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Report

Expression of multiple apoptosis-regulatory genes in human breast cancer cell lines and primary tumors

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Summary

The expression of several apoptosis-regulating genes was evaluated in 9 human breast cancer cell lines, 2 immortalized human mammary epithelial lines, 1 normal breast tissue biopsy, and 3 primary breast tumors, using a multiple antigen detection (MAD) immunoblotting method. The anti-apoptotic proteins Bcl-2, Bcl-X_L, Mcl-1, and BAG-1 were present at immunodetectable levels in 7, 10, 10, and 9 of the 11 lines. Comparing these 11 cell lines among themselves revealed that steady-state levels of Bcl-2, Bcl-X_L, Mcl-1, and BAG-1 were present at relatively higher levels in 4, 6, 5, and 5 of the lines, respectively. In contrast, the pro-apoptotic proteins Bax and Bak were detected in all 11 cell lines, and were present at relatively higher levels in 10 and 5 of the 11 lines, respectively. The Interleukin-1β converting enzyme (ICE) homolog CPP32 (Caspase-3) was expressed in 10/11 breast cell lines. High levels of p53 protein, indicative of mutant p53, were found in 8 of the 11 lines and correlated inversely with Bax expression ($p = 0.01$). Bcl-2 and BAG-1 protein levels were positively correlated ($p = 0.03$). Immunoblot analysis of primary adenocarcinomas revealed expression of the anti-apoptotic proteins Bcl-2, Bcl-X_L, Mcl-1, and BAG-1, as well as the pro-apoptotic proteins Bax, Bak, and CPP32, in at least 2 of the 3 tumors examined. Immunohistochemical analysis was also performed for all of these proteins using 20 paraffin-embedded breast cancer biopsy specimens that all contained residual normal mammary epithelium in combination with both invasive cancer and carcinoma *in situ*. All of these apoptosis-regulating proteins were detected in primary breast cancers, though the percentage of immunopositive tumor cells varied widely in some cases. Comparisons of the intensity of immunostaining in normal mammary epithelium and invasive carcinoma suggested that Bcl-2 immunointensity tends to be lower in cancers than normal breast epithelium ($p = 0.03$), whereas CPP32 immunointensity was generally higher in invasive cancers ($p < 0.0001$). Taken together, the results demonstrate expression of multiple apoptosis-modulating proteins in breast cancer cell lines and primary tumors, suggesting complexity in the regulation of apoptosis in these neoplasms of mammary epithelial origin.

Introduction

The clonal selective growth advantage that underlies all neoplasms [1] can arise either because of increases in the rates at which cells proliferate or because of decreases in the frequency with which they

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expire due to programmed cell death (reviewed in 2). While the loss of cell cycle checkpoint controls and the activation of certain proliferation-promoting oncogenes can render tumor cells relatively more sensitive than normal cells to the cytotoxic effects of radiation and chemotherapeutic drugs, the inactivation of cell death-promoting or the activation of cell death-blocking genes can produce a radio- and chemoresistant state that probably accounts in large part for the failure of many current anti-cancer therapies [3]. Escape from the normal mechanisms of cell death regulation probably also underlie, at least in part, the phenomenon of hormone-independence in carcinomas that arise from tissues such as breast and prostate. Though the molecular mechanisms that control the relative sensitivity or resistance of malignant cells to physiological and iatrogenic apoptotic stimuli remain to be fully elucidated, members of the Bcl-2 family of proteins appear to play a prominent role. These proteins control a distal step in an evolutionarily conserved pathway for programmed cell death and apoptosis [4]. Moreover, alterations in expression of Bcl-2 and some of its homologs have been described in human tumors and can have prognostic significance in some cases (reviewed in 5).

Though the precise mechanism of action of Bcl-2 family proteins remains unresolved, biochemical and genetic data suggest that these proteins control, probably indirectly, the activation of a family of cysteine proteases with homology to the Interleukin-1β Converting Enzyme (ICE). These proteases appear to be universal effectors of apoptotic cell death [6]. Among the various mammalian members of the ICE protease family, CPP32 is probably the most clearly associated with cell death (reviewed in 7). Proteolytic activation of CPP32 can be induced in tumor cell lines by various chemotherapeutic drugs and is blocked by overexpression of Bcl-2 and Bcl-X_L (reviewed in 6).

A variety of experimental observations suggest that dysregulation of normal cell turnover mechanisms plays an important role in the pathogenesis of adenocarcinomas of the breast [7–11]. Breast cancer accounts for about 160,000 new cases of malignancy per year in the United States alone, and is the second leading cause of cancer-associated death for women in developed countries [12]. At present, relatively little is known about which apoptosis-regulating genes are expressed in tumors of mammary epithelial origin. Mutations that inactivate the tumor suppressor p53 (a pro-apoptotic protein) occur commonly in advanced breast cancers, and have been correlated with poor clinical outcome [13]. Similarly, reduced levels of Bax, a pro-apoptotic gene which is a direct transcriptional target of p53 [14], have been associated with poor responses to chemotherapy and shorter overall survival in women with metastatic breast cancer [15]. Conversely, the anti-apoptotic protein Bcl-2 is found at high levels in early-stage breast cancers, particularly those that are estrogen receptor (ER) positive and p53 immunonegative (i.e., lacking mutant p53) (reviewed in 16). Other than Bcl-2 and Bax, however, no published reports exist to date which have explored the expression of other members of the Bcl-2 family in breast cancers. Also unaddressed is the expression of apoptosis-regulating proteins such as BAG-1 that lack homology with Bcl-2, but which can interact with certain Bcl-2 family proteins and modulate their functions [17]. Expression of ICE mRNA has been reported to increase in normal mammary epithelial cells when deprived of adhesion via integrins to extracellular matrix proteins [18], but it remains unresolved whether other ICE-family proteins such as CPP32 are expressed in breast cancers. In this report, we used monospecific antibodies, immunoblotting, and immunohistochemical methods to analyze the relative levels of the Bcl-2, Bcl-X_L, Mcl-1, Bax, Bak, BAG-1, and CPP32 proteins in human breast cancer cell lines and primary tumors.

Materials and methods

Immunoblotting

Frozen normal mammary tissue and 3 primary breast adenocarcinoma specimens were obtained from the UCSD Tumor Bank, ground to a fine powder in liquid nitrogen using a mortar and pestle, and then homogenized in RIPA buffer [16]. Normal mammary cell lines MCF-10A and 184-A1N4 were

obtained from Dr. D. Salomon (NCI, Bethesda, MD). Other lines were obtained from ATCC. All cell lines were maintained in culture using either DMEM (high glucose) or RPMI medium supplemented with 10% fetal bovine serum, 1 mM L-glutamine, antibiotics, and other additives as per the supplier's recommendations. Detergent-lysates were prepared from subconfluent cultures of cells, normalized for total protein content (50 μg per lane), and subjected to SDS-PAGE and subsequent immunoblotting to nitrocellulose membranes as described [18]. Filters were sequentially incubated with various polyclonal rabbit antisera (Bcl-2, Bcl-X, Mcl-1, Bax, Bak, CPP32, Raf-1) [15, 19–23] or monoclonal antibodies (BAG-1, p53) [24] at 0.1% (v/v), followed by enhanced chemiluminescence (ECL)-based detection using a novel multiple antigen detection (MAD) immunoblotting method that allows for multiple reprobings of blots without antibody stripping [25]. Data were quantified by scanning densitometry and expressed in arbitrary units reflecting the area under the tracings. Statistical comparisons were made by simple correlation analysis using the JMP package of biostatistics programs (SAS Institute, Inc.).

Immunocytochemistry

For immunohistochemical assays, 20 breast cancer biopsies were randomly chosen that had been predetermined to contain residual normal mammary epithelium along with invasive carcinoma and carcinoma *in situ* (n = 6). Tissue sections (0.5 μm) were prepared from the paraffin blocks and immunostained with polyclonal rabbit antisera specific for Bcl-2, Bcl-X, Mcl-1, Bax, Bak, or CPP32, or with monoclonal antibodies specific for BAG-1, using a diaminobenzidine (DAB)-based detection method as described in detail [21, 22, 26, 27]. Nuclei were counterstained with hematoxylin. As controls, the immunostaining procedure was performed in parallel using preimmune serum or a mouse IgG control monoclonal antibody (Dako, Denmark) to verify specificity of the results. Also, in some cases, the antiserum was preadsorbed with relevant peptide or protein antigens, further confirming the immunospecificity. The approximate percentages of immunopositive cells were scored separately for all three components: normal, *in situ*, invasive cancer. The intensity of the immunostaining was also evaluated in side-by-side comparisons of invasive cancer and carcinoma *in situ* with adjacent areas of normal mammary epithelium, scoring the intensity as greater than, less than, or equal to normal adjacent mammary epithelium (T > N; T < N; T = N). In addition, intensity was scored on an arbitrary 4-point scale as: 0, negative; 1, weak; 2, moderate; and 3, strong. Statistical comparisons were performed for percent immunopositivity data by unpaired t-test and for intensity by Pearson chi-square analysis using a 2×3 matrix.

Results

Figure 1 shows an example of the immunoblot results obtained for the cell lines and Table 1 summarizes the findings. The 26-kDa Bcl-2-α protein was detected in 7 of the 11 cell lines (MCF-10A, MD-MB-A231, MD-MB-A468, BT-474, BT-549, MCF-7, T-47D), though the levels of Bcl-2 were quite low in 3 cases of these (MCF-10A, MDA-MB-468, T-47D). No Bcl-2-β protein (∼ 22 kDa) was seen. Six of the breast lines contained small amounts of the ∼ 30 kDa form of Bcl-2, including MDA-MB-231, MDA-MB-468, BT-20, BT-549, MCF-7, and T-47D. This p30-Bcl-2 band co-migrated in SDS-PAGE experiments with the ∼ 30 kDa Bcl-2 protein found in colorectal cancer cell lines, where Bcl-2 has been shown to be phosphorylated [28]. Moreover, treatment of the lysates from breast cancer cell lines with alkaline phosphatase converted the mobility of the p30-Bcl-2 protein to the usual 26-kDa size (data not shown), consistent with p30-Bcl-2 representing a phosphorylated form of the protein.

The Bcl-X_L protein, which typically migrates as a doublet at ∼ 29–30 kDa [22], was easily detected in all of the cell lines, except BT549 which contained only a very faint Bcl-X_L band. Considerably higher relative levels of Bcl-X_L were found in both of the immortalized mammary epithelial cell lines (MCF-10A, 184-A1N4) and in the MDA-MB-231,

MDA-MB-468, BT-474, and T-47D carcinoma cell lines. No Bcl-X_S or other isoforms of Bcl-X were detected in these cell lines. Mcl-1 was present at readily detectable levels in lysates derived from all cell lines, except T-47D which contained only a very weak intensity band corresponding to Mcl-1. The highest levels of Mcl-1 were found in BT-20 and BT-474 cells, with intermediate relative levels in the BT-549 and MCF-7 cancer cell lines and in the MCF-10A immortalized mammary epithelial cell line. The Bcl-2 binding protein, BAG-1, was detected in all cell lines, except Hs-578 and ZR-75-1. The relative levels of BAG-1 protein were variable, with BT-474 and MCF-7 having the highest amounts of this anti-apoptotic protein.

The pro-apoptotic Bcl-2 family proteins Bax and Bak were expressed in all 11 cell lines. The lowest relative levels of Bax were found in BT-474 cells and the highest were in MCF7 cells. Relatively lower levels of Bak were present in 184-A1N4, MDA-MB-231, MDA-MB-468, BT-20, BT-474, and BT-549 cells, whereas relatively higher amounts of Bak were found in MCF-10A, Hs-578, MCF-7, T-47D, and ZR-75-1 cells. Among the 11 breast lines, only MCF-7 failed to express CPP32, which was present as the expected $\sim$ 32 kDa proenzyme in all other cell lines (Figure 10). Reprobing the blots with an antiserum specific for the serine/threonine protein kinase Raf-1 verified loading of approximately equivalent amounts of intact proteins for all cell lines (Figure 1).

Immunoblot analysis of the p53 protein in these 11 cell lines using a monoclonal antibody that reacts with both wild-type and mutant p53 proteins revealed the presence of immunodetectable p53 in 8 of them. This presumably reflects the presence of stabilized mutant p53 protein, and is consistent with the DNA sequence data which is available from the

Table 1. Relative expression of Bcl-2 family proteins, BAG-1, CPP32, and status of estrogen receptor (ER) and p53 in breast cancer cell lines

Cell lines	Source	Bcl-2	Bcl-X_L	Mcl-1	BAG-1	Bax	Bak	p53	CPP32	ER
MCF-10A	Fibrocystic breast disease normal ductal cells	1.0	1.0	1.0	1.0	1.0	1.0	wt	1.0	–
184-A1N4	Mammary epithelium	0	2.0	0.4	3.5	1.3	0.3	wt	1.3	n.d.
MDA-MB-231	Adenocarcinoma	4.1	1.5	0.2	1.3	0.9	0.5	mut	2.7	–
MDA-MB-468	Infiltrating adeno-carcinoma	0.9	1.7	0.2	2.3	0.7	0.6	mut	2.9	–
BT-20	Adenocarcinoma	0	0.4	1.0	0.4	0.7	0.4	mut	4.1	–
BT-474	Ductal carcinoma	5.9	1.6	1.1	3.9	0.3	0.4	mut	4.1	+
BT-549	Papillary infiltrating ductal carcinoma	4.6	0.2	0.6	2.5	0.8	0.3	mut	2.4	–
HS-578	Ductal carcinoma	0	0.4	0.3	0	1.1	1.0	mut	3.2	–
MCF-7	Infiltrating ductal adenocarcinoma	7.6	0.4	0.8	3.5	1.6	0.9	wt	0	+
T-47D	Infiltrating ductal carcinoma	0.3	1.2	0.1	1.5	0.3	1.2	mut	2.2	+
ZR-75-1	Infiltrating ductal carcinoma	0	0.5	0.4	0	1.1	0.8	wt*	3.3	+

mut = mutant, n.d. = not determined, wt = wild-type.

Summary of apoptosis-regulating proteins and other characteristics of breast cancer cell lines. Immunoblot data were quantified by scanning densitometry and expressed in arbitrary absorbance units X mm^3 and arbitrarily normalized relative to the MCF-10A cell line ('normal' ductal cells from fibrocystic breast disease). All samples (50 μg total protein) were run in the same gel and analyzed side-by-side to avoid variations due to differences in the efficiency of protein transfer and antibody-based detection during immunoblotting experiments. For the purposes of quantification, only the p26-Bcl-2 band was considered (excludes 30 kDa phospho Bcl-2 which may be an inactive version of Bcl-2; ref. 36). Data represent the mean of two experiments (std dev < 15%).

* ZR75-1 cells contained p53 protein but have been reported to have wild-type p53 genes [29]. (wt = wild-type; mut = mutant; n.d. = not determined).

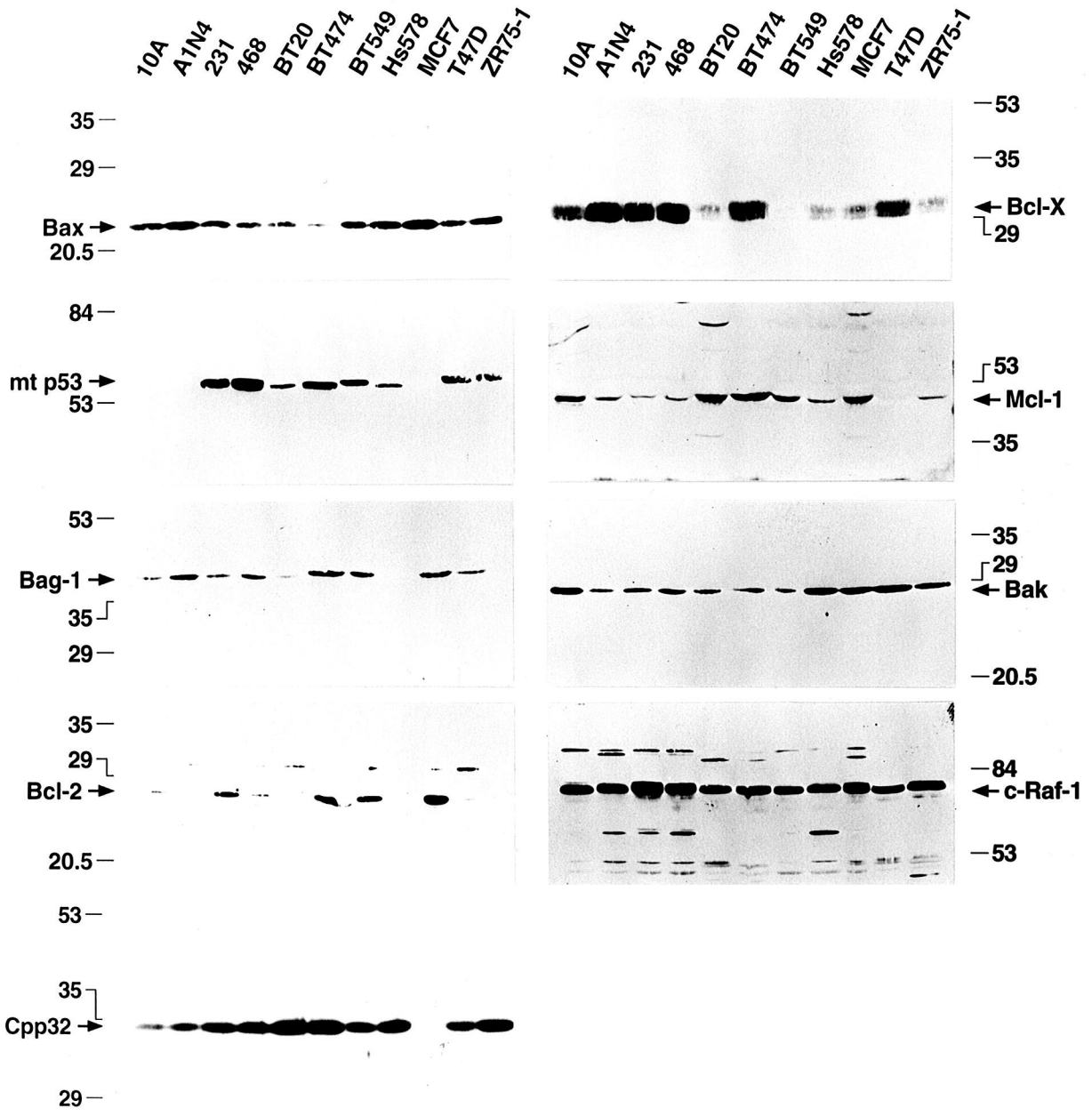

Figure 1. Immunoblot analysis of apoptosis-modulating proteins in breast cancer cell lines. Lysates from breast cancer lines (50 μg per lane) were subjected to SDS-PAGE (12% gel) and transferred to nitrocellulose paper. Antigens were detected on the blot by an ECL-based method involving sequential incubation with various antibodies as indicated [25].

literature for 8 of 9 of these cell lines [29, 30]. The one exception is ZR75-1, which has been reported to have wild-type p53 genes but which was p53 immunopositive both here and in other reports [29]. The relative levels of p53 protein in these 8 cell lines were highly variable.

Correlations of the intensity of the bands obtained by densitometric analysis of the immunoblotting results revealed statistically significant associations between p53 and Bax, which were inversely correlated ($r = -0.73$; $p = 0.01$). Bax was also inversely correlated with CPP32 ($r = -0.80$; $p =$

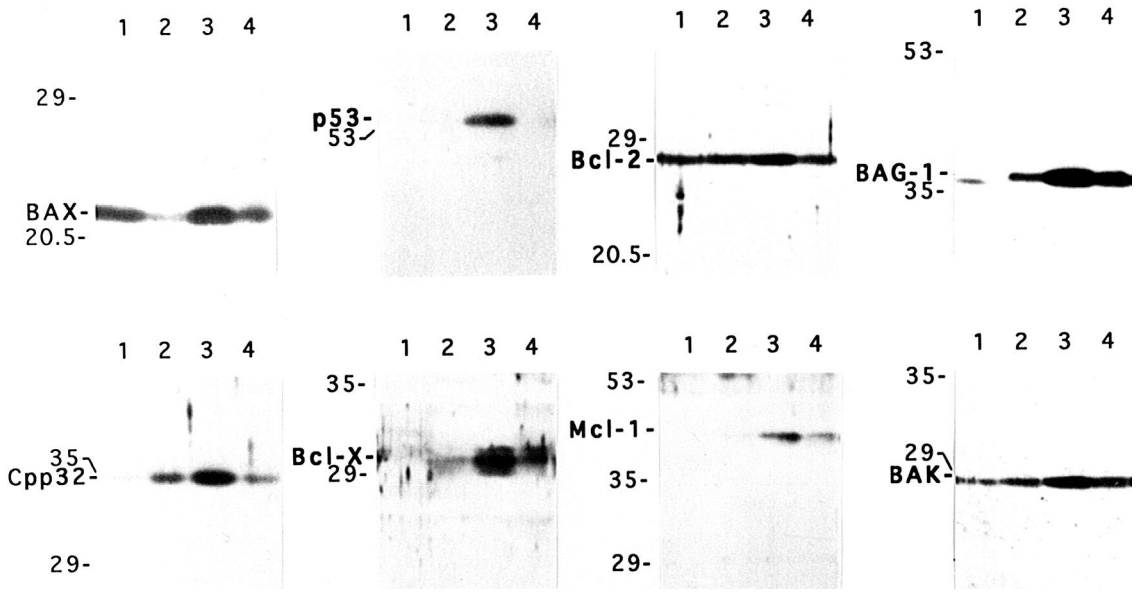

Figure 2. Analysis of apoptosis-regulatory proteins in primary breast cancers. Lysates from normal mammary tissue (lane 1) and three primary breast tumors (lanes 2–4) were normalized for total protein content (50 μg per lane) and relative levels of various apoptosis-regulating proteins were evaluated by immunoblotting.

0.003). Bcl-2 and BAG-1 were positively correlated ($r = 0.65$; $p = 0.03$). Otherwise, no statistically significant correlations were noted when all 11 cell lines were included in the analysis. When the 2 immortalized epithelial cell lines were excluded, then p53 correlated positively with Bcl-X_L ($r = 0.82$; $p = 0.007$). The correlations noted between Bcl-2/BAG-1, p53/Bax, and Bax/CPP32 remained significant ($p = 0.004$; 0.03; 0.008, respectively), even when the MCF-10A and 184-A1N4 mammary epithelial cell lines were excluded from the analysis. Significant correlations were not detected between Bcl-2 family, p53, BAG-1, or CPP32 proteins and ER status.

The expression of these apoptosis-regulating proteins was also evaluated by immunoblotting in 3 primary breast cancers, making comparisons with a single sample of normal mammary tissue (Figure 2).

Table 2. Direct comparison of immunointensity in normal mammary epithelium and neoplastic lesions

Relative intensity	Bcl-2		Bcl-X		Mcl-1		BAG-1		Bax		Bak		CPP32	
	In situ	Invasive cancer	*In situ*	Invasive cancer	*In situ*	Invasive cancer	*In situ*	Invasive cancer	*In situ*	Invasive cancer	*In situ*	Invasive cancer	*In situ*	Invasive cancer
T < N	10/19 (53%)	12/19 (63%)	4/20 (20%)	3/20 (15%)	2/20 (10%)	5/20 (25%)	3/17 (18%)	3/20 (15%)	2/18 (11%)	4/20 (20%)	3/14 (22%)	3/20 (15%)	0/14 (0%)	0/20 (0%)
T = N	2/19 (11%)	1/19 (5%)	9/20 (45%)	8/20 (40%)	18/20 (90%)	15/20 (75%)	6/17 (35%)	8/20 (40%)	4/18 (22%)	4/20 (20%)	9/14 (64%)	12/20 (60%)	3/14 (21%)	3/20 (15%)
T > N	7/19 (36%)	6/19 (32%)	7/20 (35%)	9/20 (45%)	0/20 (0%)	0/20 (0%)	8/17 (47%)	9/20 (45%)	12/18 (67%)	12/20 (60%)	2/14 (14%)	5/20 (25%)	11/14 (79%)	17/20 (85%)

Immunointensity was compared between normal mammary epithelium (N) and adjacent carcinoma *in situ* or invasive cancer (T) using 20 breast cancer biopsies. In a few cases, immunostaining was either unsuccessful or no invasive carcinoma or carcinoma *in situ* was present in the tissue section used for evaluation, thus resulting in denominators of < 20. The intensity scoring only included immunopositive cells, and did not take into consideration any cells which were immunonegative.

Bcl-2 protein was present at similar levels in all 3 tumor specimens and the normal mammary tissue sample. In contrast, Bcl-X_L protein was detected by immunoblotting in all 3 cancer specimens but not in the normal mammary tissue specimen. Mcl-1 was present at easily detectable levels in 2 of the 3 breast carcinoma specimens but was just barely detectable in the other tumor and was undetectable in normal mammary tissue. BAG-1 was observed in all specimens, but was clearly higher in the 3 carcinomas compared to the specimen of normal mammary epithelium. Only one of the primary tumors contained immunodetectable p53 protein, suggesting the presence of mutant p53 in this specimen. Bax protein was present in normal mammary epithelium and all 3 tumor specimens but was markedly reduced compared to normal mammary tissue in one of the three tumors, consistent with a previous study which demonstrated reduced Bax in ~ one-third of breast adenocarcinomas [15]. Bak was expressed in all samples, with slightly higher levels in the three primary tumors compared to normal mammary gland. Finally, CPP32 protease was immunodetectable in all samples, but was clearly higher in the 3 carcinoma specimens compared to the normal mammary gland sample (Figure 2).

Taken together, these data confirm the results obtained using cell lines, establishing that primary adenocarcinomas of the breast have the potential to express multiple apoptosis-regulatory genes. No

Table 3. Immunointensity scores for normal breast epithelium, carcinoma *in situ*, and invasive breast cancer.

Intensity	Bcl-2			Bcl-X			Mcl-1			BAG-1		
	N	*In situ*	Invasive cancer	N	*In situ*	Invasive cancer	N	*In situ*	Invasive cancer	N	*In situ*	Invasive cancer
0	0/19 (0%)	2/19 (11%)	2/19 (11%)	0/20 (0%)	2/20 (10%)	1/20 (5%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	1/17 (0%)
1	1/19 (6%)	6/19 (31%)	7/19 (37%)	13/20 (65%)	10/20 (50%)	6/20 (30%)	15/20 (75%)	17/20 (85%)	20/20 (100%)	9/20 (45%)	9/20 (45%)	6/17 (35%)
2	9/19 (47%)	4/19 (21%)	5/19 (26%)	7/20 (35%)	6/20 (30%)	12/20 (60%)	5/20 (25%)	3/20 (15%)	0/20 (0%)	10/20 (50%)	3/20 (15%)	7/17 (41%)
3	9/19 (47%)	7/19 (37%)	5/19 (26%)	0/20 (0%)	2/20 (10%)	1/20 (5%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	1/20 (5%)	8/20 (40%)	4/17 (24%)
p-value	–	0.05	0.03	–	NS	NS	–	NS	0.02	–	0.01	NS

Intensity	Bax			Bak			CPP32		
	N	*In situ*	Invasive cancer	N	*In situ*	Invasive cancer	N	*In situ*	Invasive cancer
0	1/20 (5%)	0/19 (0%)	0/20 (0%)	0/20 (0%)	0/14 (0%)	0/20 (0%)	2/20 (10%)	0/14 (0%)	0/20 (0%)
1	9/20 (45%)	5/19 (26%)	7/20 (35%)	7/20 (35%)	7/14 (50%)	9/20 (45%)	14/20 (70%)	2/14 (14%)	2/20 (10%)
2	9/20 (45%)	9/19 (47%)	7/20 (35%)	12/20 (60%)	6/14 (43%)	9/20 (45%)	4/20 (20%)	8/14 (57%)	8/20 (40%)
3	1/20 (5%)	5/19 (26%)	6/20 (30%)	1/20 (5%)	1/14 (7%)	2/20 (10%)	0/20 (0%)	4/14 (29%)	10/20 (50%)
p-value	–	NS	0.03	–	NS	NS	–	0.01	0.0001

Immunointensity scores for normal breast epithelium, carcinoma *in situ*, and invasive breast cancer. The intensity of the immunostaining was evaluated in usual mammary epithelium (N), carcinoma *in situ* or invasive cancer from 20 breast cancer biopsies, scoring the intensity on an arbitrary 4-point scale as 0, negative; 1, weak; 2, moderate; 3, strong. In a few cases, the immunostaining was either unsuccessful or no invasive carcinoma or carcinoma *in situ* was present in the tissue section used for evaluation, thus resulting in demoninators of < 20. Statistical comparisons were performed for intensity by Pearson chi-square analysis using a 2×3 matrix. NS = no significance.

quantitative relations between normal and malignant breast tissue should be inferred however from the immunoblot analysis presented in Figure 2, given that (a) paired samples of tumor and adjacent normal tissue were not employed; (b) these tissue specimens are heterogeneous with respect to their proportions of epithelial and stromal cells; and (c) the relative levels of some Bcl-2 family proteins may fluctuate in normal mammary epithelium with the estrous cycle [31].

Immunohistochemical analysis of the expression of these apoptosis-regulatory proteins was also performed using 20 breast cancer biopsy specimens which were chosen for their concomitant presence of residual normal mammary epithelium which permitted direct comparisons between normal and malignant cells. Co-existing carcinoma *in situ* was also present in these twenty biopsies. Table 2 summarizes the results, where direct side by side comparisons were made between the intensity of the immunostaining among the immunopositive cells (ie. not considering any immunonegative tumor cells) within normal mammary epithelium, carcinoma *in situ*, and invasive cancers.

Though this type of analysis precluded a statistical evaluation, it appeared that Bcl-2 immunointensity was only rarely equivalent in neoplastic and normal cells (T = N in 5% of invasive cancers and 11% of carcinomas *in situ*. Rather, in most invasive cancers (12/19 [63%)]) and carcinoma *in situ* lesions (10/19 [53%]), the intensity of Bcl-2 immunostaining was lower in the neoplastic cells compared to the normal cells (Table 2). In contrast, the intensity of Bcl-X staining in invasive cancers and carcinoma *in situ* lesions was generally equal to or greater than normal mammary epithelium (17/20 [85%] invasive cancers and 16/20 [80%] *in situ* lesions). Though the antibody employed for these studies can potentially react with both the anti-apoptotic Bcl-X_L and pro-apoptotic Bcl-X_S proteins [22], the immunoblot analysis of breast cancer lines and primary tumor specimens suggests that the immunostaining is likely to be accounted for by Bcl-X_L protein. Mcl-1, another anti-apoptotic member of the Bcl-2 family, was mostly expressed at levels similar to normal mammary epithelium (T = N in 75% invasive and 90% of carcinomas *in situ*), but was sometimes present at lower immunointensity levels in neoplastic cells (Table 2). In contrast, in nearly half of invasive cancers (9/20 [45%]) and carcinomas *in situ* (8/17 [47%]), the intensity of immunostaining for the anti-apoptotic protein BAG-1 was higher than normal. Among the pro-apoptotic proteins examined here (Bax, Bak, CPP32), the clearest trend was observed for the cell death protease CPP32 (Caspase-3), which was expressed at levels higher than adjacent normal mammary epithelium in 17/20 (85%) invasive carcinomas and 11/14 (79%) carcinomas *in situ*. This apparent upregulation of CPP32 in breast cancers reflected the observation that normal mammary epithelium expresses CPP32 at relatively low levels, based on immunohistochemical analysis [32]. Surprisingly, the intensity of Bax immunoreactivity was not uncommonly higher in neoplastic cells compared to normal epithelium (12/20 [60%] invasive cancers and 12/18 [67%] carcinomas *in situ*) (Table 2), but there were also often many tumor cells present which failed to express Bax altogether which were not factored into the immunointensity scoring (see below).

An alternative method for scoring relative intensities of immunostaining in normal, *in situ*, and invasive cancer was employed, which lends itself to statistical analysis (Pearson correlations). In this approach, an arbitrary 4-point scale was used to score immunointensity as negative, weak, moderate or strong (Table 3). Compared to normal mammary epithelium, the intensity of Bcl-2 immunostaining was significantly reduced in invasive cancers ($p = 0.03$) and carcinomas *in situ* ($p = 0.05$). The intensity of Mcl-1 was also significantly lower in carcinomas than in normal mammary epithelium ($p < 0.02$) but not in carcinomas *in situ*. However, unlike Bcl-2, the differences were attributed entirely to slightly higher (2+ vs 1+) intensity immunostaining in 5 (25%) of the 20 normal epithelial samples (Table 3), and thus must be interpreted with caution. In contrast to the anti-apoptotic proteins, the intensity of Bax immunostaining was significantly higher among invasive carcinomas than normal epithelium ($p = 0.03$), but here again, the differences could be attributed to a small proportion of cases (6/20 carcinomas versus 1/20 normals with strong intensity). However, the intensity of CPP32 immunostaining was clearly

higher among invasive cancers and some carcinoma *in situ*. For example, half (10/20 [50%]) of the invasive cancers exhibited strong (3+) CPP32 immunointensity, compared to none of the normal breast epithelial samples. No statistically significant differences in the intensity of immunostaining were noted during comparisons of normal mammary epithelium and invasive breast cancer for Bak or bcl-x when the immunointensity was scored using the 0–3 scale. Finally, the intensity of BAG-1 was significantly higher among carcinoma *in situ* than normal epithelium ($p = 0.01$) but not in invasive cancer.

Comparisons of the approximate percentages of immunopositive tumor and normal cells were also performed using the same immunostained slides. The percentages of immunonegative cells varied widely among invasive cancers and carcinomas *in situ* for all apoptosis-regulatory proteins examined, whereas the results for normal mammary epithelium were far more homogeneous (data not shown). Consequently, statistically significant differences between normal epithelium and both invasive cancers and carcinomas *in situ* were obtained for all proteins examined ($p < 0.05$). The biological significance of these results however remains to be determined. No significant differences were noted with regards to the percentages of immunopositive cells within invasive cancers compared to carcinoma *in situ* lesions, with the exception of BAG-1. The percentages of BAG-1 immunopositive cells were slightly lower within invasive cancer ($83 \pm 3\%$ [range 50–95%] versus $91 \pm 3\%$ [range 70–95%]) ($p = 0.05$).

Discussion

The data presented here indicate that several Bcl-2 family proteins, as well as the Bcl-2 binding protein BAG-1 and the ICE-family protease CPP32 (Caspase-3), are expressed in the majority of breast cancer cell lines and primary tumors. The multitude of apoptosis-regulating proteins expressed in these tumor cells and the complex interactions among these proteins suggest that the relative resistance to apoptosis in individual cases of breast cancer will be difficult to predict from merely monitoring whether or not any particular one of these proteins is present. This complexity may underlie, for example, the paradoxical observation that Bcl-2 expression is generally associated with more favorable outcome in women with both node-negative and node-positive disease, as well as in breast cancer patients with metastatic disease (reviewed in 33). Namely, assessment of Bcl-2 alone may fail to give a complete picture of the overall repertoire of Bcl-2 family and other apoptosis-modulating proteins in these neoplasms.

With some notable exceptions, the relative levels of the Bcl-2 family proteins, BAG-1, CPP32, and mutant p53 in breast cancer cell lines were not regulated in discernible patterns that would suggest commonalties in the mechanisms that control the expression of these apoptosis-modulating proteins or functional links between them. Thus, for the most part, these various apoptosis-controlling genes are probably independently regulated. This observation suggests that the genetic and environmental factors that account for dysregulation of programmed cell death pathways in breast cancers are likely to be multifactorial.

Though the data should be viewed as highly preliminary given the small number of tumor lines examined and the semi-quantitative immunoblotting method used to assess the relative levels of apoptosis-modulating proteins, it is of interest that an inverse correlation was observed between p53 and Bax. Assuming that higher levels of p53 derived from stabilizing mutations that inactivate the protein, this finding is consistent with the previous discovery that the Bax gene promoter contains typical consensus sequences for p53-binding and can be directly transcriptionally activated by p53 [14]. However, the veritable absence of immunodetectable wild-type p53 protein in breast cancer cell lines such as MCF-7 in which both alleles of p53 have been reported to be unmutated [34], suggests that other factors besides p53 account for the relatively higher expression of Bax in tumor lines with wild-type p53. Moreover, in a recent comparison of Bax and nuclear p53 immunostaining in primary breast cancers, no significant correlation was found between the expression of these two apoptosis-regulating proteins [35]. Nevertheless, it may be of biological and

clinical importance that some tumors which have mutant p53 also have reduced Bax, the predicted outcome of which would be a highly apoptosis-resistant state. Thus, more advanced breast cancers with mutant p53 may be resistant to chemotherapy and radiation-induced apoptosis not only because they would fail to upregulate wild-type p53 and consequently trans-activate the Bax gene promoter when confronted with genotoxic stress, but also because their basal expression of Bax tends to be lower than tumors with wild-type p53. In addition, when the two immortalized mammary epithelial lines were omitted and only the 9 breast cancer lines were included in the statistical analysis, an association between mutant p53 and higher levels of Bcl-X_L protein was also found. Thus, more advanced tumors with p53 gene mutations may also have a tendency to produce more Bcl-X_L, which could further contribute to reduced sensitivity to apoptotic stimuli.

The finding of Bax protein in all 9 breast cancer cell lines examined here is at odds with recent reports that breast cancer cell lines and primary adenocarcinomas of the breast almost uniformly contain little or no detectable Bax mRNA [36, 37]. Probably the most reasonable explanation for this discrepancy is that detection of mRNA can be more difficult and less sensitive than protein detection methods. Translational or post-translational control of Bax expression however could also contribute to the reported differences, and indeed examples of differential regulation of Bax protein half-life have been described [38].

This report provides the first evidence of BAG-1 expression in breast cancers. Interestingly, the intensity of BAG-1 immunostaining was often higher in invasive cancers compared to normal epithelium. The BAG-1 protein binds to and modulates the chaperone activity of $\sim$ 70 kDa family heat shock proteins [39]. BAG-1 has also been reported to interact with activated progesterone and other steroid hormone receptors [40], suggesting that it may perform other functions in cells besides cooperating with Bcl-2 in the suppression of cell death. BAG-1 also can bind to and increase the enzymatic activity of the serine/threonine protein kinase Raf-1, through a Ras-independent mechanism [41]. How these various activities of the BAG-1 protein impact on the pathogenesis and progression of breast cancers remains to be determined. However, the frequent expression of this multifunctional protein in breast cancers suggests the need to evaluate the effects of BAG-1 on cell death, proliferation, and chemoresistance in future studies of adenocarcinomas of the breast.

It may be of interest that of all the breast cancer cell lines (n = 9), immortalized mammary epithelial lines (n = 2), and primary normal and malignant breast biopsy specimens (n = 4) tested here, only one was immunonegative for CPP32, i.e., the MCF-7 breast cancer line. MCF-7 is probably the most commonly used cell line model for studying breast cancer cell behavior. The absence of CPP32 protein in MCF-7 cells, therefore, suggests that (a) CPP32 expression may become down-regulated or entirely lost in some breast cancers as a mechanism for gaining a survival advantage; and (b) MCF-7 may be aberrant compared to most other breast cancers, which apparently retain expression of this ICE-family protease which has been implicated in the execution-phase of apoptosis. In this regard, the immunohistochemical analysis of invasive carcinomas and *in situ* cancers performed here also demonstrated the presence of CPP32 immunostaining in all specimens studied, further suggesting that the absence of CPP32 in MCF7 cells may be unusual. Consequently, this finding raises some caveats about the excessive use of this particular tumor line as a model for understanding the apoptotic responses of breast cancers.

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References

1. Nowell PC: The clonal evolution of tumor cell populations. Science 194: 23–28, 1976
2. Reed JC: Bcl-2 and the regulation of programmed cell death. J Cell Biol 124: 1–6, 1994
3. Reed JC: Bcl-2: Prevention of apoptosis as a mechanism of drug resistance. Hematology/Oncology Clinics of North America 9: 451–474, 1995
4. Vaux DL, Haecker G, Strasser A: An evolutionary perspective on apoptosis. Cell 76: 777–779, 1994
5. Reed JC: Regulation of apoptosis by Bcl-2 family proteins and its role in cancer and chemoresistance. Curr Opin Oncol 7: 541–546, 1995
6. Patel T, Gores GJ, Kaufmann SH: The role of proteases during apoptosis. FASEB J 10: 587–597, 1996
7. Steck S, McDonnell T, Sneige N, El-Naggar A: Flow cytometry analysis of apoptosis and Bcl-2 in primary breast carcinomas: clinical and biological implications. Cytometry 24: 116–122, 1996
8. Neville MC, Daniel CW: The Mammary Gland: Development, Regulation and Function. Plenum Publishing, 1987
9. Wang TTY, Phang JM: Effects of estrogen on apoptotic pathways in human breast cancer cell line MCF-7. Cancer Res 55: 2487–2489, 1995
10. Shibata M-A, Maroulakou IG, Jorcyk CL, Gold LG, Ward JM, Green JE: p53-independent apoptosis during mammary tumor progression in C3(1)/SV40 large T antigen transgenic mice: suppression of apoptosis during the transition from preneoplasia to carcinoma. Cancer Res 56: 2998–3003, 1996
11. Pullan S, Wilson J, Metcalfe A, Edwards GM, Goberdhan N, Tilly J, Hickman JA, Dive C, Streuli CH: Requirement of basement membrane for the suppression of programmed cell death in mammary epithelium. J Cell Science 109: 631–642, 1996
12. Wingo PA, Tong T, Bolden S: Cancer Statistics, 1995. CA, Cancer J Clinic 45: 8–30, 1995
13. Thor AD, Moore DH II, Edgerton SM, Kawasaki ES, Reihsaus E, Kynch HT, Marcus JN, Schwartz L, Chen L-C, Mayall BH, Smith HS: Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. J Natl Cancer Inst 84: 845–855, 1992
14. Miyashita T, Reed JC: Tumor suppressor p53 is a direct transcriptional activator of human bax gene. Cell 80: 293–299, 1995
15. Krajewski S, Blomvqvist C, Franssila K, Krajewska M, Wasenius V-M, Niskanen E, Reed JC: Reduced expression of pro-apoptotic gene Bax is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. Cancer Res 55: 4471–4478, 1995
16. Krajewski S, Chatten J, Hanada M, Womer R, Reed JC: Immunohistochemical analysis of the Bcl-2 oncoprotein in human neuroblastoma. Lab Invest 71: 42–54, 1995
17. Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA, Reed JC: Cloning and functional analysis of BAG-1: a novel Bcl-2 binding protein with anti-cell death activity. Cell 80: 279–284, 1995
18. Boudreau N, Sympson CJ, Werb Z, Bissell MJ: Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science 267: 891–893, 1995
19. Krajewski S, Bodrug S, Gascoyne R, Berean K, Krajewska M, Reed JC: Immunohistochemical analysis of mcl-1 and bcl-2 proteins in normal and neoplastic lymph nodes. Amer J Pathol 145: 515–525, 1994
20. Krajewski S, Gascoyne RD, Zapata JM, Krajewska M, Kitada S, Chhanabhai M, Horsman D, Berean K, Piro LD, Fugier-Vivier I, Liu YJ, Wang HG, Reed JC: Immunolocalization of the ICE/Ced-3-family protease, CPP32 (Caspase-3), in non-Hodgkin's lymphomas (NHLs), chronic lymphocytic leukemias (CLL), and reactive lymph nodes. Blood 89: 3817–3825, 1997
21. Krajewski S, Krajewska M, Reed JC: Immunohistochemical analysis of *in vivo* patterns of *Bak* expression, a pro-apoptotic member of the Bcl-2 protein family. Cancer Res 56: 2849–2855, 1996
22. Krajewski S, Krajewska M, Shabaik A, Wang H-G, Irie S, Fong L, Reed JC: Immunohistochemical analysis of *in vivo* patterns of Bcl-X expression. Cancer Res 54: 5501–5507, 1994
23. Reed JC, Yum S, Cuddy MP, Turner BC, Rapp UR: Differential regulation of the p72–74 Raf-1 kinase by RAS and SRC oncoproteins. Cell Growth & Diff 2: 235–243, 1991
24. Takayama S, Kochel K, Irie S, Inazawa J, Abe T, Sato T, Druck T, Huebner K, Reed JC: Cloning of cDNAs encoding human BAG1 protein and localization of the human BAG1 gene to chromosome 9p12. Genomics 35: 494–498, 1996
25. Krajewski S, Zapata JM, Reed JC: Detection of multiple antigens on Western blots. Anal Biochem 236: 221–228, 1996
26. Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang H-G, Reed JC: Immunohistochemical determination of *in vivo* distribution of bax, a dominant inhibitor of bcl-2. Am J Pathol 145: 1323–1333, 1994
27. Reed JC, Krajewski S, Kitada S, Miyashita T: Methods of measuring Bcl-2 gene expression. In: Herzenberg LA et al (ed) Handbook of Experimental Immunology, 5th edition, Vol. 3, pp 128.1–128.11, 1996
28. Guan RJ, Moss SF, Arber N, Krajewski S, Reed JC, Holt PR: 30 kDa phosphorylated form of Bcl-2 protein in human colon. Oncogene 12: 2605–2609, 1996
29. Bartek J, Iggo R, Gannon J, Lane DP: Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene 5: 893–899, 1990
30. Runnebaum IB, Nagarajan M, Bowman M, Soto D, Suku-

mar S: Mutations in p53 as potential molecular markers for human breast cancer. Proc Natl Acad Sci USA 88: 10657–10661, 1991

31. Sabourin JC, Martin A, Baruch J, Truc JB, Gompel A, Poitout P: bcl-2 expression in normal breast tissue during the menstrual cycle. Int J Cancer 59: 1–6, 1994
32. Krajewska M, Wang H-G, Krajewski S, Zapata J, Shabaik A, Gascoyne R, Reed JC: Immunohistochemical analysis of *in vivo* patterns of expression of CPP32 (Caspase-3), a cell death protease. Cancer Res 57: 1605–1613, 1997
33. Reed JC: Balancing cell life and death: Bax, apoptosis, and breast cancer. J Clin Invest 97: 2403–2404, 1996
34. Casey G, Lo-Hsueh M, Lopez ME, Vogelstein B, Stanbridge EJ: Growth suppression of human breast cancer cells by the introduction of a wild-type p53 gene. Oncogene 6: 1791–1797, 1991
35. Krajewski S, Thor AD, Edgerton SU, Moore DH, Krajewska N, Reed JC: Immunohistochemical analysis of Bax and Bcl-2 in p53-immunopositive breast cancers. Clin Cancer Res 3: 199–208, 1996
36. Bargou RC, Daniel PT, Mapara MY, Bommert K, Wagener C, Kallinich B, Royer HD, Dörken B: Expression of the bcl-2 gene family in normal and malignant breast tissue: low bax-α expression in tumor cells correlates with resistance towards apoptosis. Int J Cancer 60: 854–859, 1995
37. Wagener C, Bargou RC, Daniel PT, Bommert K, Mapara MY, Royer HD, Dörken B: Induction of the death-promoting gene bax-alpha sensitizes cultured breast-cancer cells to drug-induced apoptosis. Int J Cancer 67: 138–141, 1996
38. Miyashita T, Kitada S, Krajewski S, Horne B, Delia D, Reed JC: Overexpression of the Bcl-2 protein increases the half-life of p21-Bax. J Biol Chem 270: 26049–26052, 1995
39. Takayama S, Bimston DN, Matsuzawa S, Freeman BC, Aime-Sempe C, Xie Z, Morimoto RJ, Reed JC: BAG-1 modulates the chaperone activity of Hsp70/Hsc70. EMBO J in press, 1997
40. Zeinet M, Gehring U: A protein that interacts with members of the nuclear hormone receptor family: identification and cDNA cloning. Proc Natl Acad Sci USA 92: 11465–11469, 1995
41. Wang H-G, Takayama S, Rapp UR, Reed JC: Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. Proc Natl Acad Sci USA 93: 7063–7068, 1996