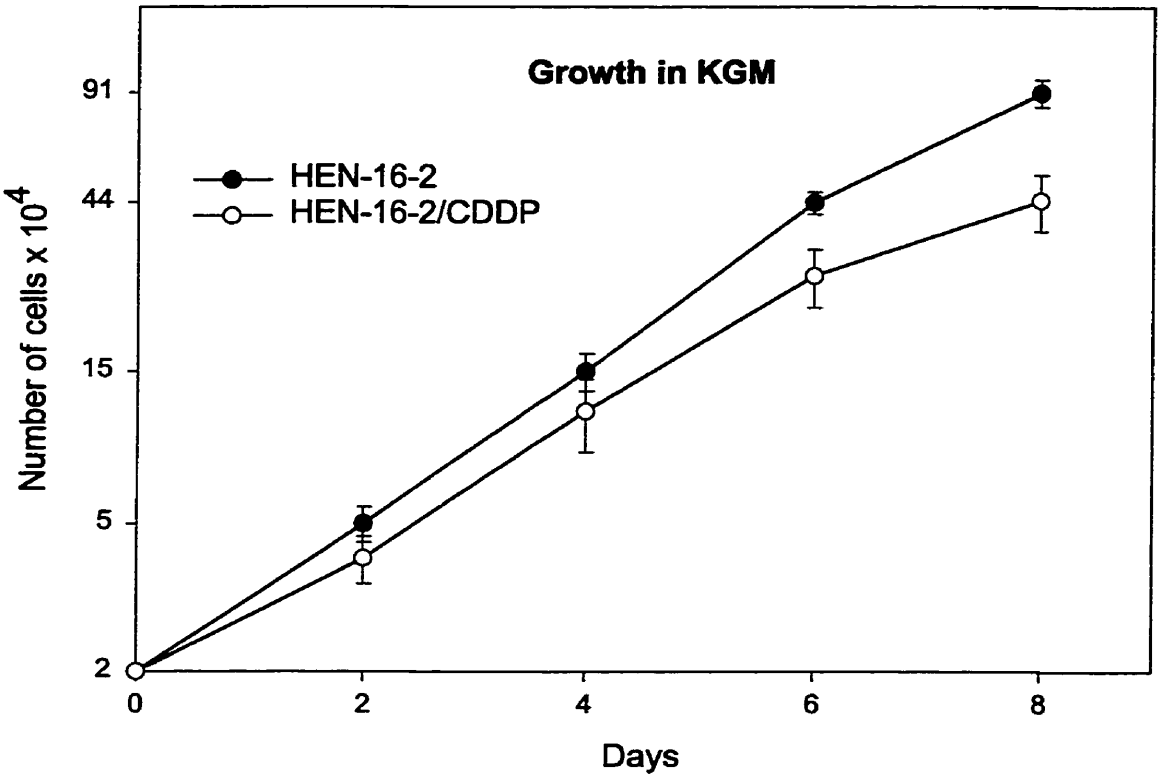
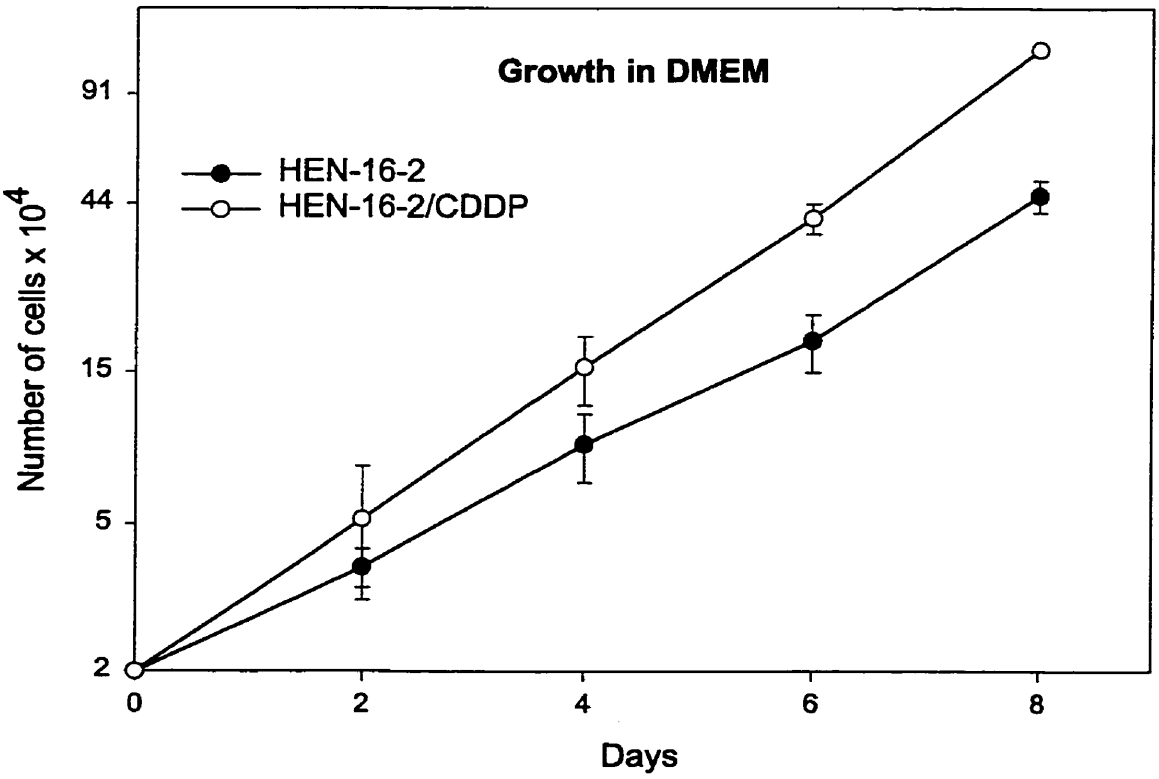


Figure 3.12. Morphology of HEN-16-2 and HEN-16-2/CDDP in monolayer culture. A. HEN-16-2 in KGM. B. HEN-16-2/CDDP in KGM. C. HEN-16-2 in DMEM. D. HEN-16-2/CDDP in DMEM plus 10% FCS. All panels show light microscopy photos at the same magnification.

Figure 3.13. Growth rates of HEN-16-2 and HEN-16-2/CDDP cells in monolayer culture. Cells were grown in 60-mm plates in the indicated media. Each value represents the mean $\pm$ the standard deviation from three individual experiments.


Growth in KGM
HEN-16-2
HEN-16-2/CDDP
Number of cells x 10^4
91
44
15
5
2
0
2
4
6
8
Days


Growth in DMEM
HEN-16-2
HEN-16-2/CDDP
Number of cells x 10^4
91
44
15
5
2
0
2
4
6
8
Days

contrast, a different pattern was observed in serum-supplemented high calcium level DMEM. HEN-16-2 immortalized cells proliferated more slowly than the multidrug-resistant cells. The doubling times of HEN-16-2 and HEN-16-2/CDDP in DMEM were significantly different ($p < 0.05$) (44 hours and 34 hours, respectively). Further, there was a significant difference ($p < 0.05$) in the saturation densities of MDR cells and their parental cells in KGM (306×10^3 and 144×10^3 cells/cm^2, respectively); and in DMEM plus 10% FCS (333×10^3 and 55×10^3 cells/cm^2, respectively) (Table 3.1).

Table 3.1. Growth characteristics of HEN-16-2/CDDP compared with HEN-16-2 cells

Growth characteristic[1]	HEN-16-2	HEN-16-2/CDDP
Doubling time		
KGM	37 ± 6	44 ± 10
DMEM + FCS	44 ± 6	34 ± 2*
Saturation density		
KGM	144 ± 10	306 ± 65*
DMEM + FCS	55 ± 3	333 ± 32*
Anchorage-independent growth	-	+

[1]The values for the doubling time (hours), saturation density ($\times 10^3$ cells/cm^2), are the mean $\pm$ the standard deviation for three independent experiments. Anchorage-independent growth assays were conducted for three independent experiments. *, $p < 0.05$, is the significance of the difference between the values for HEN-16-2/CDDP and HEN-16-2 cells.

To further characterize the oncogenic phenotype, anchorage-independent growth in soft agar assays were performed, in which HEN-16-2 and HEN-16-2/CDDP cells were assayed for growth in soft agar for 2-3 weeks. HeLa cells and HEN-16-2T (Yang *et al.*, 1996a) served as positive controls. Generally, the formation of colonies could be observed after one week of incubation, and the colonies were unequivocally identified

after two weeks. The immortalized cells, HEN-16-2, remained as single cells and did not form colonies in soft agar (Figure 3.14B), whereas MDR cells, HEN-16-2/CDDP, formed colonies (Figure 3.14A) that were similar to those formed from HEN-16-2T (Figure 3.14C), but were smaller than those formed from HeLa (Figure 3.14D).

3.5. Tumorigenicity of MDR endocervical cells

HEN-16-2, HEN-16-2/CDDP and positive control HeLa cells were injected into nude mice. Table 3.2 summarizes the results for each cell line.

Table 3.2. Tumorigenicity of HEN-16/CDDP compared with HEN-16-2 in nude mice

Cell line	Passage	Tumor incidence (No. tumors/no. injections)	Tumor size (cm^2)
HEN-16-2/CDDP	50-80	6/6	1.5-1.8
HEN-16-2	50-80	0/6	N/A
HeLa	N/A	2/2	2.0-2.5

All injections of HEN-16-2/CDDP cells led to tumor formation in nude mice. The tumors were generally apparent after three weeks and continued to grow until the mice were sacrificed (Figure 3.15). HeLa cells also gave rise to tumors in all injected nude mice. None of the untreated immortalized cells induced tumors in nude mice after 2 months, indicating that induction of tumors in nude mice was due to cisplatin treatment.

Two tumors, formed from injection of HEN-16-2/CDDP cells into nude mice, were examined histologically. Both tumors were invasive squamous cell carcinomas characterized by the loss of normal epithelial cell arrangement, growth into the mouse

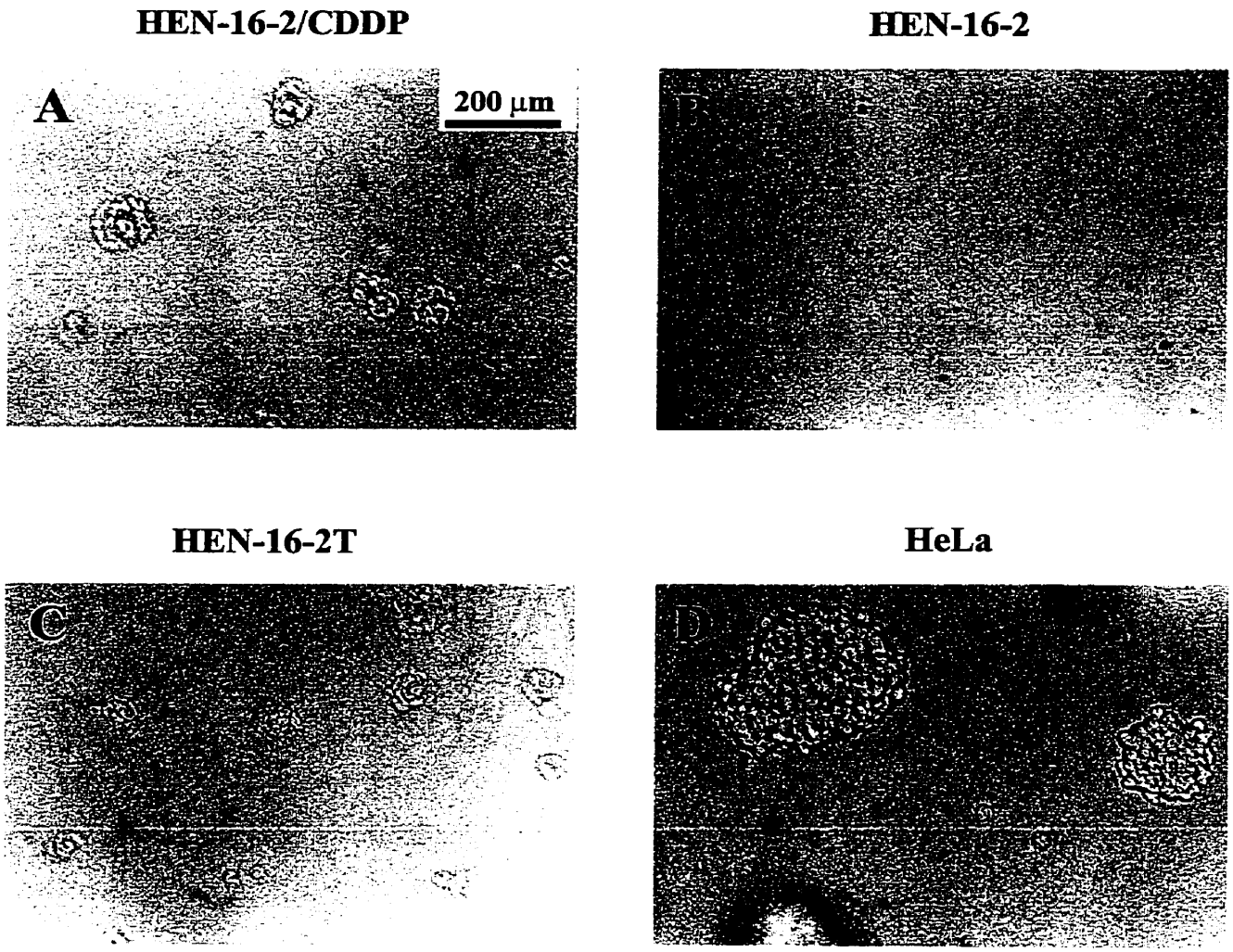


Figure 3.14. Anchorage-independent (soft agar) growth of HEN-16-2/CDDP. A. HEN-16-2/CDDP. B. HEN-16-2. C. HEN-16-2T. D. HeLa.

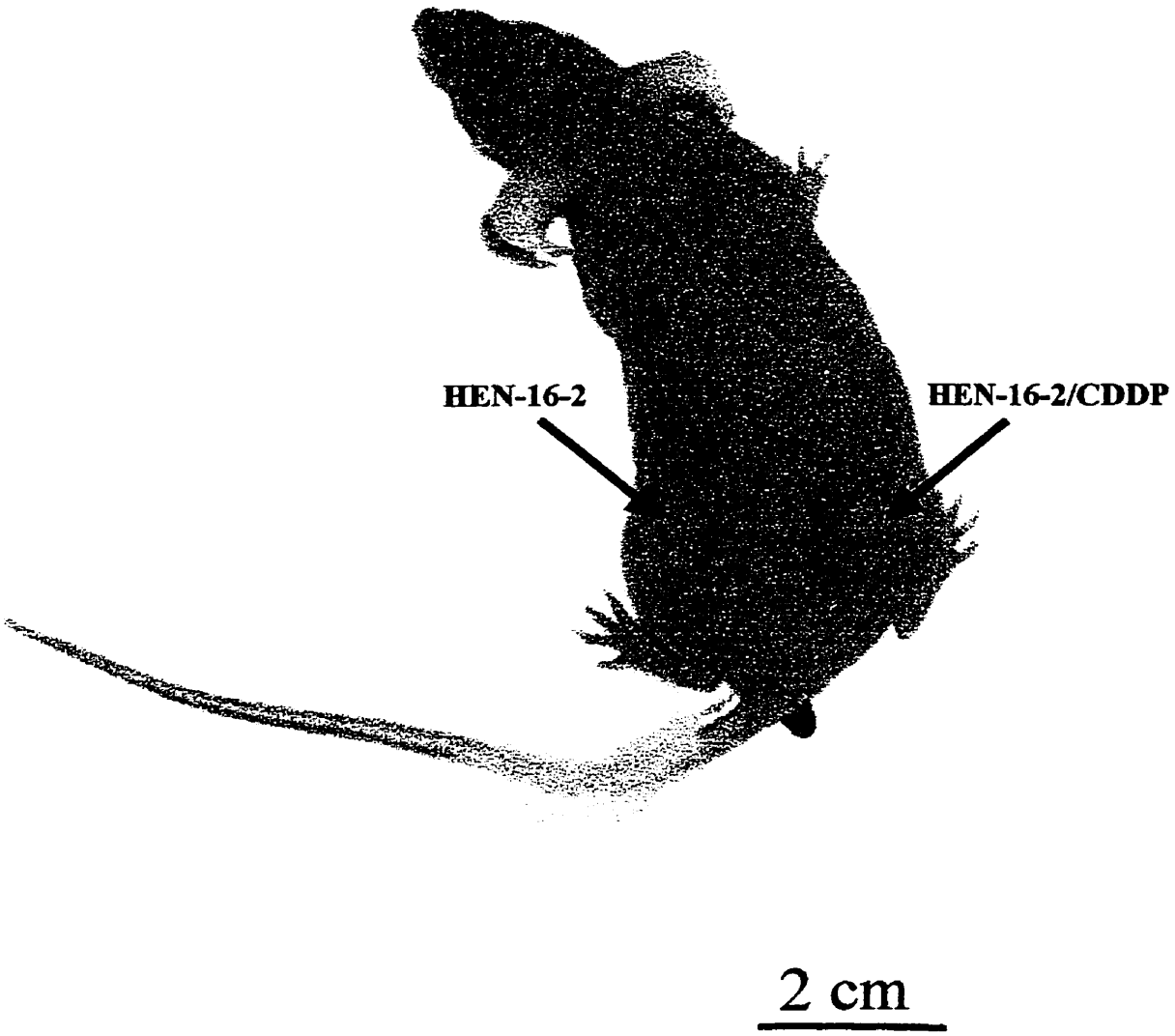


Figure 3.15. HEN-16-2/CDDP tumorigenesis on nude mouse.

substratum, high nucleus/cytoplasm ratio and high densities of chromtain staining (Figure 3.16).

3.6. Expression of apoptosis-related cellular genes

The levels of the apoptosis-promoting proteins p53, Bak, and Bax were examined by Western blot analysis. There was no significant difference in the levels of expression of p53, Bak, or Bax between HEN-16-2 and HEN-16-2/CDDP (Figure 3.17 and Table 3.3).

Table 3.3. Expression of apoptosis-regulating proteins in HEN-16-2/CDDP compared with HEN-16-2[1]

Protein	HEN-16-2	HEN-16-2/CDDP
p53	1.0 ± 0.3	0.8 ± 0.5
Bak	1.0 ± 0.1	0.9 ± 0.2
Bax	1.0 ± 0.3	1.0 ± 0.3
Bcl-2	1.0 ± 0.4	0.9 ± 0.3
Bcl-X_L	1.0 ± 0.1	1.7 ± 0.2*
BAG-1 p50	1.0 ± 0.2	22.9 ± 6.1*
BAG-1 p46	1.0 ± 0.1	1.2 ± 0.4
BAG-1 p33	1.0 ± 0.3	3.3 ± 0.8*

[1]The levels of cellular proteins were quantified relative to those of HEN-16-2 after normalization to actin control. Protein expression was quantified by measuring the optical density of bands at medium exposure on X-ray films. The data represent the mean $\pm$ the standard deviation of three experiments. *, $p < 0.05$, is the significance of the difference between the values for HEN-16-2/CDDP and HEN-16-2 cells.

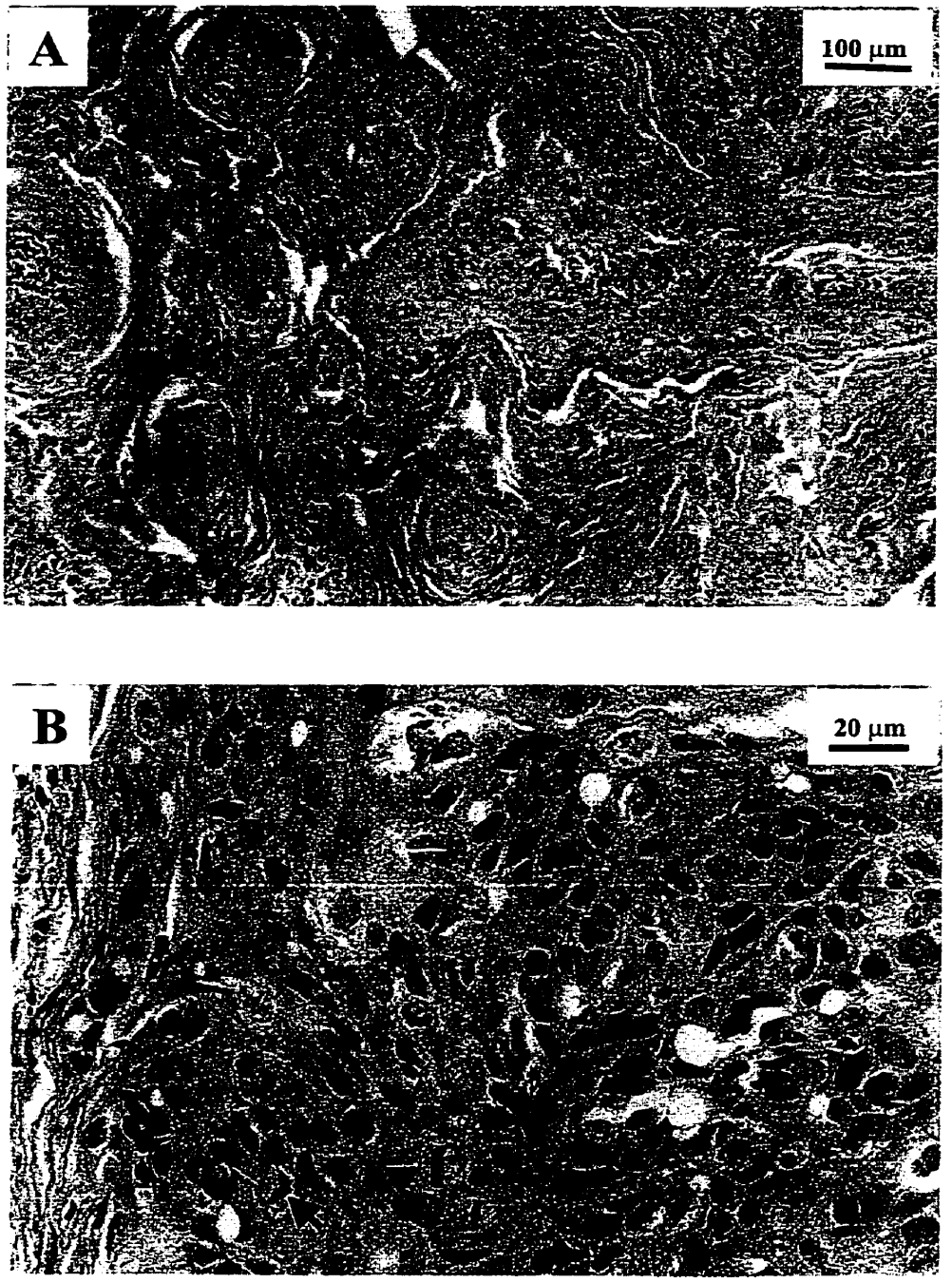


Figure 3.16. Histology of HEN-16-2/CDDP tumor. A. Squamous cell carcinomas formed in nude mice. B. Higher magnification showing dysplastic cells in a second tumor, demonstrating mitotic cells (arrowheads) proximal to the mouse substratum on the left.

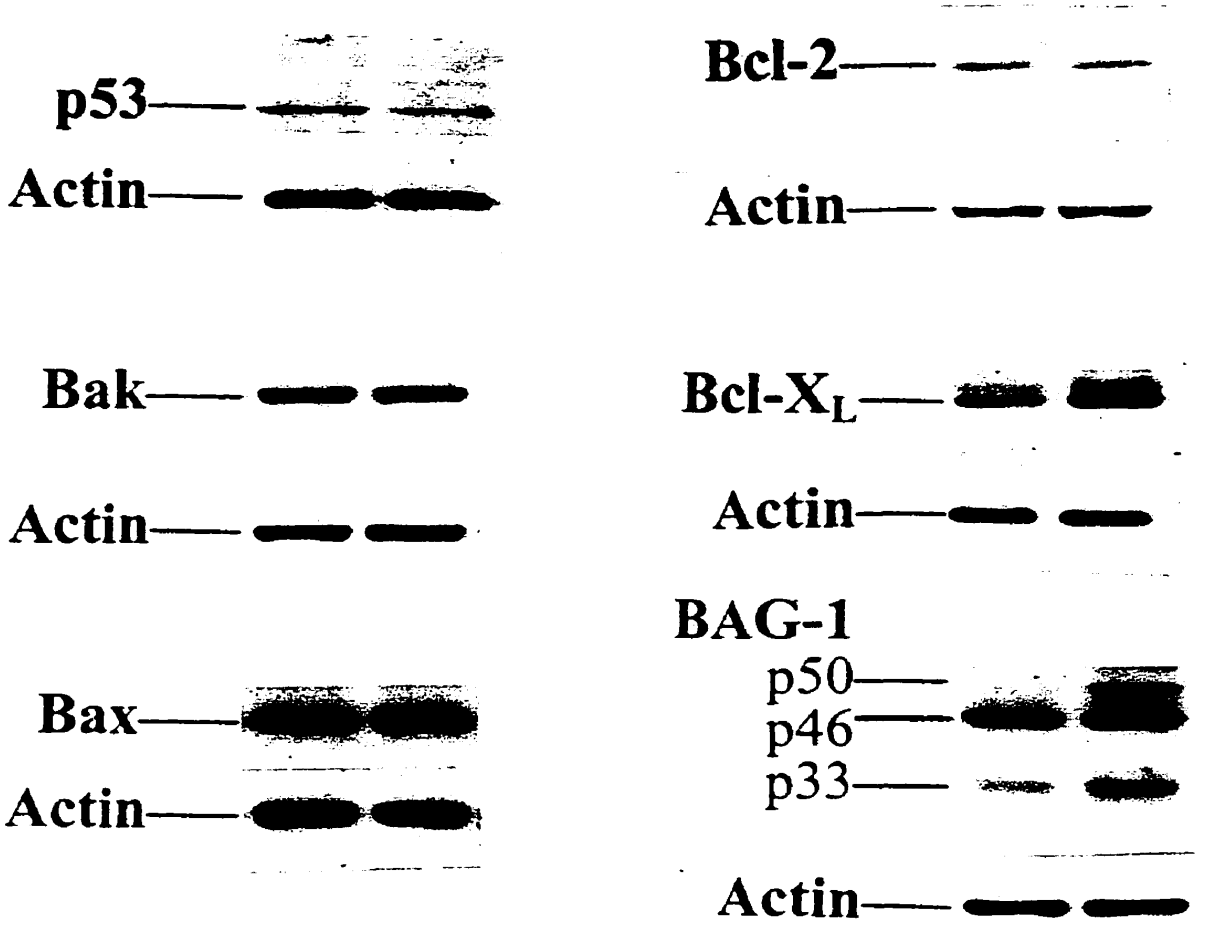


Figure 3.17. Expression of apoptosis-associated proteins in HEN-16-2/CDDP and HEN-16-2 cells. Western blot analysis is shown for HEN-16-2 (left lanes) and HEN-16-2/CDDP (right lanes). β-actin was an internal control.

The expression of the anti-apoptotic protein Bcl-2 and BAG-1 p46 isoform was not significantly different between HEN-16-2 and HEN-16-2/CDDP cells (Figure 3.17 and Table 3.3). However, there was a significantly ($p < 0.05$) higher (1.7-fold) level of the expression of Bcl-X_L in HEN-16-2/CDDP cells than in HEN-16-2 cells (Figure 3.17 and Table 3.3). The expressions of BAG-1 isoforms p50 and p33 were increased significantly ($p < 0.05$) from HEN-16-2 cells to HEN-16-2/CDDP cells, 22.9-fold and 3.3-fold respectively (Figure 3.17 and Table 3.3). The shortest isoform of BAG-1, p29, was not detectable in either of these two cell lines.

3.7. Effect of overexpressing BAG-1 in C33A cervical cells on the sensitivity to apoptotic stimuli

Having determined that a high-level of anti-apoptotic BAG-1 expression was associated with insensitivity of HEN-16-2/CDDP cells to different cytotoxic drugs and other apoptotic stimuli, a potential role of BAG-1 in the direct regulation of apoptosis in cervical cells was examined. The BAG-1 p50 stably transfected C33A cell line, C33A-BAG-1, was kindly provided by Dr. Xiaolong Yang (Yang *et al.*, 1999b). C33A-BAG-1 cells overexpressing BAG-1 (Figure 3.18) were found to be more resistant to cell death induced by cisplatin than C33A transfected with a control vector (C33A-NEO) (Figure 3.19). Similarly, C33A-BAG-1 cells were more resistant to cell death induced by doxorubicin (Figure 3.20) and etoposide (Figure 3.21A) than the control cells. Moreover, C33A-BAG-1 cells were found to have a higher survival rate when exposed to heat shock or UV (Figure 3.21B) than the control cells. However, parallel studies indicated that

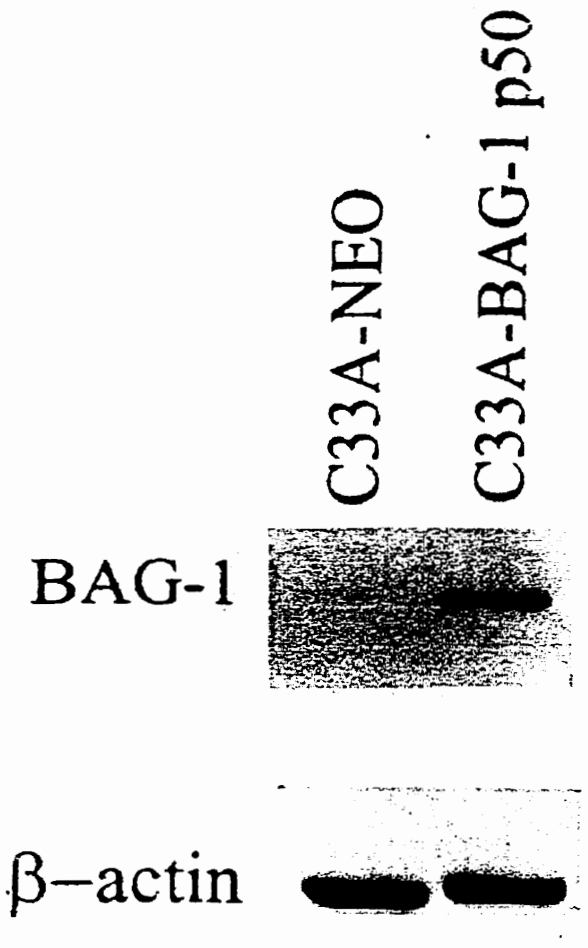
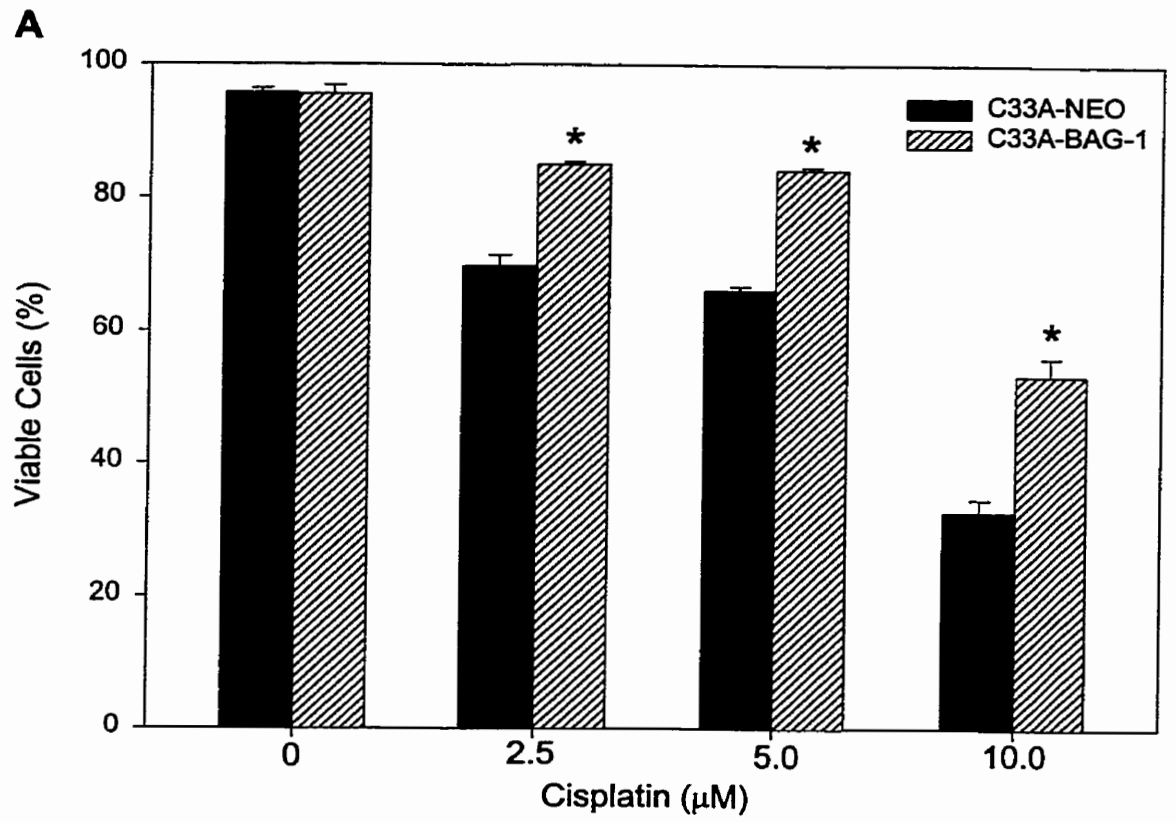


Figure 3.18. BAG-1 expression levels in C33A-BAG-1 versus C33A-NEO cells. β-actin was an internal control.

Figure 3.19. Effect of BAG-1 on dose- and time-dependent induction of apoptosis by cisplatin in C33A-BAG-1 versus C33A-NEO cells. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean ± the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between stably transfected NEO control cells and full-length BAG-1 overexpressing cells. A. Dose-dependence. Cells were treated with the indicated concentrations of cisplatin for 48 h. B. Time-dependence. Cells were exposed to 5 μM cisplatin for the indicated periods.


A
100
80
60
40
20
0
Viable Cells (%)
C33A-NEO
C33A-BAG-1
*
*
*
0
2.5
5.0
10.0
Cisplatin (μM)

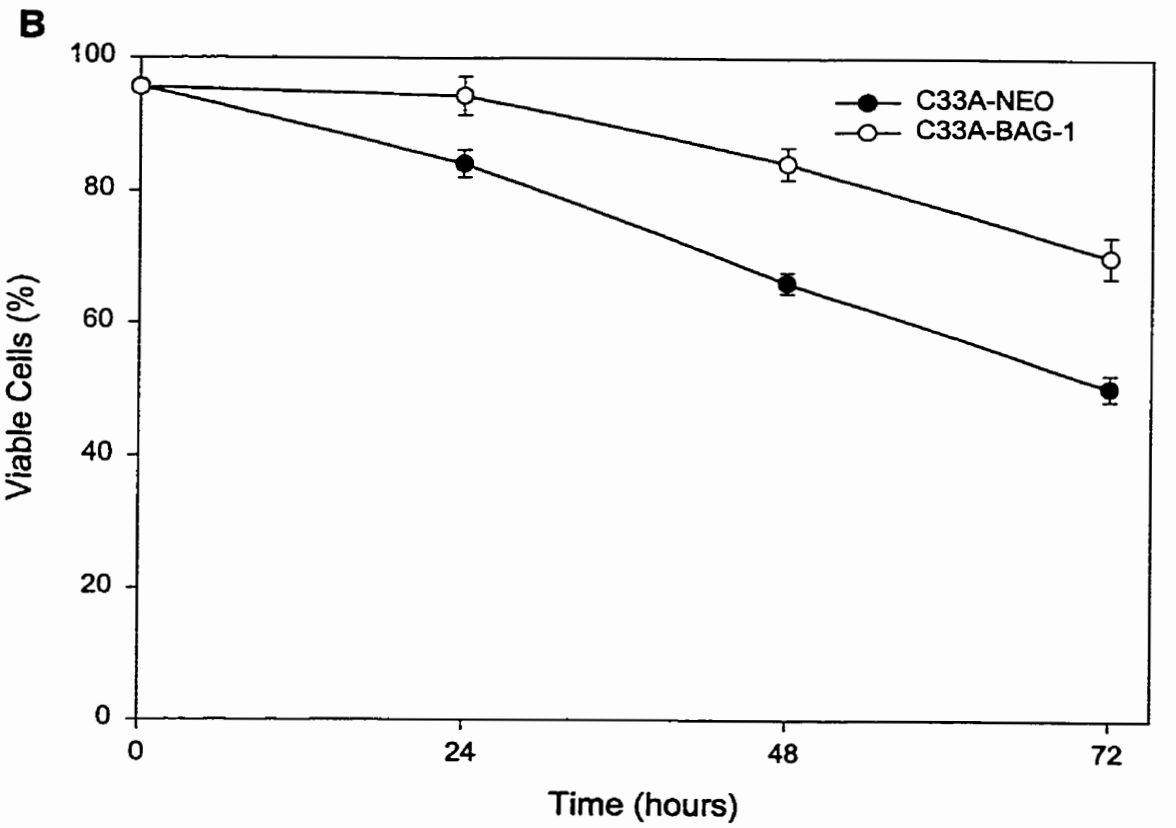

B
100
80
60
40
20
0
Viable Cells (%)
C33A-NEO
C33A-BAG-1
0
24
48
72
Time (hours)

Figure 3.20. Effect of BAG-1 on dose- and time-dependent induction of apoptosis by doxorubicin in C33A-BAG-1 versus C33A-NEO cells. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean ± the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between stably transfected NEO control cells and full-length BAG-1 overexpressing cells. A. Dose-dependence. Cells were treated with the indicated concentrations of doxorubicin for 48 h. B. Time-dependent. Cells were exposed to 1 μM doxorubicin for the indicated periods.

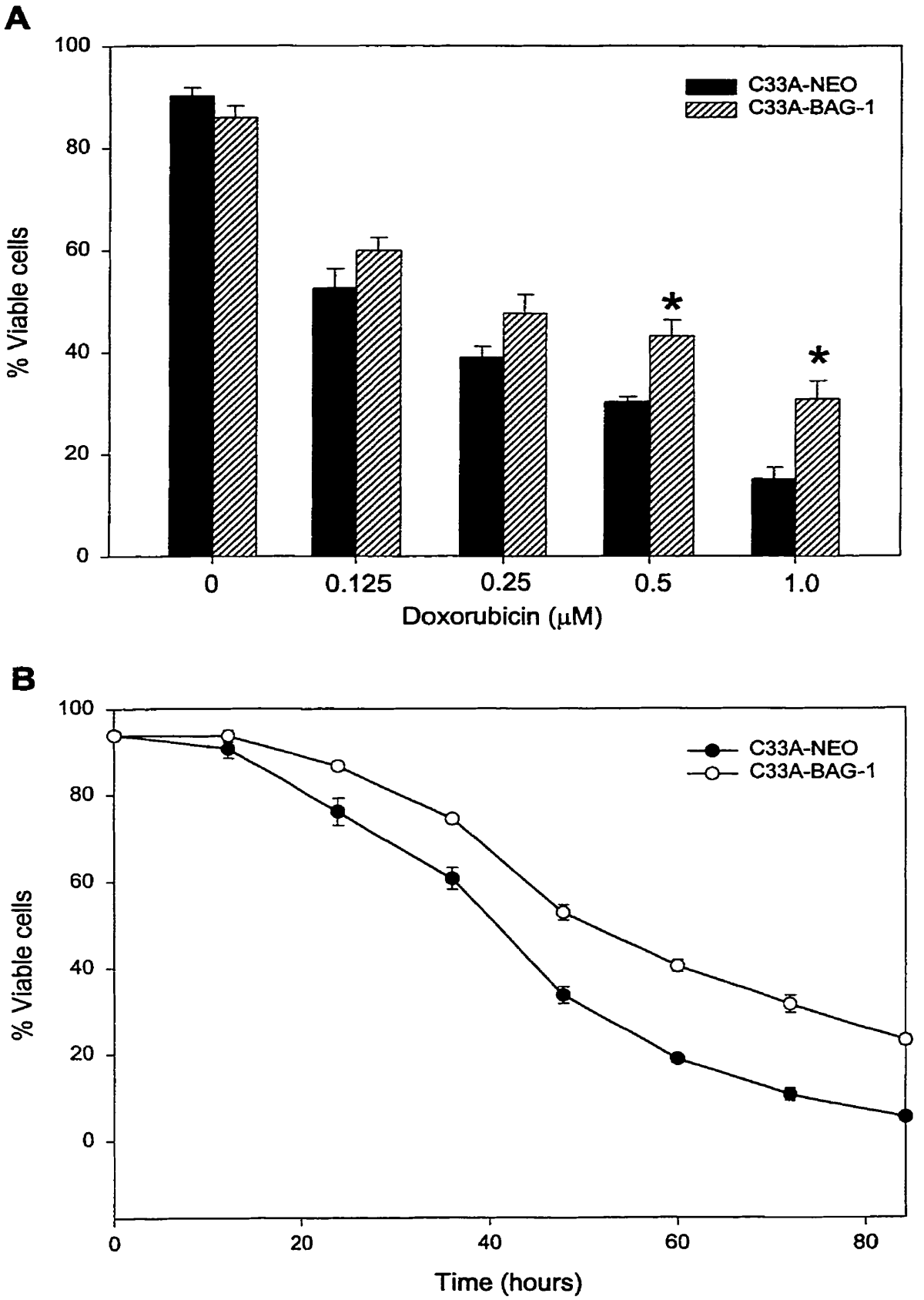
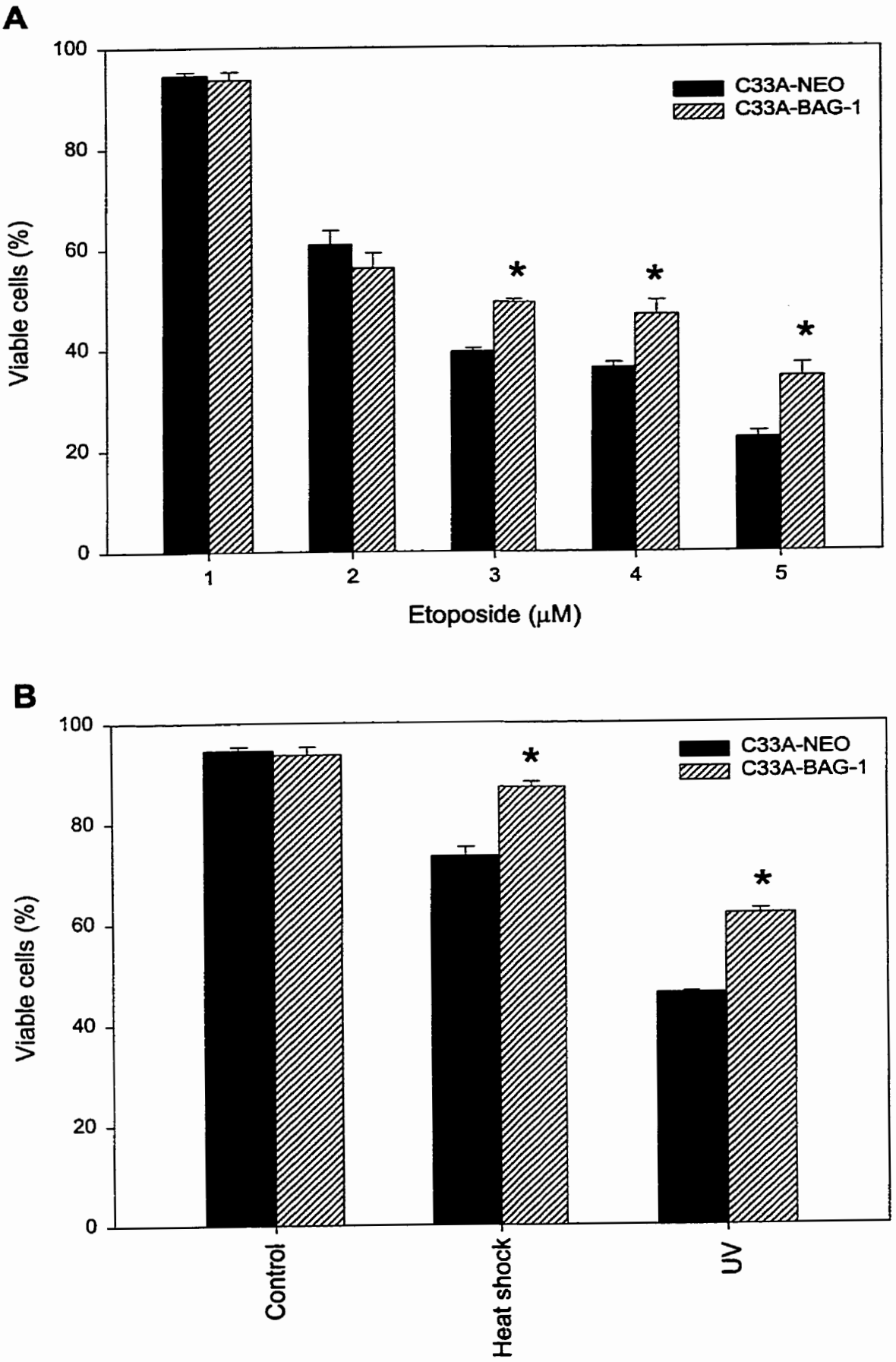

A
100
80
60
40
20
0
% Viable cells
C33A-NEO
C33A-BAG-1
*
*
0
0.125
0.25
0.5
1.0
Doxorubicin (μM)
B
100
80
60
40
20
0
% Viable cells
C33A-NEO
C33A-BAG-1
0
20
40
60
80
Time (hours)

Figure 3.21. Effect of BAG-1 on induction of apoptosis by etoposide, UV irradiation and heat shock in C33A-BAG-1 versus C33A-NEO cells. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean ± the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between stably transfected NEO control cells and full-length BAG-1 overexpressing cells. A. Dose-dependent induction of apoptosis by etoposide. Cells were treated with the indicated concentrations of etoposide for 48 h. B. Induction of cell death by UV irradiation and heat shock. Cells were subjected to heat shock at 45 °C for 45 minutes or 50 mJ/cm^2 UV irradiation, and then were returned to normal culture for 48 hours.


A
100
80
60
40
20
0
Viable cells (%)
C33A-NEO
C33A-BAG-1
*
*
*
1
2
3
4
5
Etoposide (μM)
B
100
80
60
40
20
0
Viable cells (%)
C33A-NEO
C33A-BAG-1
*
*
Control
Heat shock
UV

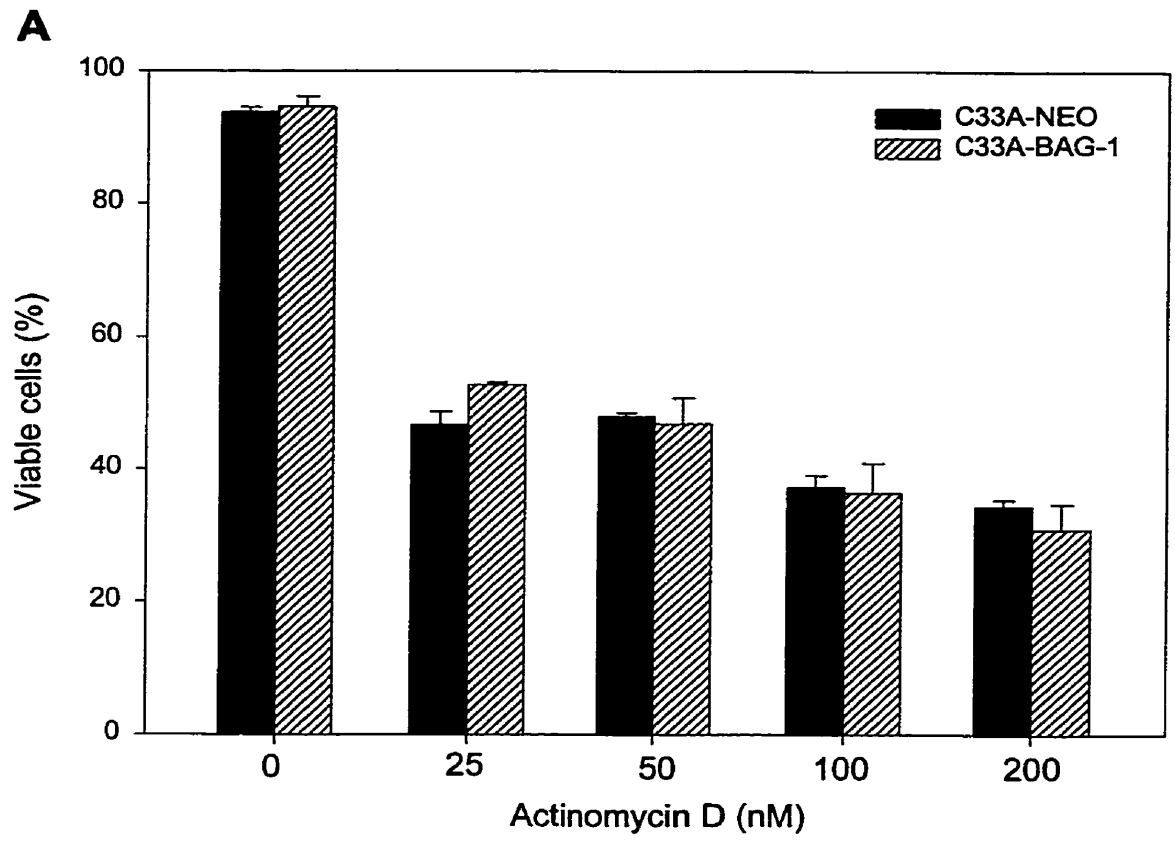
overexpression of BAG-1 failed to protect the cervical C33A cells from apoptosis induced by actinomycin D or paclitaxel (Figure 3.22).

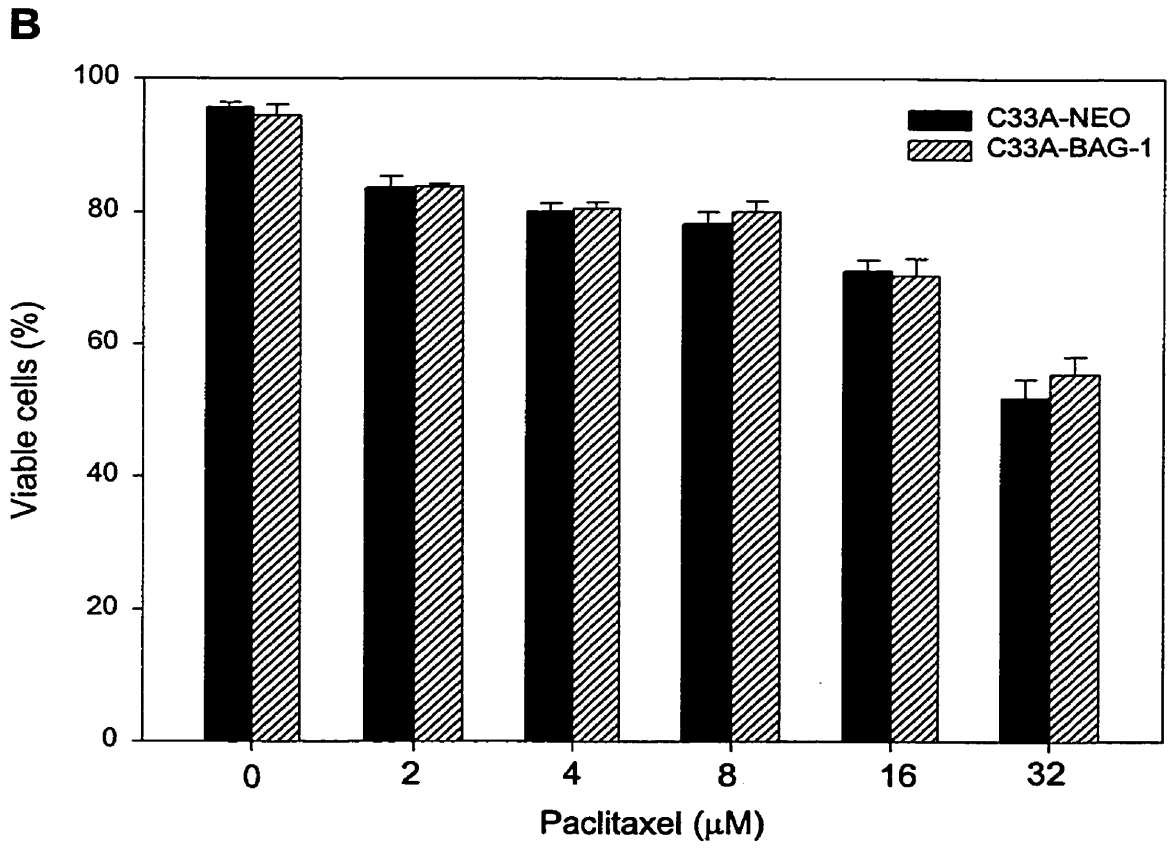
3.8. Isolation and identification of BAG-1-interacting proteins

Previous studies have indicated that BAG-1 interacts with various proteins and modulates their functions (see section 1.4; Figure 1.7). It appears that BAG-1 plays an important role in signal transduction pathways in apoptosis. However, how BAG-1 accomplishes these multiple functions is still unclear. To further characterize BAG-1 and understand its role in regulating apoptosis, screening a human keratinocyte cDNA library was conducted to clone novel BAG-1-interacting proteins using the yeast two-hybrid system.

Because BAG-1 full-length (p50) had not been cloned in the laboratory before the cDNA library screening, BAG-1 p46 isoform was used as bait for interacting proteins in the yeast two-hybrid system (Figure 3.23). 18 positive clones were isolated from approximately 2.5×10^6 yeast transformants. After verification of 17 positive clones (Figure 3.24), both strands of the cDNA obtained were sequenced and analyzed for sequence homology through the Basic Alignment Search Tool (BLAST) in the National Center for Biotechnology Information Sequence Databases. One 2406 bp and two 1822 bp cDNA sequences obtained from positive clones were found to be *Hsp70* (Figure 3.25A and Table 3.4). Two 2589 bp cDNA sequences obtained from positive clones was found to be *Hsp70-2* (Figure 3.25B and Table 3.4). One 1832 bp and ten 1530 bp cDNA sequences obtained from positive clones were found to be *Hsc70* pseudogene (Figure

Figure 3.22. Effect of BAG-1 on induction of apoptosis by actinomycin D and paclitaxel in C33A-BAG-1 versus C33A-NEO cells. Cells were seeded and incubated with the indicated concentrations of actinomycin D or paclitaxel for 48 hours. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean $\pm$ the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between stably transfected NEO control cells and full-length BAG-1 overexpressing cells. A. By actinomycin D. B. By paclitaxel.


A
100
80
60
40
20
0
Viable cells (%)
C33A-NEO
C33A-BAG-1
0
25
50
100
200
Actinomycin D (nM)


B
100
80
60
40
20
0
Viable cells (%)
C33A-NEO
C33A-BAG-1
0
2
4
8
16
32
Paclitaxel (μM)

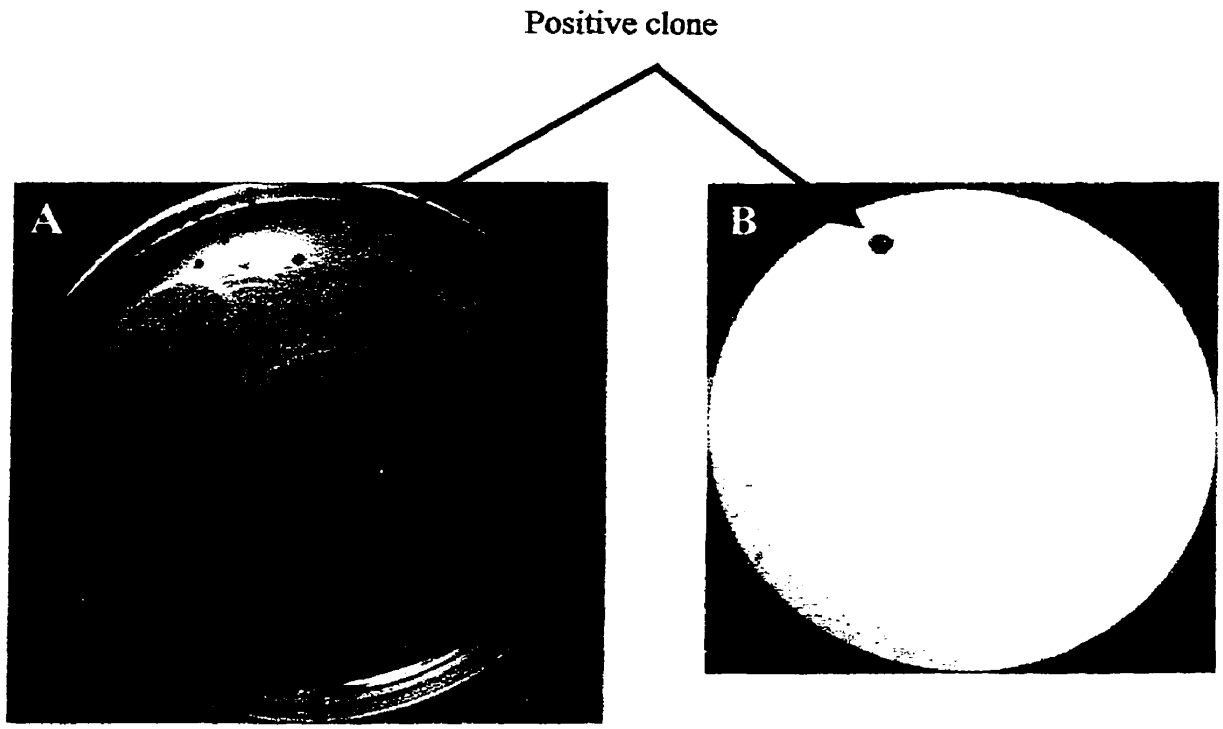


Figure 3.23. Representative result of screening and identifying possible BAG-1-interacting-protein positive clones from the yeast two hybrid system. A. Yeast colones on SD/-Trp/-Leu/-His agar plate. B. β-galatosidase filter lift assay result (blue color) for the same colony indicated on A and B by arrows.

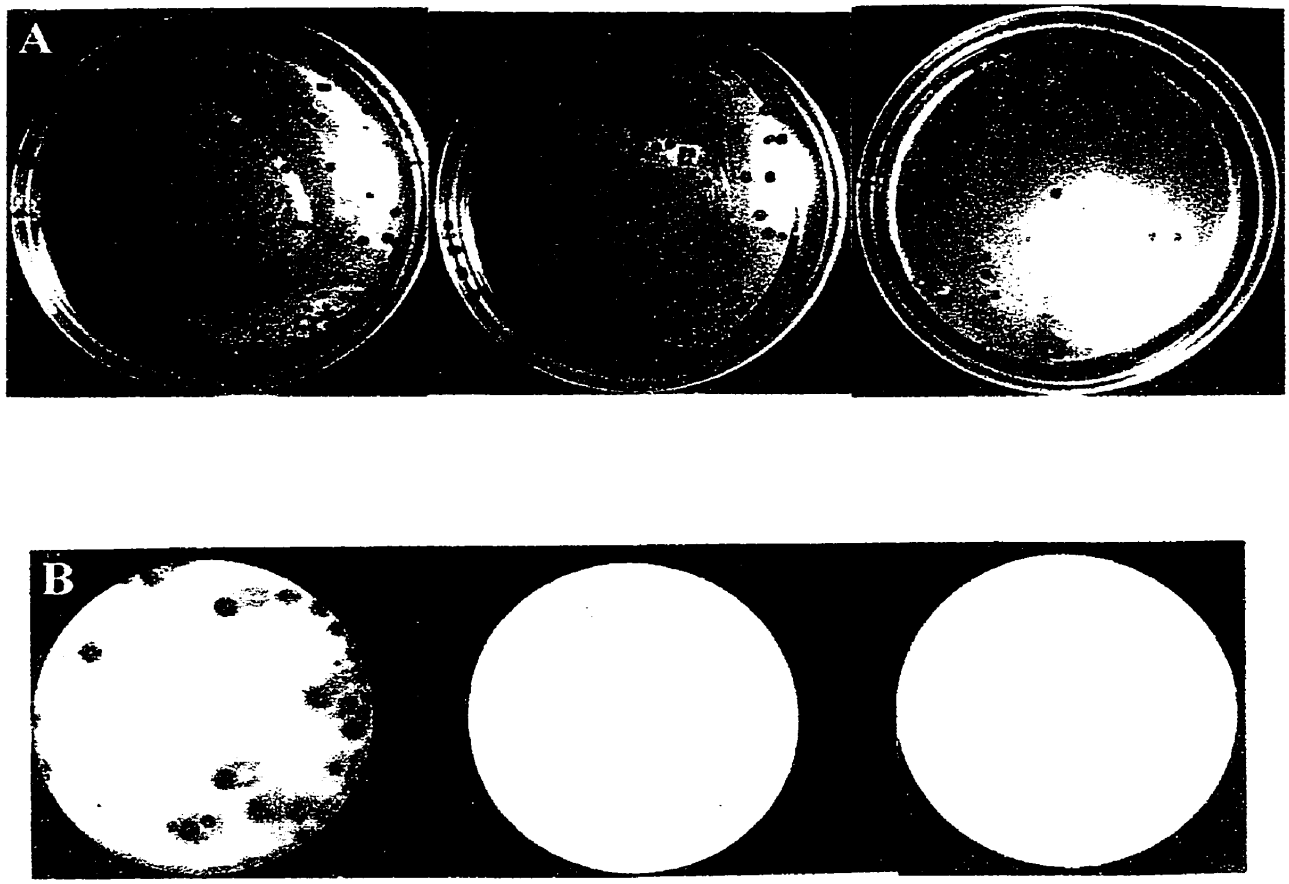


Figure 3.24. Representative results of verification of BAG-1-interacting-protein positive clones from the yeast two hybrid system. BAG-1 was the bait and lamin and pAS2-1 vector plasmids was negative controls to verify the specificity of the interaction between BAG-1 and Hsp70Y. A. Yeast colones on SD/-Trp/-Leu/-His agar plates. B. β-galatosidase filter lift assay result.

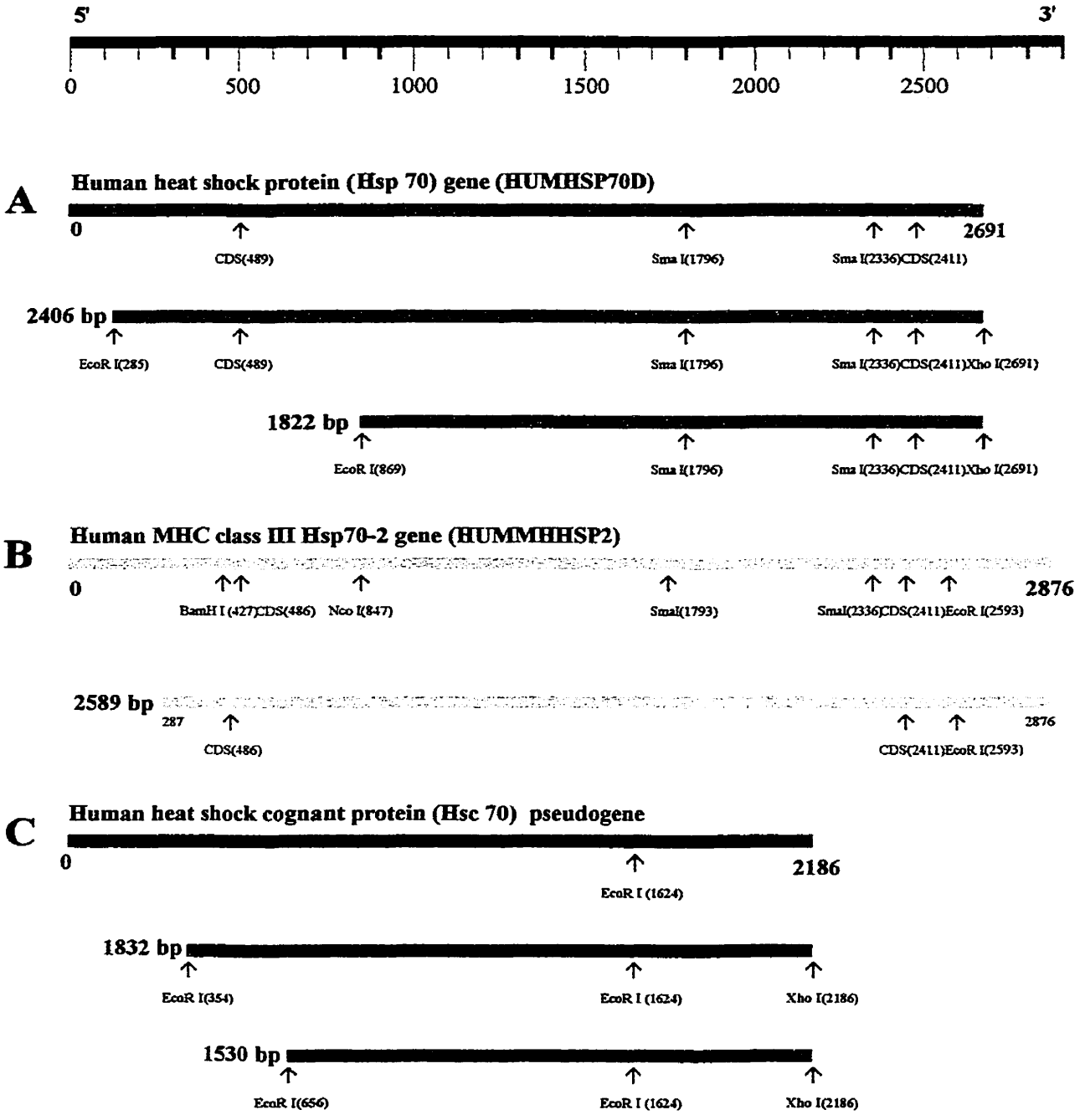


Figure 3.25. BAG-1-interacting-protein cDNAs clones indentically to previously known sequences. A. Hsp70 (top) and two cDNA clones (bottom). B. Hsp70-2 (top) and a cDNA clone . C. Hsc70 pseudogene (top) and two cDNA clones (bottom).

3.25C and Table 3.4). Furthermore, one 5340 bp cDNA sequence was found to be identical to Hsc70 at the both 5' and 3' ends. However, the total length of the cDNA is much longer than Hsc70 mRNA, indicating that this cDNA may be different from *Hsc70* and any other *Hsp70* genes. Therefore, this 5340 bp cDNA was a candidate novel heat shock protein (Hsp) gene cDNA and arbitrarily named *Hsp70Y* (Figure 3.26 and Table 3.4).

Table 3.4. Positive BAG-1-interacting cDNA clones identified from Keratinocyte MATCHMAKE cDNA library using yeast two-hybrid system

Identity	Clone numbers	Size (bp)
Hsp70	1	2406
	2	1822
Hsp70-2	2	2589
Hsc70 pseudogene	1	1832
	10	1530
Hsp70Y	1	5340

3.9. Mutation analysis of BAG-1 functional domain for interaction with Hsp70 and Hsp70-2

3.9.1. *In vitro* interaction

The BAG-1 domains involved in the interaction of BAG-1 with Hsp70 and Hsp70-2 were characterzed using *in vitro* protein binding assays. GST-Hsp70 and GST-Hsp70-2

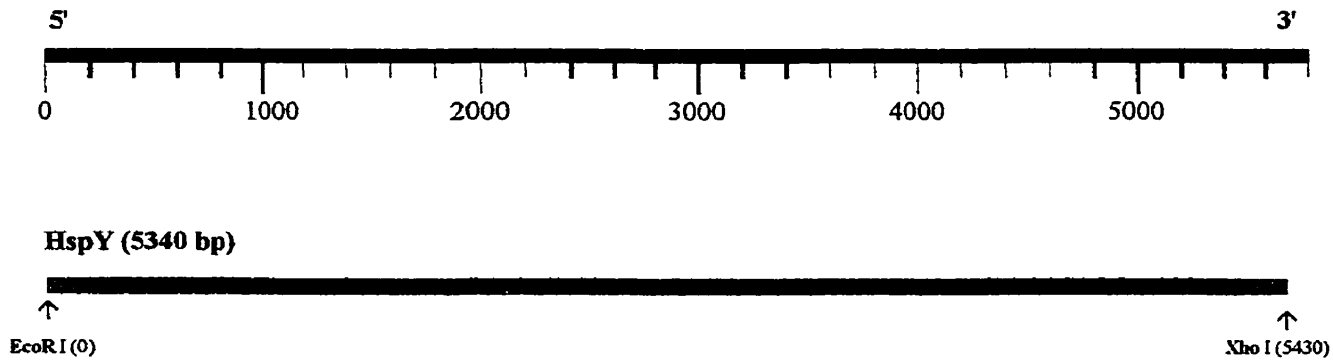


Figure 3.26. A candidate novel heat shock protein family member identifed as BAG-1-interacting-protein.

were prepared (Figure 3.27). GST-BAG-1 wild-type and mutated fusion proteins were kindly provided by Dr. Xiaolong Yang. ^{35}S-labeled *in vitro* transcribed/translated (IVT) proteins were prepared (Figure 3.28A and B). GST-fusion proteins immobilized on glutathione sepharose were tested for binding to ^{35}S-labeled IVT proteins. IVT-Hsp70 or IVT-Hsp70-2 bound *in vitro* to GST-BAG-1 p46 (amino acids 72 to 345), GST-BAG-1 p33 (amino acids 116 to 345), GST-BAG-1 p29 (amino acids 139 to 345), GST-BAG-1ΔN4 (amino acids 200 to 345), but not to GST-BAG-1ΔC1 (amino acids 72 to 320), GST-BAG-1ΔC2 (amino acids 72 to 219), GST-BAG-1ΔC3 (amino acids 72 to 156), or GST negative controls (Figure 3.29 and 3.30). Similarly, GST-Hsp70, or GST-Hsp70-2 bound to IVT-BAG-1 p50 (amino acids 1 to 345) but not to GST negative control (Figure 3.31). These results indicated that all of the four isoforms of BAG-1 are able to interact with Hsp70 or Hsp70-2 and the carboxyl-terminal 145 amino acids (amino acids 200 to 345) are important in the mediation of the interaction.

Hsp70 family members contain two major domains: the amino-terminal 44 kDa ATPase domain and carboxyl-terminal domain (Sriram *et al.*, 1997). To determine whether the Hsp70 ATPase domain was responsible for the interaction between BAG-1 and Hsp70, sequences encoding this Hsp70 ATPase domain were generated by PCR and inserted into the expression vector pCR3.1. IVT-Hsp70 ATPase domain was found to interact with GST-BAG-1 p50 (Figure 3.31).

3.9.2. *In vivo* interaction

The yeast two-hybrid system is highly sensitive, and can be used to assay the

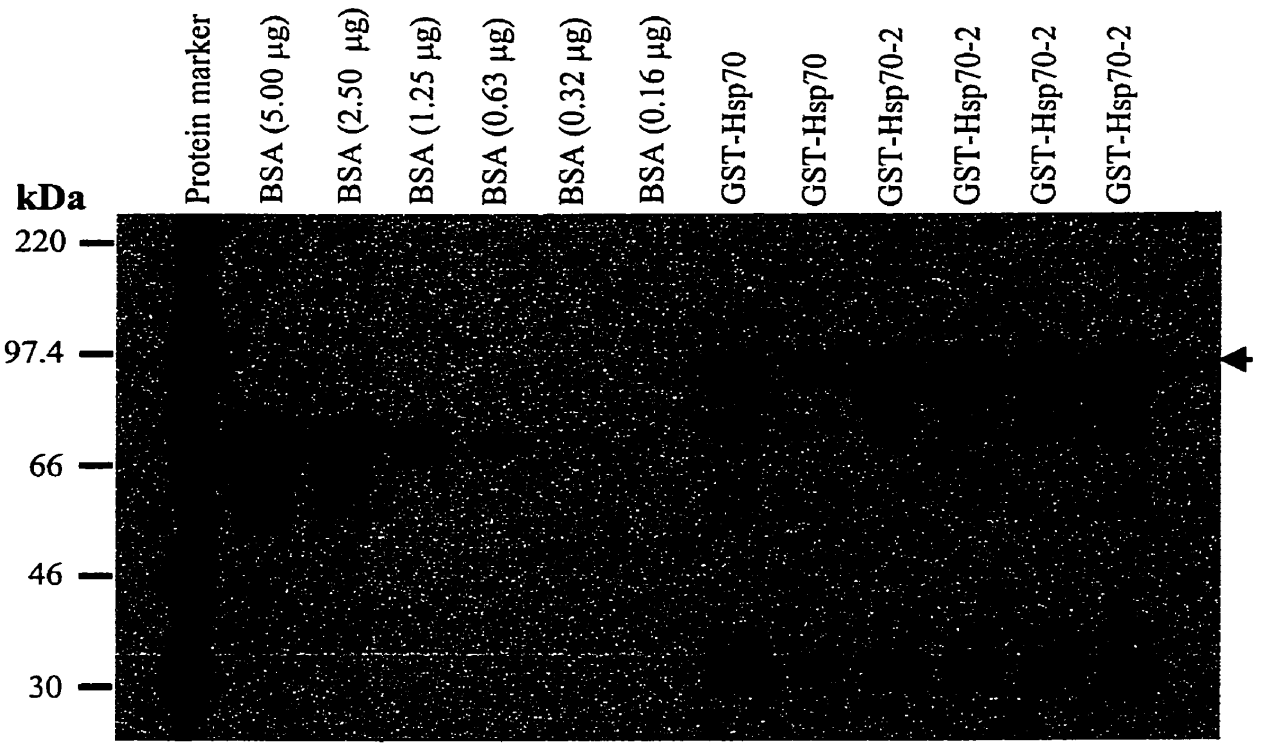


Figure 3.27. GST-Hsp70 and GST-Hsp70-2 preparation. Bovine serum albumin (BSA) was used to determine the concentration of GST-Hsp70 or GST-Hsp70-2. A Coomassie Brilliant Blue R250 stained SDS-polyacrylamide gel is shown.

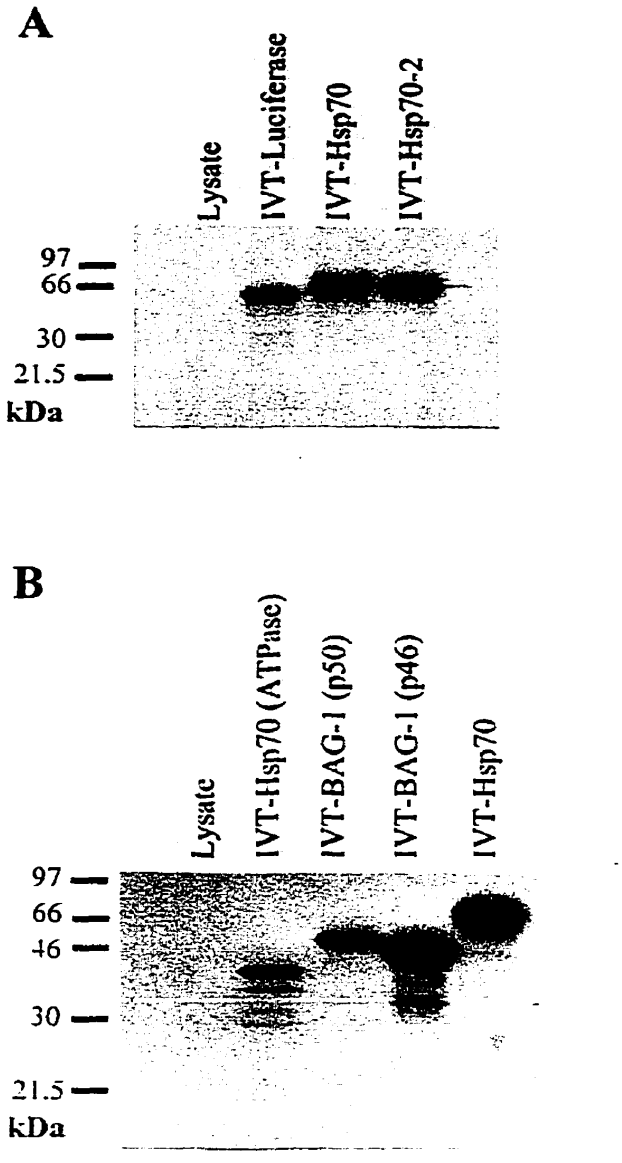


Figure 3.28. *In vitro* transcription/translation of proteins used in BAG-1-Hsp70s *in vitro* interaction assays. A. *In vitro* transcripted/translated Hsp70 (IVT-Hsp70) and Hsp70-2 (IVT-Hsp70-2). Lysate was loaded as a negative control, and IVT-luciferase was positive control. B. *In vitro* transcripted/translated ATPase domain of Hsp70 [IVT-Hsp70 (ATPase)], BAG-1 p50 [IVT-BAG-1 (p50)], and BAG-1 p46 [IVT-BAG-1 (p46)]. Lysate was loaded as a negative control, and IVT-Hsp70 was positive control.

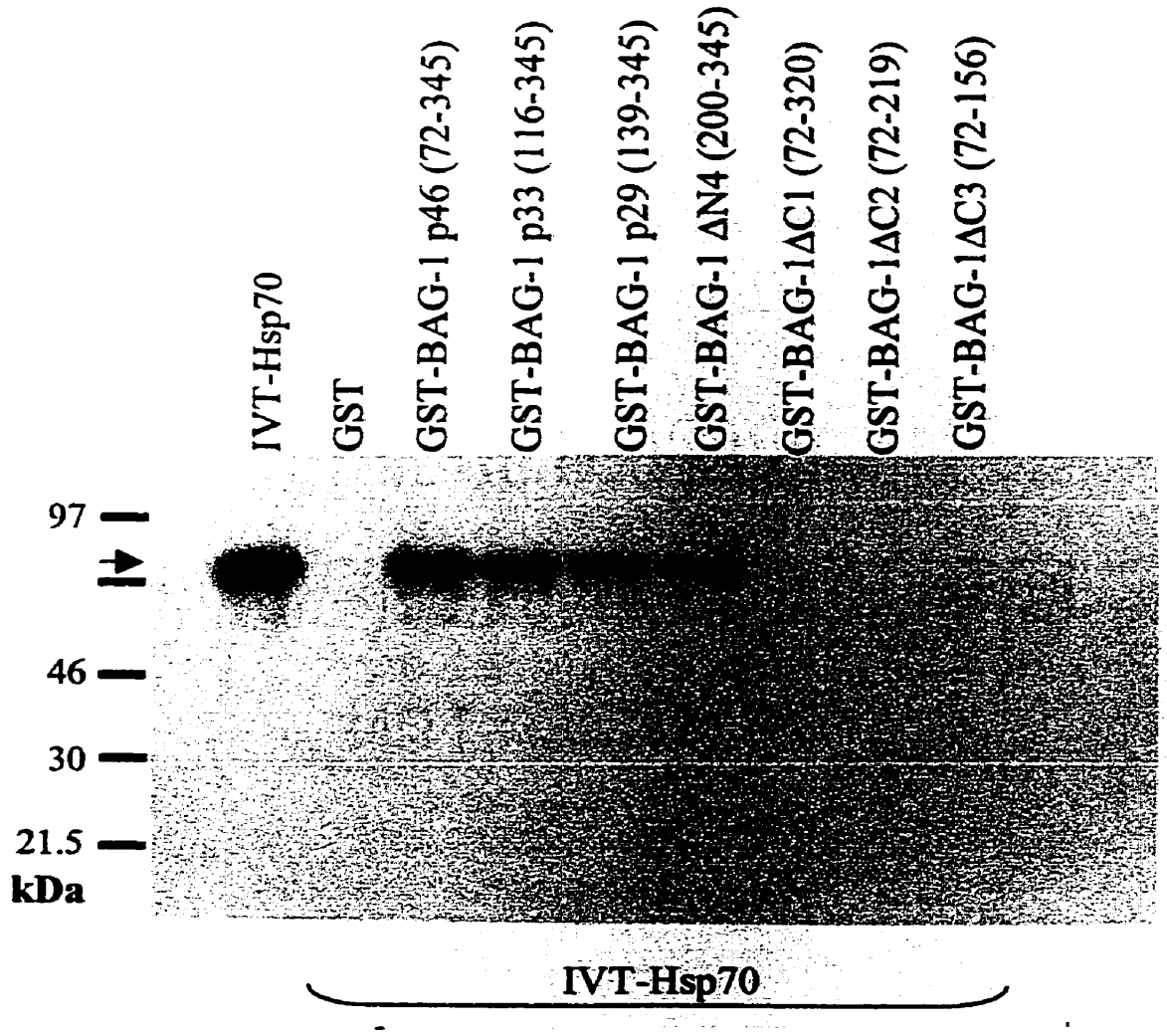


Figure 3.29. *In vitro* assay of interaction between Hsp70 and BAG-1. GST protein was used as a negative control. IVT-Hsp70 was directly loaded as positive control.

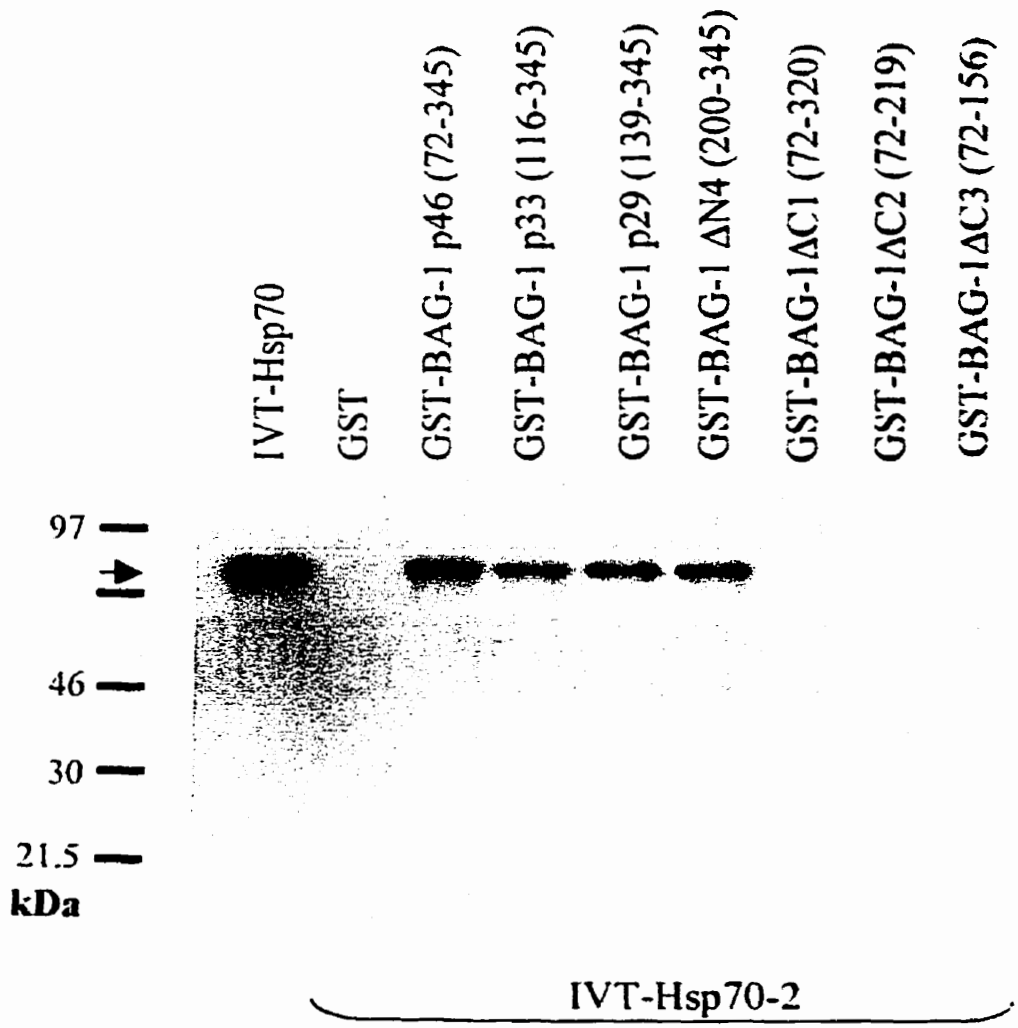


Figure 3.30. *In vitro* assay of interaction between Hsp70-2 and BAG-1. GST protein was used as a negative control. INV-Hsp70 was directly loaded as positive control.

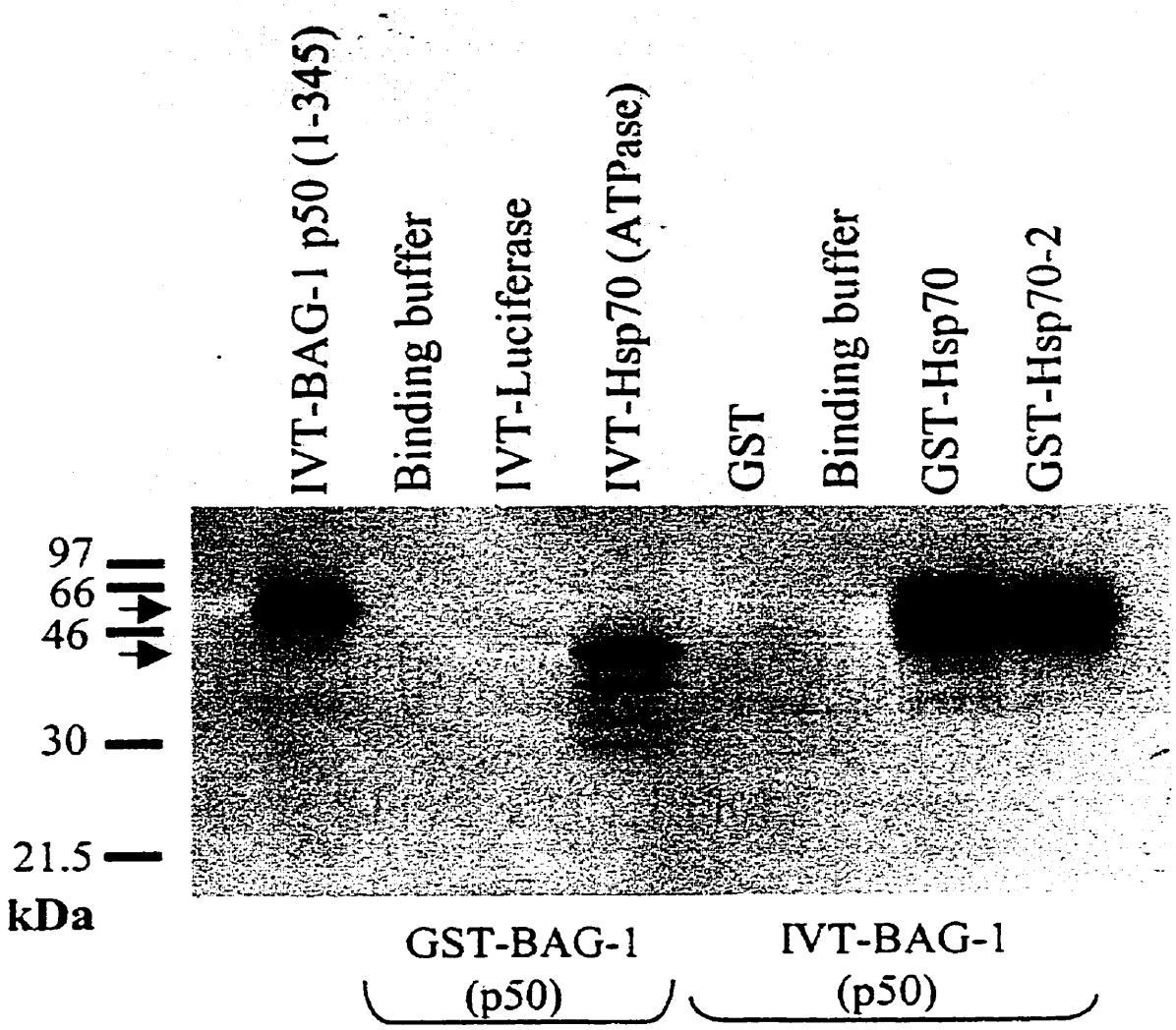


Figure 3.31. *In vitro* assay of interaction between BAG-1 and Hsp70 ATPase domain. Binding buffer, IVT-Luciferase, or GST protein were used as negative controls. IVT-BAG-1 p50 was directly loaded as positive control.

interaction of short peptides with proteins. Moreover, the assays are performed in eukaryotic yeast cells, which produce proteins with a conformation more closely resembling human proteins than those produced in the bacterial system. To further characterize the structural domain of BAG-1 responsible for the interaction between BAG-1 and Hsp70 or Hsp70-2, BAG-1 p50, p46, p33 and p29 isoforms, and a series of nested BAG-1 deletion mutations were generated by PCR (Figure 3.32) and inserted in-frame with the GAL4 BD to the pAS2-1 plasmid. The interaction of the different isoforms and deletion mutations of BAG-1 with Hsp70 was detected by the yeast two-hybrid system (Figure 3.33). Table 3.5 summarizes the interactions in the yeast two-hybrid system. These results indicated that the carboxyl-terminal 30 amino acids from 315 to 345 are responsible for the interaction between BAG-1 and Hsp70 or Hsp70-2.

3.10. Effect of BAG-1 on Hsp70s-mediated protein refolding activity *in vivo*

To examine whether the interaction of BAG-1 with Hsp70 chaperones affects Hsp70 chaperone-mediated protein refolding function, C33A-BAG-1 and C33A-NEO cells were transiently transfected with plasmids expressing luciferase and β-galactosidase. The luciferase and β-galactosidase expressing cells were subjected to heat-shock to partially inactivate luciferase and β-galactosidase enzymatic activity. After returning the cells to 37 °C for 30 minutes, luciferase activity in C33A-BAG-1 cells was 1.68 fold compared to that before recovery. After 60 minutes, luciferase activity was 1.83 fold that prior to recovery. In comparison, luciferase activity in C33A-NEO cells was 1.37 fold and 1.42 fold at 30 minutes and 60 minutes after recovery. Thus,

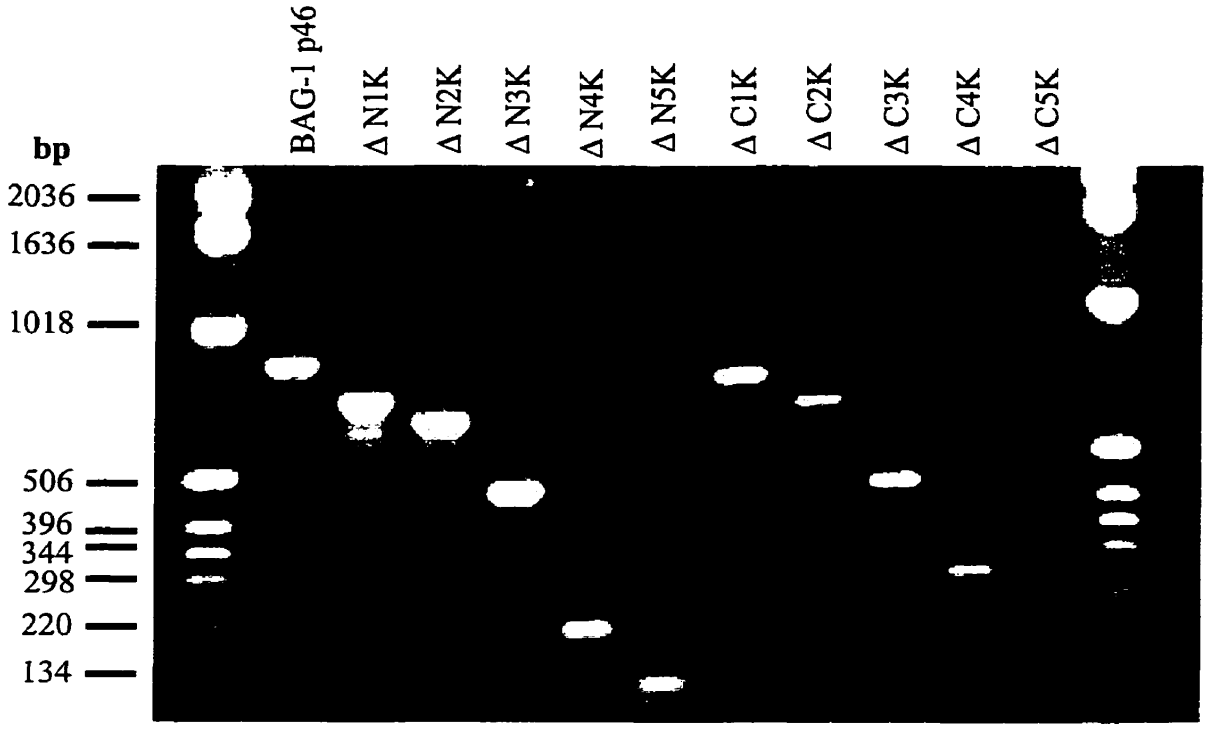


Figure 3.32. Analysis of PCR products of BAG-1 isoforms and deletion mutants. An ethidium bromide-stained agarose gel is shown.

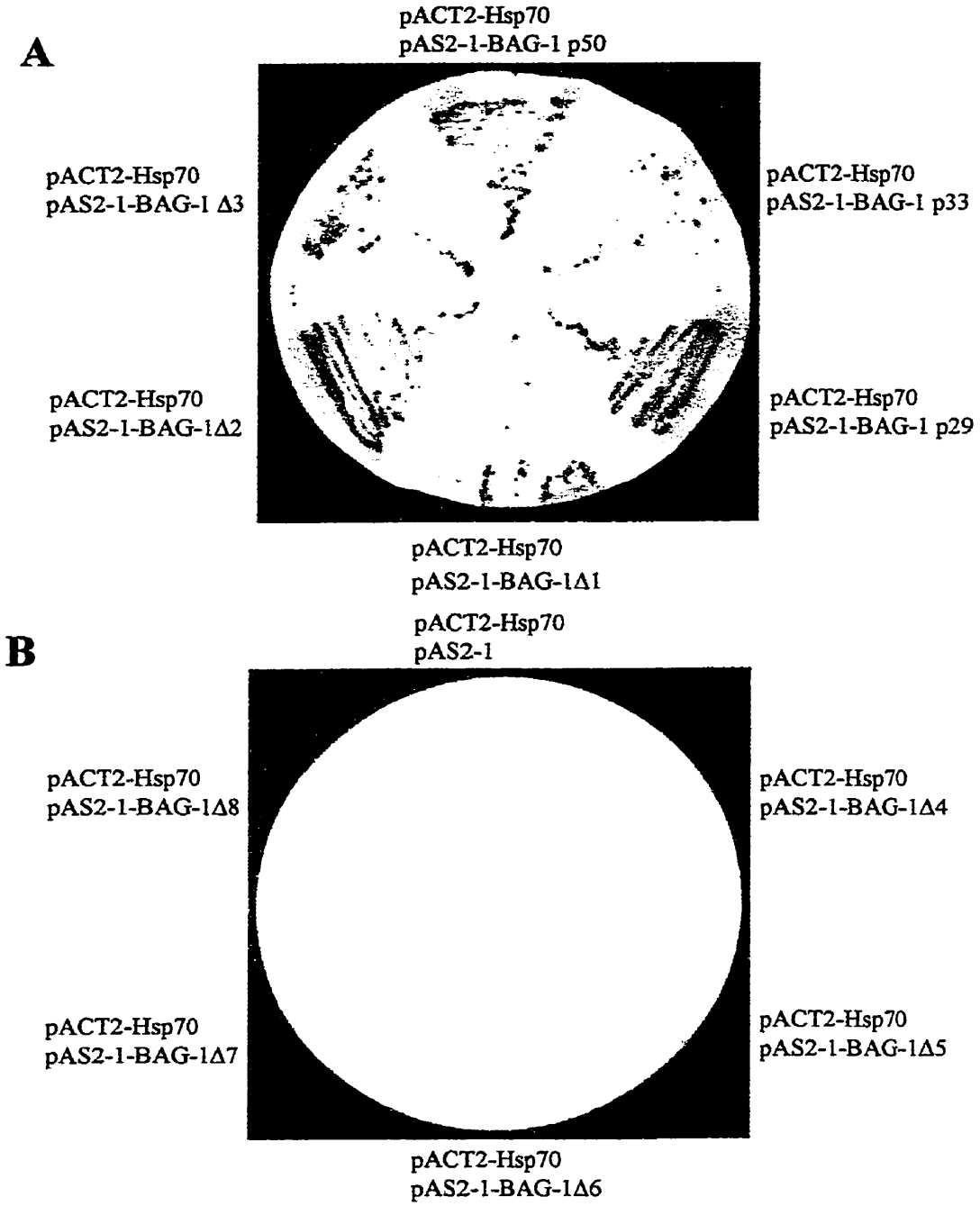


Figure 3.33. *In vivo* interaction between BAG-1 deletion mutants and Hsp70. β-galactosidase filter lift assays. A. Interactions between Hsp70 and BAG-1 isoforms and BAG-1Δ1, Δ2 and Δ3 mutants were detected. B. No interaction between Hsp70 and BAG-1 Δ4 to Δ8 mutants was detected.

Table 3.5. Deletion mutation analysis of the functional domain of human BAG-1 binding with Hsp70 or Hsp70-2 in the yeast two-hybrid system

Plasmid (amino acids)	-	**pACT2**	**pACT2-Hsp70**	**pACT2-Hsp70-2**
-	ND	-	-	-
pAS2-1 (-)	-	-	-	-
pAS2-1-BAG-1 p50 (1-345)	-	-	+	+
pAS2-1-BAG-1 p46 (72-345)	-	-	+	+
pAS2-1-BAG-1 p33 (116-345)	-	-	+	+
pAS2-1- BAG-1 p29 (139-345)	-	-	+	+
pAS2-1-BAG-1Δ1 (200-345)	-	-	+	+
pAS2-1-BAG-1Δ2 (286-345)	-	-	+	+
pAS2-1-BAG-1Δ3 (315-345)	-	-	+	+
pAS2-1-BAG-1Δ4 (72-320)	-	-	-	-
pAS2-1-BAG-1Δ5 (72-291)	-	-	-	-
pAS2-1-BAG-1Δ6 (72-215)	-	-	-	-
pAS2-1-BAG-1Δ7 (72-156)	-	-	-	-
pAS2-1-BAG-1Δ8 (72-121)	-	-	-	-

overexpression of BAG-1 in C33A-BAG-1 cells (Figure 3.18) led to a small, but significant increase in luciferase refolding (Figure 3.34A). Similarly, the expression of BAG-1 led to a small but significant increase in the refolding of β-galactosidase, compared with that of C33A-NEO control cells (Figure 3.34B). β-Galactosidase activity in C33A-BAG-1 was found to recover 1.59 fold BAG-1 after refolding for 30 minutes and 1.90 fold after refolding for 60 minutes, whereas β-galactosidase activity in C33A-NEO recovered only 1.05 fold and 1.28 fold at 30 and 60 minutes, respectively (Figure 3.34B).

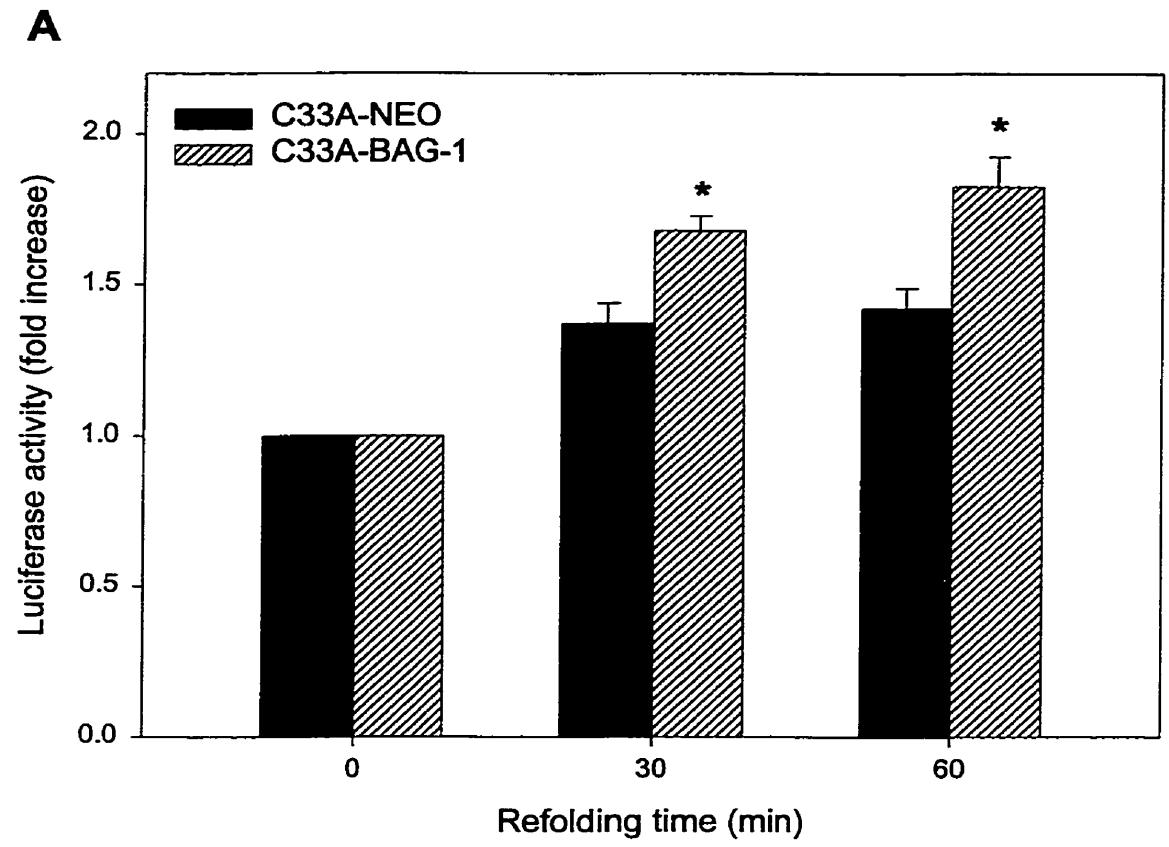
3.11. Effect of overexpression of Hsp70 or Hsp70-2 in cervical C33A cells on sensitivity to apoptotic stimuli

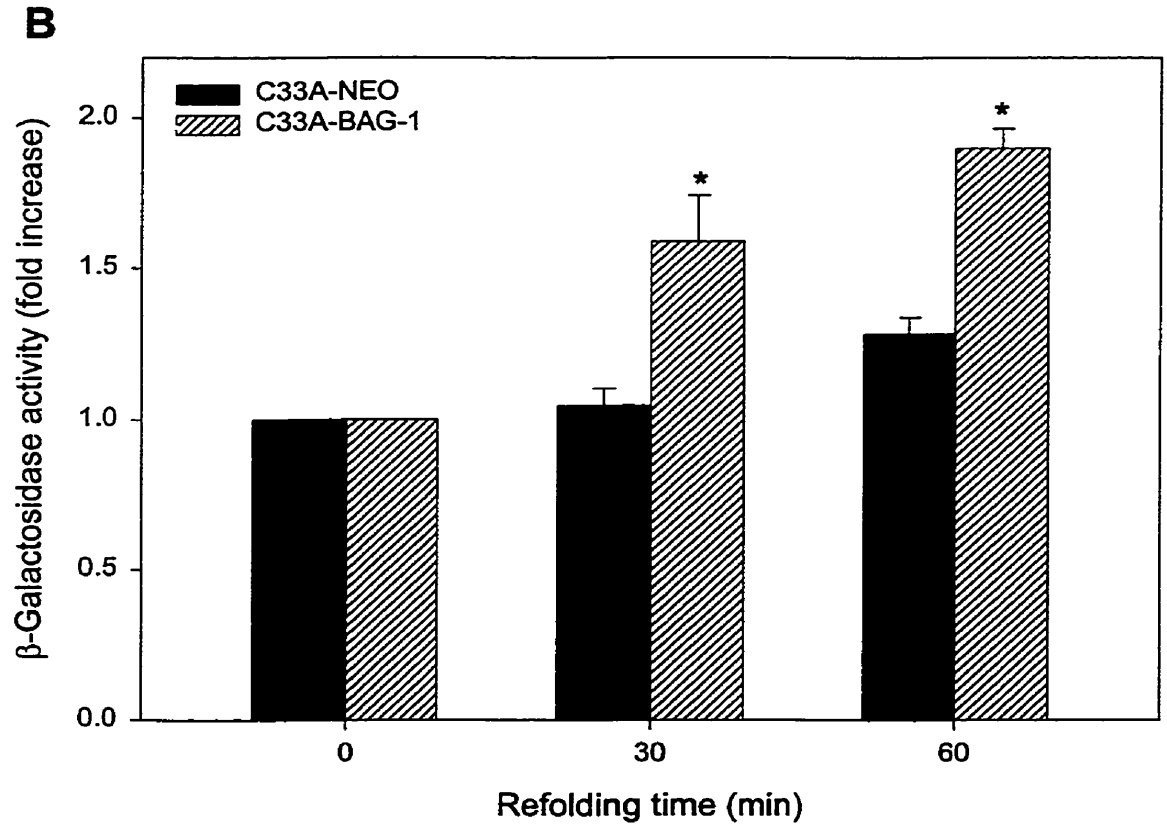
Although induction of hsp70 protein synthesis led to an enhancement of apoptosis in human leukemia cells (Chant *et al.*, 1996) and other cell types (Murdoch, 1995; Furlini *et al.*, 1994, Galea-Lauri *et al.*, 1996), Hsp70 chaperones are thought to play cytoprotective roles in most types of cells during times of stress by inhibiting apoptosis (Polla *et al.*, 1996).

To determine the role of Hsp70 in cervical cell apoptosis, C33A cells were transfected with a plasmid expressing Hsp70 (pCR3.1-Hsp70) or Hsp70-2 (pCR3.1-Hsp70-2), or the control plasmid (pCR3.1), and stable clones overexpressing Hsp70s were obtained (Figure 3.35).

When challenged with 45 minutes of heat shock at 45°C, C33A cells overexpressing Hsp70s displayed enhanced survival compared with control transfectants

Figure 3.34. Effect of BAG-1 on *In vivo* protein refolding in C33A cells following heat shock. BAG-1 promotes Hsp70s mediated refolding of denatured luciferase and β-galactosidase *in vivo*. C33A-NEO and C33A-BAG-1 cells were transiently transfected with pSV-luciferase and pSV-β-galactosidase. One day after transfection, cells were distributed into cell culture tubes and cultured for one more day, and then subjected to 42°C heat shock for 45 minutes. After returning cells to 37°C culture for 30 minutes or 60 minutes to allow protein refolding, the cells were lysed for detection of enzymatic activity. The luciferase or β-galactosidase activities before heat shock were taken as 100%. Results represent the mean ± the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in relative enzymatic activity between stably transfected NEO control cells and full-length BAG-1 overexpressing cells. A. luciferase. B. β-galactosidase.


A
C33A-NEO
C33A-BAG-1
Luciferase activity (fold increase)
2.0
1.5
1.0
0.5
0.0
*
*
0
30
60
Refolding time (min)


B
C33A-NEO
C33A-BAG-1
β-Galactosidase activity (fold increase)
2.0
1.5
1.0
0.5
0.0
*
*
0
30
60
Refolding time (min)

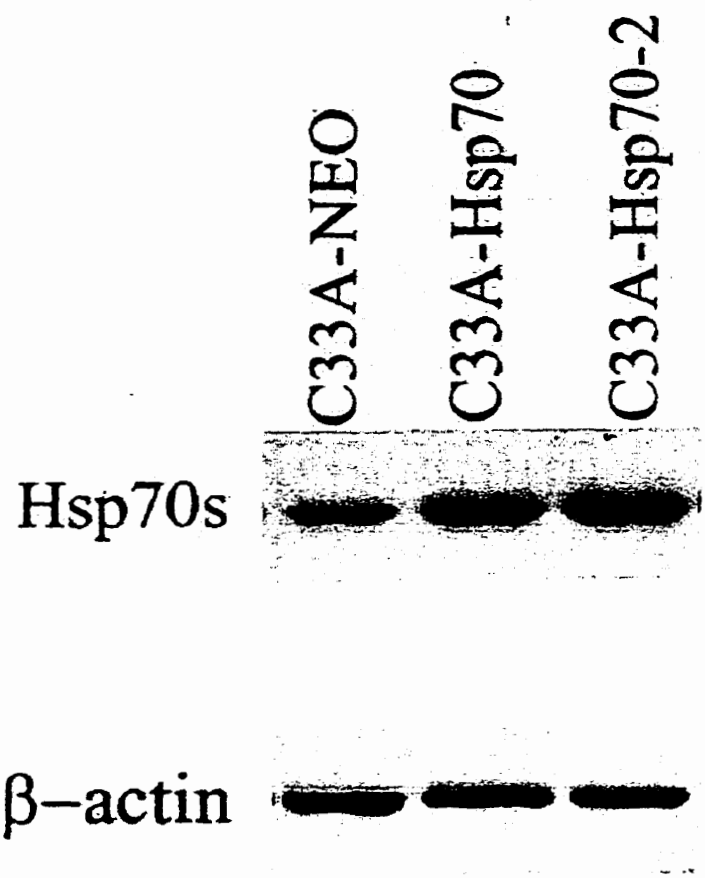


Figure 3.35. Hsp70s protein expression in C33A-hsp70 and C33A-hsp70-2. β-actin was an internal control.

(Figure 3.36). It was also found that Hsp70s overexpression led to the inhibition of cell death induced by various chemotherapeutic drugs and UV irradiation (Figure 3.36).

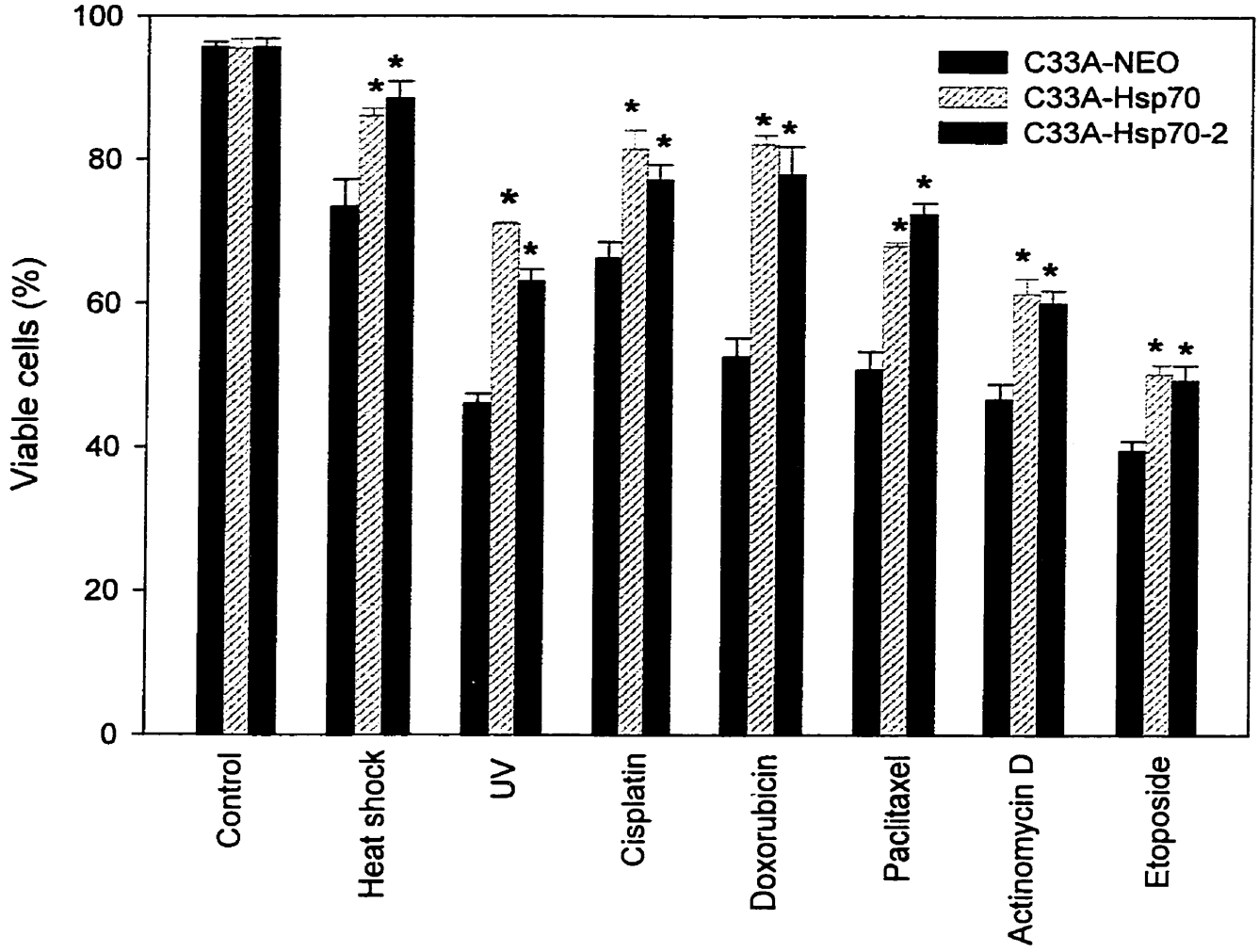


Figure 3.36. Effect of overexpressing of Hsp70s on induction of apoptosis by different agents. Cells exposed to heat shock at 45 °C for 45 minutes or 50 mJ/cm^2 UV irradiation and then returned to 37 °C culture for 48 hours. Cells were also treated with 5 μM cisplatin, 0.25 μM doxorubicin, 40 μM paclitaxel, 25 nM actinomycin D or 20 μM etoposide for 48 hours. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean ± the standard deviation from three independent experiments. *, $p < 0.05$, is the statistical significance of the difference in cell viability between stably transfected NEO control cells and full-length BAG-1 overexpressing cells.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

4.1. Cisplatin treatment of human endocervical cells immortalized by HPV16 and the multidrug-resistant phenotype

Cisplatin was discovered in the 1800s. Its biologic activity was first noted by Rosenberg in 1961. After multiple preclinical trials, it was released for clinical use in 1972 (Eustace, 1980). Since that time, it has become one of the most commonly used chemotherapy drugs and it is efficacious in a multitude of cancers.

Cisplatin is one of the most effective chemotherapeutic agents in treating cervical cancer. However, the response to cisplatin is generally short in duration, and acquired drug resistance is the greatest obstacle to the success of chemotherapy.

The cellular mechanisms of drug resistance depend upon altered levels or function of key gene products. These alterations may result from changes that occur at any point along the pathways of gene expression and regulation. Indeed, multiple molecular processes have been shown to be involved in examples of drug resistance, including altered drug influx and efflux, altered drug metabolism, altered drug targets, and altered cellular response to their damage (see section 1.2). The prevalence of these changes reflects the phenotypic and genetic instability of cancer cells under the mutagenic pressure of antineoplastic agents. This acquired drug resistance may result from the selection of clones originally insensitive to the drug or from the induction of resistance through the disruption or modulation of gene expression.

The endocervix is the origin of over 95% of cervical tumors. A human endocervical *in vitro* system has been used to study the role of HPV in the oncogenesis of endocervical cancer. It has provided the direct evidence that linked HPV to immortalization and further transformation of the HPV-immortalized cervical cells by carcinogens including smoking (Yang 1996a). *In vitro* endocervical MDR systems, however, have not yet been established to mimic and study acquired MDR in endocervical cancers.

The sensitivity to chemotherapeutic drugs is determined by a variety of cellular factors, including drug uptake and retention, ability to repair damage and propensity to undergo irreversible growth arrest or apoptosis. Drug treatment can inbibit tumor growth in at least two distinct ways: irreversible growth arrest and apoptosis. Irreparable damage to chromosomes prevents cell division, and therefore cells will no longer be able to generate progeny. This process occurs in all cell types and is sometimes referred to as mitotic death. In some cell types, the cellular damage may trigger apoptosis. For both effects of drug treatment, the indicator for success or failure of anti-cancer therapy is whether cells can survive the onslaught of drug treatment and retain the capacity to divide. Therefore, drug resistance of tumors is often determined by clonogenic survival of drug-treated cells.

As was expected, our clonogenic survival assays indicated that the cisplatin-treated endocervical cells acquired resistance to cisplatin (Figure 3.1A). Moreover, these cisplatin-resistant cells also acquired cross-resistance to paclitaxel (Figure 3.1B), an anticancer drug structurally and functionally different from cisplatin. Therefore, cisplatin

treatment of human endocervical cells immortalized by HPV16 conferred an MDR phenotype.

An *in vitro* endocervical cell system mimicking drug-resistance acquisition *in vivo* will be advantageous to study the molecular mechanism underlying the development of drug resistance. However, *in vitro* cell monolayers and *in vivo* epithelia are markedly different. The status and expression of HPV genes, three-dimensional cell-cell interactions and other cofactors, such as hormones, growth factors and the host immune surveillance, are some key differences (Herrington, 1995). Despite this limitation, the *in vitro* model system in this study remains a useful model to analyze the cellular mechanisms of acquired MDR in cervical cancer, particularly in understanding the role of apoptosis in endocervical cells carcinogenesis and acquired MDR.

4.2. Response of endocervical MDR cells and parental cells to various apoptotic stimuli

Clinical studies with patients suffering from acute myeloid leukemia have identified a correlation between high levels of Bcl-2 protein and a poor prognosis for the outcome of chemotherapy (Campos *et al.*, 1993). Also, experiments have shown that over-expression of Bcl-2 or the absence of p53 can significantly increase clonogenic survival of at least some types of tumour cells after radiation or drug treatment (Lowe *et al.*, 1993a; b; Strasser *et al.*, 1994). These results provide evidence that abnormalities in apoptosis control can influence the sensitivity of cancer cells to chemotherapeutic drugs (section 1.2.2.6). Overexpression of anti-apoptotic proteins in some types of cells causes

resistance to nearly all apoptotic stimuli, conferring an MDR phenotype that differs from other types of drug-resistance mechanisms (Reed, 1998b).

To determine whether the inhibition of apoptosis is the major mechanism of the MDR of endocervical cells, HEN-16-2/CDDP, further experiments were conducted to study the response of multidrug resistant endocervical cells and their parental cells to various apoptotic stimuli, including therapeutic drugs, heat shock, UV light irradiation and anti-Fas antibody.

4.2.1. Induction of apoptosis by cancer chemotherapeutic agents

In a study investigating the mechanism of action of etoposide (a topo II inhibitor), it was found that etoposide induced internucleosomal DNA fragmentation (Kaufmann, 1989). This observation raised the possibility that etoposide causes apoptosis. Since then, the spectrum of chemotherapy agents causing apoptosis has expanded, and the evidence supporting the role of apoptosis in chemotherapy continues to accumulate. The chemotherapeutic agents that have been identified as apoptosis-inducing include etoposide, dexamethasone, cisplatin, paclitaxel, 5-FU, doxorubicin ATRA and 4-HPR (Kaufmann, 1989; Walker *et al.*, 1991; Shinomiya *et al.*, 1994; Havrilesky *et al.*, 1995; Huschtscha *et al.*, 1996). The occurrence of apoptosis has been documented by demonstration of endonucleosomal DNA breakdown and other biochemical and morphologic criteria of apoptosis.

In vivo studies have also provided evidence that chemotherapeutic agents induce apoptotic tumor cell death. For example, a retinoic acid-treated T-cell lymphoma was

shown to undergo apoptosis *in vivo* (Su *et al.*, 1993). In a study of esophageal squamous cell carcinoma, it was shown that both radiation and chemotherapy (5-FU, cisplatin, and bleomycin) induced apoptotic cell death *in vivo*, as determined by examination of biopsy specimens (Moreira *et al.*, 1995). *In vitro* and *in vivo* studies clearly show the induction of apoptosis by chemotherapeutic agents in various cell lines and tumors. In this study, compared with HEN-16-2, endocervical MDR HEN-16-2/CDDP cells showed a significant inhibition of apoptosis induced by cisplatin (Figure 3.2 and 3.3), actinomycin D (Figure 3.5), doxorubicin (Figure 3.6), etoposide (Figure 3.7), paclitaxel (Figure 3.8), 5-FU (Figure 3.9A), staurosporine (Figure 3.9B), ATRA (Figure 3.10A) and 4-HPR (Figure 3.10B). These result suggest that inhibition of apoptosis might be responsible for the MDR phenotype of HEN-16-2/CDDP cells.

4.2.2. Induction of apoptosis by UV irradiation

A variety of extrinsic and intrinsic signals can trigger apoptosis, including environmental stress such as UV (Sachs and Lotern, 1993; Buttke and Sandstrom, 1994; Kyriakis *et al.*, 1994; Thompson, 1995). In this study, HEN-16-2/CDDP showed inhibition of apoptosis induced by 50 mJ/cm^2 dose UV (Figure 3.11), further suggesting that the phenotype of this MDR of human endocervical cells may be due to the inhibition of apoptosis, rather than by overexpression of Pgp or other classic MDR proteins.

4.2.3. Induction of apoptosis by heat shock

The spectrum of tissue susceptibility to apoptosis induction by heat shock is

essentially similar to cancer therapeutic agents and radiation (Allan and Harmon, 1986; Barry *et al.*, 1990; Sellins and Cohen, 1991; Takano *et al.*, 1991;). In this study, HEN-16-2/CDDP cells were more resistant than parental cells to apoptosis induced by heat shock at 45 °C for 45 minutes (Figure 3.11). This result supports the concept that inhibition of apoptosis may cause the MDR phenotype found in human endocervical cells.

4.2.4. Induction of apoptosis by anti-Fas antibody

Fas (also called CD95), a receptor for Fas ligand (FasL), belongs to the TNF receptor family, and transduces the FasL apoptotic signal into cells (Nagata and Golstein, 1995). The molecular mechanism for Fas-induced apoptosis is currently being elucidated. Aggregation of the Fas receptor by binding to FasL, or by crosslinking with an anti-Fas antibody induces the formation of a death-inducing signaling complex (DISC) of proteins composed of Fas, an adaptor called FADD, and the inactive proform of caspase-8 (Kischkel *et al.*, 1995; Nuñez *et al.*, 1998). Oligomerization of procaspase-8 in DISC seems to induce self-processing into the mature, active, p20 and p10 subunits of caspase-8 protease. The activated caspase-8 is then released from DISC, and activates other downstream caspases by proteolytic cleavage of their zymogen forms (Medema *et al.*, 1997).

In many cells, overexpression of anti-apoptotic proteins, such as Bcl-2 and Bcl-X_L, inhibits apoptosis induced by a variety of stimuli, including anti-Fas antibody (Cory, 1995; Korsmeyer, 1995; Reed, 1997). Consistently, the cell death rate induced by anti-

Fas antibody is less in HEN-16-2/CDDP cells than HEN-16-2 cells (Figure 3.11), further suggesting that resistance of HEN-16-2/CDDP to multiple drugs is due to the dysregulation in apoptosis pathways, but less likely due to the enhanced drug efflux, enhanced drug detoxification, altered drug targets, or altered cellular damage repair.

4.3. Evidence of tumorigenicity of cervical cells by anticancer drug cisplatin

It has been well recognized that cervical carcinogenesis is a multistage process in which HPV and other co-factors are necessary for the full malignant transformation of primary cervical cells (zur Hausen, 1991; 1994; Herrington, 1995). Many carcinogens, such as those in cigarette smoke, have been demonstrated to be risk factors for cervical cancer (Phillips and Smith, 1994; Yang, *et al.*, 1996a; Nakao *et al.*, 1996).

The HEN-16-2/CDDP cell line possesses the general growth characteristics of cervical tumors (Li *et al.*, 1992): faster growth in medium containing serum and a high calcium level, higher saturation density, and anchorage-independent growth (Figures 3.13, 14 and Table 3.1). Moreover, HEN-16-2/CDDP cells formed tumors in nude mice, whereas a similar passage of untreated HEN-16-2 cells remained non-tumorigenic in nude mice (Figure 3.15 and Table 3.2). These results provided the first *in vitro* evidence that cisplatin treatment can further transform HPV-immortalized endocervical cells, supporting the hypothesis that cisplatin has carcinogenic potential (Greene, 1992).

The carcinogenic potential of drugs used in cancer therapy has been recognized for decades (Haddow *et al.*, 1948). In laboratory animals, cisplatin has been found to produce malignancies, notably acute leukemia (Kaldor *et al.*, 1988, Barnhart and Bowden, 1985;

Kempf and Ivankovic, 1986). Cisplatin-associated second malignancies were found to develop in many cancer patients (see review Green, 1992).

DNA damage upon cisplatin treatment may occur in both neoplastic and non-neoplastic cells. Lethal injury to neoplastic cells is obviously the desired effect; however, non-neoplastic cells may also be killed or damaged. If the nonlethal DNA damage in normal cells can be faithfully repaired, the cells return to normal. However, if the cellular damage cannot be repaired, normal cells will undergo apoptosis. Alternatively, the damaged cells may have undergone initial stages of oncogenic progression. Such cells may enter the cell cycle again without DNA damage repair, thus mutation occurs in these cells. Then, if the accumulated mutations cause an upregulation of cell proliferation or downregulation of apoptosis, new primary neoplastic cells can arise. Carcinoma *in situ* can then progress to invasion and metastasis if the host defenses are compromised.

When the HPV16 E6 protein is expressed in cervical cells, baseline p53 protein levels are reduced dramatically following ubiquitin-mediated proteolysis through interaction with E6 (section 1.3.2). This causes the loss of cell cycle arrest following DNA damage (Kessis *et al.*, 1993; Demers *et al.*, 1994; Foster *et al.*, 1994; Hickman *et al.*, 1994; Slebos *et al.*, 1994). Inactivation of p53 by the HPV16 E6 protein was found to increase the rate of mutagenesis (Havre *et al.*, 1995). Thus, the inactivation of p53 function by HPV16 E6 in HEN-16-2 cells allows the survival of cells mutated by cisplatin treatment that would otherwise be eliminated by apoptosis.

Therefore, high-risk HPVs may indirectly contribute to cervical tumorigenesis by

promoting genomic instability and the accumulation of mutations in HPV-infected cells after treatment with cisplatin.

In view of the fact that many human premalignancies contain disabled or dysregulated genes such as p53 that effectively induce apoptosis or Bcl-2 that effectively block apoptosis, clonogenic cells surviving such genotoxic treatment *in vivo* are likely to induce mutations contributing to progression of a benign disease as a paradoxical consequence of attempts to eradicate a malignant one. Therefore, the use of carcinogenic drugs for cancer treatment needs clear justification according to the susceptibility of cells to the DNA-damage-induced apoptosis versus oncogenic mutation. The benefits from the cancer therapy should be carefully evaluated. Abrogation of apoptosis provides a double hazard of tumorigenicity and multidrug-resistance in the face of genotoxic therapy, such as cisplatin.

4.4. Role of apoptosis-related cellular genes in multidrug resistance and tumorigenesis of human endocervical cells

Recent clinical studies have shown a strong correlation between apoptosis and progression of premalignant cervical lesions (Isacson *et al.*, 1996; Shoji *et al.*, 1996). Cellular, but not HPV, genes regulating apoptosis such as the *bcl*-2 family, are considered to be important for this correlation (Shoji *et al.*, 1996; Pillai *et al.*, 1996; Yang *et al.*, 1998b). However, the precise role of apoptosis-associated genes in this oncogenic progression is still poorly understood. One purpose of this study was to determine whether the expression of apoptosis-associated proteins varied during the process of

acquired MDR and tumorigenesis induced by the anticancer drug cisplatin. The expression of these anti-apoptotic proteins, Bcl-X_L and BAG-1 p50 and p33 isoforms, increased significantly in HEN-16-2/CDDP, indicating that these proteins may be involved in the aquired MDR and tumorigenesis of human endocervical cells. In contrast, the levels of the apoptosis-promoting proteins p53, Bak, and Bax varied little when the expression of each was compared in HEN-16-2 and HEN-16-2/CDDP.

Deletions or mutations of the tumor suppressor gene, p53, have been detected in a majority of various common human cancers (Hollstein *et al.*, 1991; Levine *et al.*, 1991; Levine, 1997). p53 was found to play an important role in apoptosis; for example, the p53 protein is required for DNA damage-induced apoptosis in lymphocytes and colonic epithelial cells (Clarke *et al.*, 1993; Lowe *et al.*, 1993a; Clarke *et al.*, 1994; Strasser *et al.*, 1994). Loss of p53 function was reported to cause resistance to apoptosis induced by DNA-damaging reagents in various human cells (Zhan *et al.*, 1994). Conversely, overexpression of p53 was shown to induce apoptosis in certain cell types (Oren, 1994; Yonish-Rouach *et al.*, 1991; 1995). It has become clear that the p53 response varies not only according to the insulting stimulus but also according to the tissue and cell type involved (Midgley *et al.*, 1995; MacCallum *et al.*, 1996). Although much data regarding p53 is now available (over 10,000 p53-related papers published since 1992), the precise mechanism of p53 function is still uncertain.

Since p53 expression varied little in HEN-16-2 and HEN-16-2/CDDP (Figure 3.17 and Table 3.3), expression levels of Bax and Bak were examined. They are Bcl-2 family members that antagonize the function of Bcl-2 and promote apoptosis under conditions of

stress (Oltvai *et al.*, 1993). Bax was shown to be downstream of p53 and transactivated by p53 (Miyashita *et al.*, 1994; 1995). Like p53, Bax can function as a tumor-suppressor gene by inducing apoptosis in tumor cells (Yin *et al.*, 1997). In support of this hypothesis, inactivation of Bax accelerated transformation of epithelial cells in transgenic mice expressing a truncated SV40 large T antigen that inactivates the tumor suppressor pRB but not p53 (Yin *et al.*, 1997). Bax and Bcl-2 appear to have intrinsic independent functions as effectors of apoptosis and survival, respectively. The evidence includes observations of mutants of Bax and Bcl-2 that are incapable of dimerizing, but still display antagonistic activity towards each other and remain capable of inducing or repressing apoptosis, respectively (Cheng *et al.*, 1996; Simonian *et al.*, 1996a,b; 1997; Zha and Reed, 1997; Wang *et al.*, 1998;). Experiments with knock-out mice demonstrated that Bax promoted cell death in the absence of Bcl-2 (Knudson and Korsmeyer, 1997).

No changes were found in the expression of Bax or Bak proteins when comparing HEN-16-2 with HEN-16-2/CDDP cells (Figure 3.17 and Table 3.3), suggesting that dysregulation of apoptosis may not be caused by dysregulated expression of Bax or Bak. This was consistent with results using the HEN-16-2 cell line transformed by CSC (Yang *et al.*, 1996a; 1998b).

Overexpression of Bcl-2 renders pre-B lymphocyte cells resistant to apoptosis (Vaux *et al.*, 1988). Recently, high levels of Bcl-2 mRNA or protein were found in neuroblastoma, lymphoma, breast, lung, prostate and cervical cancers (Haldar *et al.*, 1994; Ikegaki *et al.*, 1994, Bargou *et al.*, 1995, Liang *et al.*, 1995; McDonnell *et al.*,

1992; Reed *et al.*, 1991; Yang *et al.*, 1998b). However, no mutations in Bcl-2 were found. Overexpression of Bcl-2 was found to prevent cell death induced by a wide variety of apoptotic stimuli, including chemotherapeutic drugs (White, 1996; Reed, 1994). The expression of Bcl-2 was found to be progressively enhanced after primary cells were immortalized by HPV16 and again after the immortalized cells were transformed by CSC (Yang *et al.*, 1998b), suggesting that abnormal activation of the Bcl-2 gene was involved in cervical cell immortalization by HPV16 and late events of cervical tumorigenesis induced by CSC. In this study, however, the expression of Bcl-2 was found to be not significantly different between HEN-16-2 and HEN-16-2/CDDP (Figure 3.17 and Table 3.3). Therefore, in HEN-16-2/CDDP cells, dysregulation of apoptosis may be caused by altered expression of other apoptosis-related genes.

Two splice forms of Bcl-X , Bcl-X_L and Bcl-X_S, have different functions: Bcl-X_L is the longer form and has an anti-apoptotic effect, whereas Bcl-X_S is the shorter form and promotes apoptosis (Boise *et al.*, 1993). Overexpression of Bcl-X_L mRNA and protein was reported in human lung cancer cell lines, lymphomas, colorectal adenocarcinomas, gastric cancers and cervical cancers (Hirose *et al.*, 1997; Krajewska *et al.*, 1996; Kondo *et al.*, 1996; Reeve *et al.*, 1996; Xerri *et al.*, 1996; Yang *et al.*, 1998b). Bcl-X_L was the predominant form of Bcl-X expressed in human cervical cells, and it was overexpressed in both the HPV16-immortalized and the CSC-transformed cell lines; whereas the expression of Bcl-X_S protein was undetectable in all the cell lines (Yang *et al.*, 1998b). Consistent with these observations, the expression of Bcl-X_L was found to be higher in HEN-16-2/CDDP than HEN-16-2 cells (Figure 3.17 and Table 3.3).

BAG-1 is a gene which was isolated through the interaction of its protein with Bcl-2 (section 1.4). Cotransfection of BAG-1 with Bcl-2 was shown to increase the protection from cell death induced by several stimuli, including staurosporine, anti-Fas antibody, cytolytic T cells, and cytokine withdrawal (Takayama *et al.*, 1995; Clevenger *et al.*, 1997). BAG-1 is not a member of the Bcl-2 family and may have unique function in protection from apoptosis. Overexpression of BAG-1 protected GM701 immortalized fibroblasts from heat shock-induced cell death, but did not similarly protect 293 human kidney epithelial cells (Takayama *et al.*, 1997).

Recently, BAG-1 was found to be overexpressed in human cervical carcinoma cell lines and tissues. Enhanced resistance to apoptosis induced by staurosporine was found to correlate well with expression of the BAG-1 p50 isoform in human cervical cells. Further study has indicated that the overexpression of BAG-1 p50 in cervical carcinoma C33A cells enhanced the resistance to apoptosis induced by staurosporine (Yang *et al.*, 1999b). In agreement with these findings, the expression of BAG-1 p50, and p33 isoforms was higher in HEN-16-2/CDDP than in HEN-16-2. However, in HEN-16-2 cells and HEN-16-2/CDDP cells, the expression level of the p46 isoform of BAG-1 is similar (Figure 3.17 and Table 3.3), and the shortest isoform of BAG-1, p29 was undetectable (Figure 3.17).

Consistent with the effects of staurosporine, in this study, overexpression of BAG-1 p50 in cervical C33A cells conferred resistance to apoptosis induced by anticancer drugs including cisplatin, doxorubicin and etoposide (Figure 3.19, 20, 21). Also, this overexpression conferred resistance to apoptosis induced by UV and heat shock (Figure

3.21). However; it failed to protect C33A cells from apoptosis induced by two other anticancer drugs, actinomycin D and Paclitaxel (Figure 3.22). To further determine whether BAG-1 or Bcl-X_L play a direct role in the process of multidrug resistance and carcinogenesis of HPV16-immortalized endocervical cells, it would be interesting to overexpress Bcl-X_L or BAG-1 different isoforms in HEN-16 cells and assay oncogenic properties and the pheotype of drug-resistance.

The precise roles and mechanisms of BAG-1 in regulating apoptosis in cervical cells are complex and not well understood. Avenues to be considered are the functional interactions of BAG-1 with other apoptosis-related proteins (Figure 1.7).

The recognition that the expression of apoptosis-related genes can be regulated by various biologic response modifiers, such as retinoids, cytokines, and growth factors, suggests opportunities for modulating apoptosis by combination chemotherapy. Because different chemotherapeutic agents have different mechanisms of action to induce apoptosis, it would be of interest to examine their efficacies in various combinations in the induction of apoptosis in HEN-16-2/CDDP cells.

Alternatively, antisense approaches to downregulate protein expression could be envisioned. Indeed, sequence-specific down-regulation of *Bcl-2* or *Bcl-X_L* expression *in vitro* has been reported to markedly enhance sensitivity to chemotherapeutic drugs (Campos *et al.*, 1994; Kitada *et al.*, 1994; Ziegler *et al.*, 1997; Taylor *et al.*, 1999). Therefore, it would be interesting to assay whether inhibition of *BAG-1* or *Bcl-X_L* expression by antisenses could sensitize the HEN-16-2/CDDP cells to apoptotic stimuli.

Apoptosis is controlled through cellular genes including apoptosis-promoting genes

(*p53, Bad, Bak, Bax, Bcl-X_S, Bid, Bik, Bim/Bod, Blk, Bok, Hrk/Dp5, Nip3 and Nik*) and apoptosis-inhibiting genes (*Bcl-2, Bcl-w, Bcl-X_L, Bfl-1, Mcl-1*, and *BAG-1*) (for review, see Chao and Korsmeyer, 1998; Zamzami *et al.*, 1998; Reed, 1998a). Alterations in these apoptosis-regulating gene products may have important roles in carcinogenesis and MDR in HEN-16-2/CDDP cells. Therefore, it could be interesting to further examine those apoptosis-related genes that have not yet been studied in HEN-16-2/CDDP.

4.5. Identification of Hsp70 and Hsp70-2 chaperones as BAG-1 interacting proteins from a cDNA library using the yeast two-hybrid system

The anti-apoptotic protein BAG-1 has been reported to form complexes with and modulate the functions of Bcl-2, the serine/threonine-specific protein kinase Raf-1, steroid hormone receptors, such as ER, AR, and GR, retinoic acid receptor (RAR), HGF and PDGF receptors, and Siah-1A (Takayama *et al.*, 1995; Wang *et al.*, 1996; Bardelli *et al.*, 1996; Kullmann *et al.*, 1998; Liu *et al.*, 1998; Matsuzawa *et al.*, 1998; Froesch *et al.*, 1998; see section 1.4). These observations suggest a general regulatory role for BAG-1 in signal transduction pathways involved in cell survival and possibly other cellular processes as well. However, how BAG-1 accomplishes these multiple functions is still unclear.

To further characterize and understand the role of BAG-1 in regulating apoptosis, a cDNA library was screened for novel BAG-1 interacting proteins. Using BAG-1 p46 as bait in yeast two-hybrid screening, 17 human cDNA sequences of *Hsp70*, *Hsp70-2*, *Hsc70* pseudogene and *Hsp70Y* were cloned (Table 3.4; Figure 3.25 and 3.26). *Hsp70Y*

was highly homologous to Hsc70 at its amino-terminal and carboxyl-terminal ends; however, the total length of *Hsp70Y* cDNA was much longer than *Hsc70* mRNA, indicating that *Hsp70Y* was a different gene from *Hsc70* and any other *Hsp70* genes, and therefore was a novel candidate *Hsp* (Figure 3.26). Since *Hsp70Y* was 5340 bp, the analysis of this gene is still ongoing.

A variety of strategies, including Southwestern blots, phage display, and the yeast two-hybrid system, have been devised to screen large libraries for genes or fragments of genes whose products may interact with a protein of interest. The yeast two-hybrid system is an *in vivo* method that uses yeast GAL4 protein transcriptional activity as a screening method and an assay of exogenous protein-protein interactions (Fields and Song, 1989; Chien *et al.*, 1991; Fields and Sternglanz, 1994; Figure 2.3; section 2.12.1).

After the yeast two-hybrid system was developed and refined, various genes were identified through its use. In fact, Hsc70/Hsp70 was identified to be BAG-1-interacting protein by Takayama *et al.*(1997) using mouse BAG-1 as bait in the yeast two-hybrid system, at the time that I independently identified Hsp70, Hsp70-2, and Hsp70Y to be BAG-1-interacting proteins using human BAG-1 p46 as bait in the yeast two-hybrid system (see sections 2.12 and 3.7). These independently reproducible results indicate the high efficiency and specificity of the yeast two-hybrid system to screen cDNA libraries.

The yeast two-hybrid system has several advantages over other techniques for characterizing protein-protein interactions. First, it is highly sensitive, being able to detect weak and transient interactions that are not detected by other methods. Second, it enables not only identification of interacting proteins, but also the rapid cloning of genes

encoding these proteins. Moreover, because it is performed *in vivo*, the proteins produced in this eukaryotic system are probably more similar to their counterparts produced in native cells than those produced in bacterial systems.

The BAG-1 p50 isoform has 71 more amino-terminal amino acids compared to BAG-1 p46. The BAG-1 p50 isoform was reported to collaborate with AR, enhancing its transactivation function, whereas the BAG-1 p46 isoform did not (Froesch *et al.*, 1998), indicating an important functional difference between the BAG-1 p50 and p46 isoforms. Therefore, it would be valuable to screen a cDNA library using BAG-1 p50, or even using the amino-terminal fragment as bait in the yeast two-hybrid system. cDNA encoding proteins that would include, but would not be limited to, AR should be identified as BAG-1 p50 isoform amino-terminal domain binding proteins.

Although BAG-1 was initially identified as a Bcl-2-binding protein, attempts to demonstrate interactions between BAG-1 and Bcl-2 using purified proteins have not been successful. It is possible that BAG-1 interacts with Bcl-2 using Hsp70 chaperones or other proteins as adaptors.

A novel yeast system based on the yeast two-hybrid system, termed the yeast three-hybrid system, has been recently used to identify interactions among three proteins (Licitra and Liu, 1996), and this system appears to be a potentially useful tool to further characterize BAG-1. Because it is possible that BAG-1 indirectly interacts with other proteins using Hsp70 chaperones as adaptors, BAG-1 could be expressed as a fusion protein with the GAL4 BD, and Hsp70 could be conditionally expressed from the P_{met25} promoter in pBridge plasmid (Figure 4.1). The GAL4 AD fusion protein from a cDNA

library (the same cDNA library that was used in the yeast two-hybrid system), would then be screened, as for the yeast two-hybrid system (Figure 4.1).

It is also possible that interactions between Hsp70 chaperones and BAG-1 may confer a BAG-1 conformation that facilitates the direct interaction of BAG-1 with other proteins. In this case, the yeast three-hybrid method described above would be useful to identify and clone these proteins and characterize their interactions. However, Hsp70 would need to be inserted in-frame with GAL4 BD, whereas BAG-1 would be conditionally expressed from the P_{met25} promoter pBridge plasmid.

4.6. Region of BAG-1 interacting with Hsp70 and Hsp70-2

Human BAG-1 contains several structural domains that have the potential to be involved in protein-protein interactions (Dr. Xiaolong Yang, unpublished data). There is an α-helical domain located between amino acids 225 and 261. The α-helical domain is a structure with potential for mediating protein-protein interactions (Muchmore *et al.*, 1996). In addition, there is a ubiquitin-like domain located between amino acids 163 and 199. The ubiquitin-like domain is also a candidate for facilitating protein-protein interaction, since ubiquitin is involved in interactions with many proteins through this domain on target proteins (for review, see Hershko and Ciechanover, 1992). The yeast two-hybrid system results demonstrated that the BAG-1 carboxyl-terminal amino acids 315 to 345 are responsible for the interaction with Hsp70 and Hsp70-2 (Figure 3.33 and Table 3.5). These findings confirmed the result of *in vitro* binding assays (Figure 3.29;

Figure 4.1

3.30; 3.31). My results are consistent with those of other independent studies (Takayama *et al.*, 1997; Zeiner *et al.*, 1997). Recently, the BAG-1 carboxyl-terminal domain responsible for interaction with Hsp70 chaperones was demonstrated to be located in a domain conserved among BAG-1 family members (Takayama *et al.*, 1999).

4.7. BAG-1 modulation of Hsp70s chaperone activity

Exposure of cells to sublethal temperature or other stress induces the synthesis and accumulation in the cytoplasm of a set of proteins, collectively known as Hsps which subsequently makes the cells resistant to normally lethal temperatures or to other forms of cellular injury (Lindquist, 1986; Parsell and Lindquist, 1993). These phylogenetically similar and highly conserved proteins function as enhancers of cell survival and behave as molecular protein chaperones at the biochemical level. Hsps bind to nascent or misfolded polypeptides under normal conditions or to denatured proteins created under the influence of physical agents, leading either to their correct folding or to rapid elimination (Beckmann *et al.*, 1990; Parsell and Lindquist, 1993; Welch, 1993).

The family of Hsp70 molecular chaperones are known to play key roles in protecting mammalian cells. The ATP-bound form of Hsp70 binds and releases polypeptides or proteins quickly, whereas the ADP-bound form maintains tight binding to substrates (Flynn *et al.*, 1989; Palleros *et al.*, 1991; Schmid *et al.*, 1994). BAG-1 has been suggested to have the activity of a nucleotide exchange factor for Hsp70 chaperones *in vitro*, analogous to the role of GrpE in the bacterial DnaK/DnaJ cycle (Höhfeld and Jentsch, 1997). In contrast, other data indicated that BAG-1 was a negative regulator of

Hsp70 chaperone activity *in vitro* (Takayama *et al.*, 1997; Zeiner *et al.*, 1997; Bimston *et al.*, 1998). However, the *in vitro* conditions, such as ATP concentration in the reaction buffer of these experiments were different.

These contradictory results suggest that BAG-1 could either inhibit or promote the ATPase activity of Hsp70 chaperones probably dependent on the reagent composition and levels in the interaction buffer, and thus modulate the Hsp70s-mediated refolding of thermally denatured proteins. Thus far, the precise mechanisms by which BAG-1 regulates Hsp70s-mediated refolding activity *in vitro* remain unclear.

In my *in vivo* experiments, after heat shock, luciferase and β-galactosidase in C33A human cervical carcinoma cells were denatured, and had reduced levels of enzymatic activity. After returning to normal culture conditions, it appears that the expression of BAG-1 protein led to a small but significant increase in the refolding of luciferase and β-galactosidase, compared with C33A-NEO control cells (Figure 3.34A and B).

The chaperone function of the mammalian Hsp70s is modulated by their physical interactions with other proteins. This modulation involves cooperation among multiple chaperone cofactors in complexes that include Hsp70s, Hsp40, Hip, Hop, CHIP and BAG-1 (Höhfeld, 1998; Kelley, 1998; Ballinger *et al.*, 1999).

A recent study found that the carboxyl terminal domain of BAG-1, which was responsible for the interaction with Hsp70s, was conserved among several novel BAG-1 family members, including BAG-2 and BAG-3 (Takayama *et al.*, 1999). This observation suggested that the modulation of Hsp70s-mediated protein refolding activity by BAG-1 may be redundantly shared with other novel BAG-1 family members.

Moreover, different cell types have distinct intracellular contexts, which could also affect the Hsp70s-mediated protein refolding activity. To delineate a general mechanism whereby BAG-1 modulates Hsp70s-mediated protein refolding *in vivo*, other cell types will also need to be studied.

Hsp70 chaperones are multiple function proteins, and play a role in signal transduction pathways, which lead to adaption to stressful conditions in cells and organisms. Evidence has accumulated for their participation in regulating the activity of signaling proteins (Kimura *et al.*, 1995), such as the steroid receptors (Picard *et al.*, 1990; Tsai *et al.*, 1994; Nathan and Lindquist, 1995) and Ras and Raf kinases (Stancato *et al.*, 1993). Therefore, it would be interesting to investigate how BAG-1 regulates other functions of Hsp70s, such as protein complex assembly, translocation and Hsp70-associated protein-ubiquitin-proteasome pathway protein-degradation.

4.8. The role of Hsp70s in inhibition of apoptosis

Hsp70s are highly expressed in many tumor cells and have reported to be an indicator of poor therapeutic outcome in breast cancer (Mivechi and Rossi, 1990; Ferrarini *et al.*, 1992; Kaur and Ralhan, 1995; Ciocca *et al.*, 1993). Furthermore, transgenic mice overexpressing the human Hsp70 develop T-cell lymphomas (Seo *et al.*, 1996). All these data suggested that Hsp70 may play a role in tumorigenesis and drug resistance.

The role that Hsp70s play in the regulation of apoptosis is unclear. Conflicting reports on the subject possibly stem from the various mechanisms that different cells use

in response to different stimuli that induce apoptosis. After induction of Hsp70 accumlation, protective effects against Fas-stimulated apoptosis and protection from apoptosis-inducing drugs have been reported (Polla *et al.*, 1996; Mehlen *et al.*, 1996; Samali and Cotter, 1996), although the induction of Hsp70 may not protect from apoptosis (Cox *et al.*, 1994). In this study, it was found that Hsp70 or Hsp70-2 overexpression in cervical carcinoma C33A cells conferred protection from cell death induced by various stimuli, including several chemotherapeutic drugs, UV irradiation and heat shock (Figure 3.36).

The precise molecular mechanism whereby Hsp70 expression leads to cell survival or inhibition of apoptosis is not understood. It has been suggested to be due to protection from protein denaturation, misfolding and degradation (Hartl *et al.*, 1994; Craig *et al.*, 1994; Laroia *et al.*, 1999). Others have suggested that Hsp70s-mediated cell survival mechanism may arise from its assistance in the transfer of newly synthesized proteins into mitochondria helping to maintain overall mitochondrial integrity (Ungermann *et al.*, 1994; Pfanner *et al.*, 1994), which plays an important role in regulating the cell death pathway (for review, see Green and Reed, 1998; Gross *et al.*, 1999; see section 1.1.4). One study indicated that Hsp70-mediated cell survival involves the inhibition of caspase activity (Mosser *et al.*, 1997); whereas another recent study suggested that Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like effector proteases (Jäättelä *et al.*, 1998).

4.9. Future directions

To more fully understand the molecular mechanism of apoptosis in carcinogenesis and MDR in human endocervical cells, several experiments could be done using this *in vitro* HEN-16-2 and HEN-16-2/CDDP model:

1. It would be interesting to overexpress Bcl-X_L or BAG-1 different isoforms in HEN-16 cells and to determine whether BAG-1 and Bcl-X_L play a direct role in the process of multidrug resistance and carcinogenesis.
2. Assay mRNA and protein expression levels of *BAG*-1, *Bcl-X_L* and other related genes after treating both cell types with cisplatin or other agents.
3. Examine other apoptosis-related genes, which may also contribute to the inhibition of apoptosis in HEN-16-2/CDDP cells, and their role in MDR and tumorigenesis.
4. Use mRNA differential display method (Yang *et al.*, 1996b) or cDNA microarrays (Duggan *et al.*, 1999) to identify genes that are differentially expressed in the process of inhibition of apoptosis. Such studies are critical to better understand the molecular mechanisms whereby apoptosis is dysregulated in human cervical cells, since, as discussed above, MDR and tumorigenesis are driven by a series of changes in gene expression. Thus, identifying genes that are differentially expressed, especially oncogenes, tumor suppressor genes, in the process of MDR and tumorigenesis are critical to understanding the molecular mechanisms involved in apoptosis.
5. Assay whether inhibition of *BAG*-1, *Bcl-X_L* or other related gene expression by antisenses or other agents could sensitize the HEN-16-2/CDDP cells to apoptotic

stimuli.

6. Compare the results obtained for HEN-16-2 and HEN-16-2/CDDP with similar experiments using primary human ectocervical cells.

CHAPTER 5

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