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Utilizing Chimeric Proteins for Exploring the Cellular Fate of Endogenous Proteins

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We recently designed and constructed chimeric proteins for the elimination of specific cell populations. These chimeric proteins are composed of a targeting component fused to an apoptotic protein as the killing moiety. However, chimeric proteins can serve not only to eliminate cell populations, but also as "biological tools" for studying the fate of endogenous proteins. We show here that upon entering their target cell, a variety of chimeric proteins composed of an endogenous protein as their killing moiety reach the subcellular location of their endogenous counterpart. In contrast, bacterial-based killing domains head for the subcellular site of their substrate. Moreover, the chimeric protein acts similarly to the endogenous protein, while causing the cell to die. Therefore, chimeric proteins may serve as a unique tool for investigating cellular proteins and their intracellular localization, without the need to overexpress them.

Key Words: **chimeric proteins; Bax; confocal microscopy; biological tool; endogenous protein localization.**

Chimeric proteins have been designed and constructed to eliminate cell populations bearing specific receptors or binding sites (1–6). These chimeric proteins are composed of two proteins fused at the cDNA level, resulting in a chimera that contains a targeting and a killing domain. The chimera has the ability to bind via its targeting component to specific receptors on the cell surface and internalize, allowing the killing moiety to act and cause cell death (7). However, these chimeric proteins can be applied not only to kill cells, but also as "biological probes" that can be used to address basic issues in cellular biology (8). There have been many studies on the binding of the chimeric proteins to their specific receptors or binding sites. However, little is known about the fate of the chimeric protein within the cell after internalization. The specific uptake and delivery of a large quantity of protein in the form of a chimeric protein, usually one that has an endogenous counterpart, from the exterior to the interior of the cell allows us to follow-up the intracellular fate of this protein.

In an attempt to understand the processes following internalization, we examined the intracellular path followed by a variety of chimeric proteins, using confocal microscopy and the appropriate antibodies. The targeting domains included: Gonadotropin releasing hormone (GnRH) to target adenocarcinomas (2–4), interleukin-2 (IL-2) to target activated T and B cells through the IL-2 high affinity receptor ((1); Aqeilan and Lorberboum-Galski, manuscript in preparation), and the constant region of IgE (Fcϵ) to target mast cells and basophils (5, 6) (Table 1). As the killing domain we used apoptotic death proteins such as human-Bax and human-Bak or the bacterial toxin *Pseudomonas exotoxin* A (PE). Since our apoptotic-based chimeras are composed of an endogenous protein, they were studied extensively in an attempt to unravel the mechanism by which they promote cell death. PE is a bacterial protein (9, 10), rather than an intracellular one, therefore, we assumed its subcellular location, most probably, be determined by the site of its substrate. Therefore, PE-based chimeric proteins were used as a control to examine the contribution of both moieties in determining subcellular chimera localization.

We found that while the targeting moiety directs the chimeric protein to specific cell populations, it has almost no effect on the chimeric protein's intracellular localization. The intracellular fate of a chimeric protein is determined by the killing component. When we deliver a chimera composed of a killing moiety that has an endogenous counterpart, the intracellular fate of the chimera is similar to that of the of the endogenous protein. Since endogenous proteins are located in various subcellular compartments, depending on cell type,

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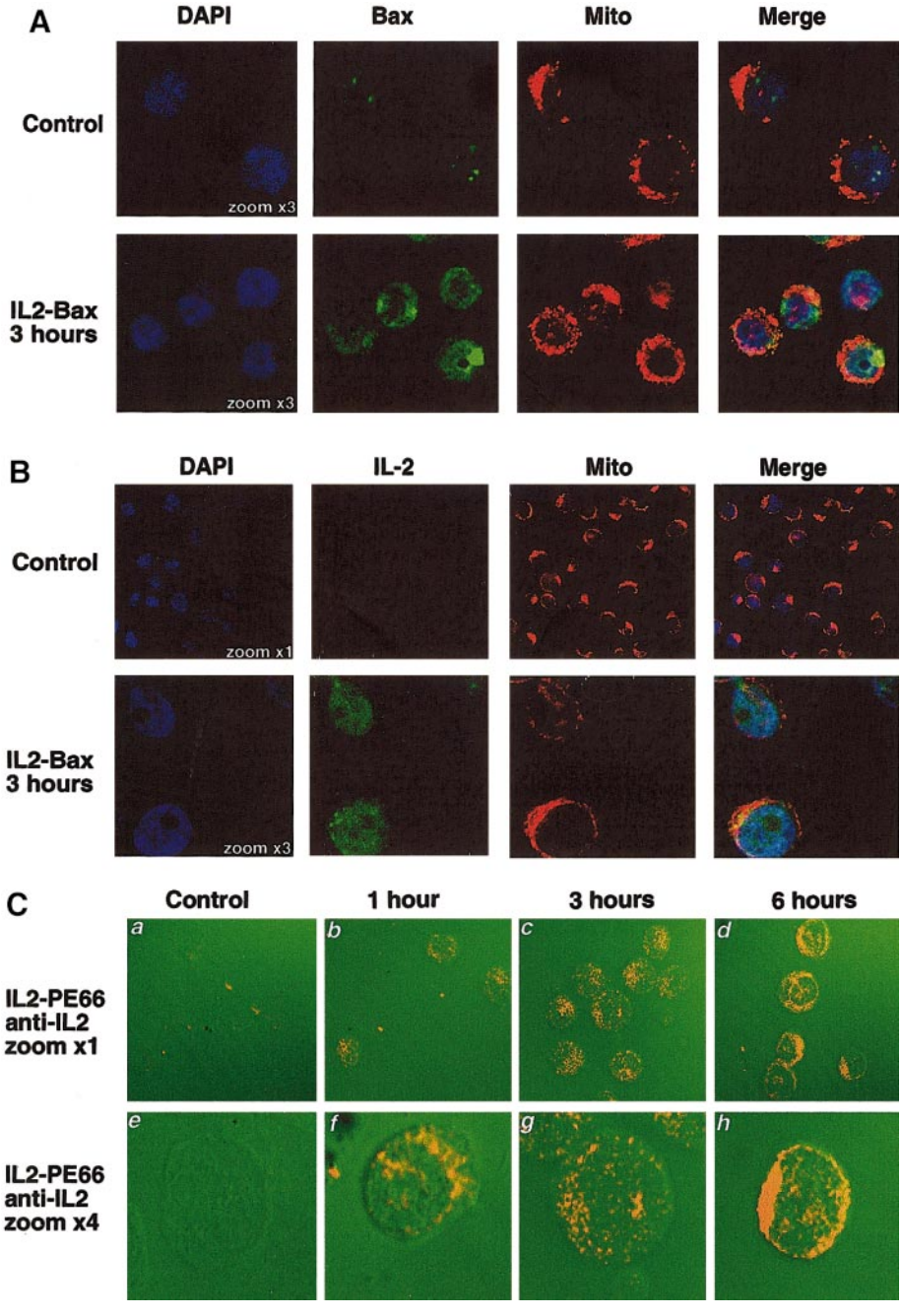

FIG. 1. IL-2 does not determine the subcellular localization of IL2-Bax. Localization of Bax or IL2 within IL2-Bax-treated HUT102 cells. Cells were incubated with IL2-Bax for 3 h, and visualized by confocal microscopy. Anti-Bax (A) or anti-IL2 (B) antibodies were visualized with FITC (green). Mitochondria were stained with Mitotracker Red CMXRos (red); DAPI (blue) was used to stain the nucleus. (C) Localization of IL2 in IL2-PE66-treated HUT102 cells. Cells were incubated with IL2-PE66^{4glu} for different periods of time. Anti-IL2 antibodies were visualized with FITC (orange).

a chimeric protein carrying the endogenous protein as a killing moiety will, therefore, also be found in different compartments. When the chimera's killing domain is composed of a foreign protein that has an endogenous substrate, the chimera indeed localizes to its substrate's subcellular compartment, like the PE-based chimeric proteins, which allocate to the cytoplasm.

Our results shed light on the long-term question of the intracellular fate of chimeric proteins after binding to a specific site on the cell surface. Furthermore, chimeric proteins provide researchers with a unique tool for investigating cellular proteins and their intracellular localization, since the killing moiety of the chimeric protein heads for the subcellular compartment of the

TABLE 1

Chimeric Proteins and Their Targets

Chimeric protein	Targeting moiety	Target cells
IL2-PE66^{4glu}/Bax	IL-2	IL-2-receptor-bearing cells
Fcε-PE40/Bax/Bak	Constant region of IgE (Fc)	Mast cells and basophils
GnRH-Bax	GnRH	Adenocarcinomas

endogenous protein and, presumably, acts similarly to the native protein.

MATERIALS AND METHODS

Cell lines. Cells were maintained and grown as described previously: HUT102 and activated lymphocytes (1); C57, HMC-1, KU812 and RBL (6); Colo205 and HeLa (2). All media and supplements were acquired from Biological Industries (Beit Ha'emek, Israel).

Expression of the chimeric proteins. The chimeric genes were expressed in *E. coli* strain BL21. Subfractionation of the expressing cells showed high enrichment for the chimeric proteins in the insoluble fraction. The fraction was denatured and renatured as described previously (1). These partially purified chimeric proteins were used in all our experiments.

Confocal microscopy. Control cells or cells treated with various chimeric proteins for different periods of time were collected and allowed to adhere to glass coverslips pretreated with 10% poly-L-lysine (Sigma Chemical Co., St. Louis, MO), except for HeLa and Colo205 cells, which were grown directly on the coverslips. HUT 102 cells were incubated with 250 nM of a mitochondrion-specific dye (Mitotracker Red CMXRos; Molecular Probes Inc., Eugene, OR). After 30 min incubation, the cells were washed with PBS. All cells were fixed in 3.7% paraformaldehyde, permeabilized with 0.2% Triton X-100 and blocked with 1% bovine serum albumin (BSA). The slides were incubated with anti-human Bax and anti-Bak (Pharmingen, San Diego, CA), anti-human IL-2 (Serotec, Raleigh, NC) or anti-IgE (Upstate Biotechnology, NY) for 40 min at 25°C and then washed with PBS. Second antibodies conjugated to FITC or Cy5 (Jackson Immunoresearch Lab. Inc., West Grove, PA) were then used for detection. Nuclei were counterstained with DAPI (1 μg/ml). The slides were examined and photographed with a Zeiss LSM 410 confocal laser scanning system attached to a Zeiss Axiovert 135M-inverted microscope with a 100× 1.2 C-aphchromate oil immersion objective lens (Carl Zeiss, Thornwood, NY). A krypton/argon laser at 488 and 568 nm lines was used for fluorescence excitation of FITC and Mitotracker Red CMXRos, respectively. A 364 Innova Enterprise ion laser was used for DAPI staining.

RESULTS AND DISCUSSION

Chimeric proteins have been used for the past several years to target and kill specific cell populations. These molecules were developed as unique agents for targeted therapy in humans (7, 11). This is not, however, the sole applicability of chimeric proteins. As these molecules must be internalized to exert their toxic activity, one can monitor the cell's ability to bind, internalize and target the killing moiety of the chimeric protein to its cellular destination (8).

Recently, we constructed chimeric proteins composed of endogenous proteins, such as proapoptotic proteins, which serve as the killing moieties (1, 4, 6). The present study was designed to validate that an endogenous protein, in the form of a chimera, behaves like its natural counterpart protein, and, therefore, can serve as a tool for tracking it. Studies were performed using confocal microscopy and antibodies against both moieties of the tested chimeric protein. All the experiments were preformed while validating the cytotoxic effect of the tested chimeras on the target cells (data not shown).

The IL-2 Moiety Does Not Direct IL2-Based Chimeric Proteins within the Cell

We have recently shown that the IL2-Bax chimeric protein specifically targets IL2-receptor bearing cells, such as the human HUT102 T-cell line and freshly activated lymphocytes, causing specific cell death via apoptosis *in vitro* (1). To follow the intracellular fate of the IL2-Bax chimeric protein, we treated HUT-102 cells with IL2-Bax and stained them with anti-Bax and anti-IL2 antibodies. We found that the chimeric protein localized to the nucleus 3 h after treatment, as evident from the colocalization of the green staining (either Bax or IL-2) and the blue staining (DAPI) (Figs. 1A and 1B). There was almost no staining of the chimera within the cytoplasm at this point (Fig. 1). Similar results were observed when we treated fresh human PHA-activated lymphocytes with IL2-Bax (Table 2; Aqeilan and Lorberboum-Galski, manuscript in preparation).

Bax, a pro-apoptotic protein of the Bcl-2 family, causes cell death (12, 13) and is generally considered a

TABLE 2

Intracellular Localization of Various Chimeric Proteins in Target Cells

Chimeric protein	Cell line	Intracellular localization		Endogenous protein localization
		1 h	24 h	
IL2-Bax	HUT102	Nucleus	Cytoplasm	Nucleus[a]
	Activated lymphocytes	Nucleus	Cytoplasm	Nucleus[a]
IL2-PE66^{4glu}	HUT102	Cytoplasm	Cytoplasm	—[b]
Fc-PE40	C57	Cytoplasm	Cytoplasm	—[b]
Fc-Bax	C57	Cytoplasm	Cytoplasm	Cytoplasm
	KU812	Cytoplasm	Cytoplasm	Cytoplasm
	RBL	Cytoplasm	Cytoplasm	Cytoplasm
Fc-Bak	C57	Cytoplasm	Cytoplasm	Cytoplasm
GnRH-Bax	HeLa	Cytoplasm	Cytoplasm	Cytoplasm
	Colo205	Nucleus	Nucleus	Nucleus[a]

[a] Although its cellular location is mainly nuclear, the Bax protein is also found in the cytoplasm.

[b] PE is a bacterial protein and, therefore, has no endogenous counterpart.

cytoplasmic protein (14). However, we demonstrate here (Fig. 1) that Bax is found also in the nucleus. On the other hand, IL-2, the targeting moiety of the chimera, may also contribute to the subcellular localization. Therefore, it was intriguing to examine which part of the IL2-Bax chimeric protein was responsible for directing it to the nucleus. To this end, we followed the internalization and localization of various IL2- or Bax-based chimeric proteins.

We first examined the cellular localization of the well characterized chimeric protein IL2-PE66^{4glu}, which targets and selectively eliminates activated T cells expressing IL2 receptors, both *in vitro* and *in vivo* (15, 16). The killing moiety in this chimera is *Pseudomonas exotoxin* A (PE) (9), a bacterial toxin, that causes cell death by inhibiting protein synthesis via ADP ribosylation of Elongation Factor-2 (EF-2). We have constructed a variety of chimeric proteins in which the different targeting moieties are fused to a full length mutated PE (PE66^{4glu}) or a truncated form of PE (PE40), both devoid of the ability to bind to PE's natural receptor (17). PE's intracellular substrate, EF-2, is a cytoplasmic protein and there is no evidence that it is present in the nucleus.

We treated HUT102 T-cells with IL2-PE66^{4glu} for various periods of time. Using antibodies against IL-2, we found that there was an increase in the amount of chimera observed within the cytoplasm (Fig. 1C), as can be seen by the augmented orange staining. This phenomenon was time-dependent, as the signal after 6 h of treatment (Fig 1C; d) was stronger than after 1 h (Fig. 1C; b).

These findings are in contrast to the location of the IL2- Bax chimeric protein, found by using the same antibodies and the same target cells. Since IL2-PE66^{4glu} differs from IL2-Bax only in its killing domain, this indicates that the killing domain is, most probably, responsible for the subcellular localization of the chimeric protein.

All Fc-Based Chimeric Proteins Are Localized in the Cytoplasm

To further investigate the role of the killing domain in determining the subcellular localization of chimeric proteins, we examined the cellular localization of the Fcε-PE40 chimeric protein in mast cell lines. This chimera was designed to target mast cells and basophils, the main inducers of allergic responses (5, 18). The constant (Fcε) portion of the mouse IgE served as the targeting moiety, thus enabling its delivery to cells exhibiting FcεRI, the high affinity receptor for IgE. Treatment with Fcε-PE40 leads to mast cells elimination *in vitro* (5) and prevents passive coetaneous anaphylaxis (PCA) *in vivo* without causing degranulation (18).

Using antibodies against IgE, we found that the Fcε-PE40 chimeric protein enters the cell, and is situated in the cytoplasm, as seen by the increase in green staining (Fig. 2A). This effect is evident both after 3 and 6 h, and is similar to the effect of IL2-PE66^{4glu} on HUT102 cells (Table 2; Fig. 1C).

Having assumed that the killing domain of the chimera directs the protein to a specific subcellular location, we examined what would happen in mast cell lines treated with two additional chimeric proteins: Fcε-Bax and Fcε-Bak (6). Both these chimeric proteins are fused to the same mouse Fcε targeting sequence as in the Fcε-PE40 chimera. However, they contain the human Bax or human Bak as the killing domain. It was conceivable that upon entering the cell, Fcε-Bax would home for the nucleus, similarly to IL2-Bax, whereas Fcε-Bak would head for the cytoplasm, since there is no evidence that Bak is located in the nucleus (19).

Mouse C57 mast cells were treated with the chimeric proteins Fcε-Bax and Fcε-Bak for various lengths of time, and examined under a confocal microscope, after staining with anti-IgE (green), -Bax (red), or -Bak (red) antibodies. Unexpectedly, we found that both Fcε-Bax and Fcε-Bak were present in the cytoplasm (Figs. 2B and 2C): When C57 cells were treated with Fcε-Bax, heightened yellow staining was seen in the cytoplasm, indicating colocalization of the two parts of the chimeric protein (Fig. 2B). Similarly, cells treated with Fcε-Bak showed costaining (yellow) of both anti-Fc and anti-Bak antibodies in the cytoplasm (Fig. 2C). It should be pointed out that these results were not confined to mouse C57 mast cells, since the same pattern was seen also in the human basophilic cell line KU812 and in the rat basophile cell line RBL (Table 2; data not shown).

Subcellular Localization of GnRH-Bax Is Determined by the Location of Endogenous Bax

Whereas Fcε-Bax localized to the cytoplasm (Fig. 2B), similarly to Fcε-PE40 (Fig. 2A), IL2-Bax was found in the nucleus (Fig. 1A) immediately following internalization. The difference between the intracellular location of Fcε-Bax and IL2-Bax may be due to the fact that these chimeric proteins were tested on different target cell lines: Fcε-Bax on the mouse C57 mast and human Basophilic cell lines, and IL2-Bax on human HUT102 T-cells and activated T lymphocytes. Endogenous proteins such as Bax may have different locations in various cell lines.

To test this hypothesis, we searched for cell lines in which endogenous Bax is known to be located in different subcellular fractions. To this end, we used a third Bax-containing chimera: GnRH-Bax (4). GnRH (Gonadotropin Releasing Hormone) is a decapeptide involved in the human reproductive system (20, 21).

We previously showed that GnRH-based chimeric proteins target and eliminate adenocarcinomas both *in vitro* and *in vivo* (2, 3, 22).

We treated two adenocarcinoma cell lines with the GnRH-Bax chimeric protein and monitored the location of the chimera, using antibodies against Bax. The two cell lines responded differently: the location of the chimeric protein was cytoplasmic in HeLa cells (Fig. 3A) but nuclear in Colo205 cells (Fig. 3B).

GnRH-Bax causes apoptotic death of both HeLa and Colo205 cells (4). Both cell lines are adenocarcinomas, although derived from different tissues. However, they differ in the cellular localization of endogenous Bax: cytoplasmic in HeLa cells (23, 24), and nuclear in Colo205 cells (25). Therefore, it appears that the chimeric protein allocates to the subcellular compartment in which the endogenous protein is situated.

It is well known that in different cell lines endogenous proteins may differ in appearance and cellular localization (26). This variance is understandable since these molecules possess natural inner-protein signals that determine their presence and location in a specific cell type. The various cells may "read" the information differently, thereby altering the localization of the protein. The two adenocarcinoma cell lines studied, which differ in the subcellular localization of their endogenous Bax, allocate the chimeric protein to different subcellular locations according to cell line.

Additional support for our conclusion that the killing domain directs the chimeric protein to its intracellular location, comes from our results with an additional chimera: GnRH-DFF40 (Ben-Yehudah *et al.*, manuscript in preparation). This chimeric protein is composed of human DFF40 (DNA Fragmentation Factor, known also as human CAD) as its killing moiety. GnRH-DFF40 caused the specific cell death of adenocarcinomas both *in vitro* and *in vivo*. DFF40 exhibits DNase activity (27) and, upon internalization into its target cell allocates to the nucleus. However, when we removed the DFF40's C′ terminus, which contains the nuclear localization sequence, the chimeric protein appeared in the cytoplasm. Once again, this suggests that the killing moiety of the chimeric protein determines the subcellular localization of the chimera.

Chimeric proteins have previously been used as biological tools to study mainly ligand–receptor interactions. With the aid of the chimeric protein IL2-PE66^{4glu}, we characterized the internalization capacity of IL2 via the different IL2R-subuints (8). Internalization was found to be mediated via the $\alpha\gamma$ form of IL2R, as well as through the $\beta\gamma$ and $\alpha\beta\gamma$ IL2R forms. However, IL2 could not be internalized through the IL2R γ subunit alone (8).

An additional chimeric protein used as a biological tool is DAB-IL2. DAB-based chimeric proteins were constructed by the fusion of the diphtheria toxin to interleukin-2. Site directed mutagenesis was used to generate point mutations in the IL-2 binding domain of the chimeric protein to study ligand receptor interactions (28). These experiments identified two residues within the IL2 that are most probably involved in the binding of IL2 to its receptor.

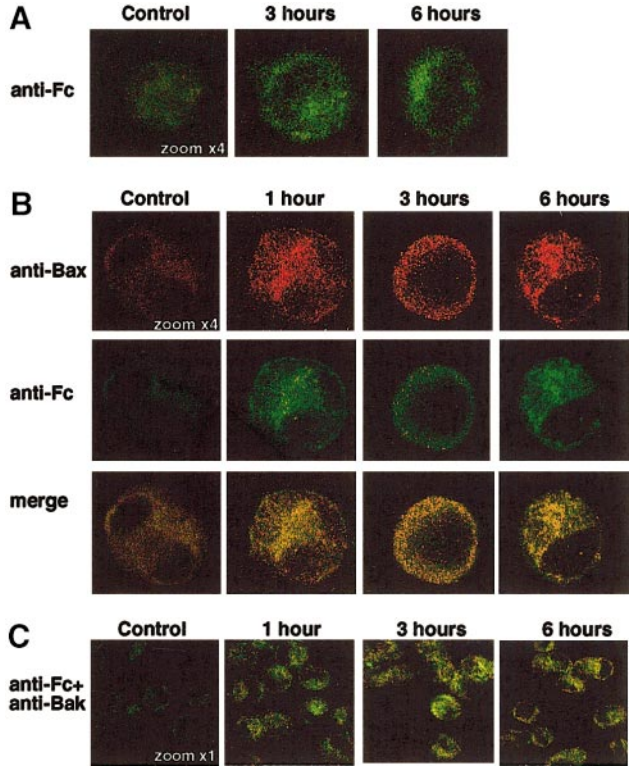

FIG. 2. Fcε-based chimeric proteins are directed to the cytoplasm. Cells treated with Fcε-PE40, Fcε-Bax, or Fcε-Bak for various periods of time were visualized by confocal microscopy using the appropriate antibodies, as described under Materials and Methods. (A) Localization of PE in Fcε-PE40-treated C57 cells. Anti-IgE antibodies were visualized with FITC (green). (B) Localization of Fc or Bax in Fcε-Bax-treated C57 cells. Anti-IgE antibodies were visualized with FITC (green); anti-Bax was visualized with Cy5 (red). (C) Localization of Fc or Bak in Fcε-Bak-treated C57 cells. Anti-IgE antibodies were visualized with FITC (green); anti-Bak was visualized with Cy5 (red); colocalization resulted in yellow staining.

To date, the most common method of studying intracellular proteins is to over- express them in cell lines. These proteins are overexpressed in their native form, or as fusion proteins, where they are linked to Green Florescent Protein (GFP) (29, 30). A comprehensive study on the subcellular localization of eight caspases was recently reported by Shikama *et al.* (31). Using confocal microscopy and specific antibodies, they were able to examine the subcellular localization of the different caspases after transfecting fusion GFP-Caspase chimeras into a number of cell lines. Recent data, however, suggest that overexpression of GFP may have a cytotoxic effect (29, 32).

In this study we present an additional approach in studying the behavior of proteins inside the cell: the use of targeting chimeric proteins. These molecules

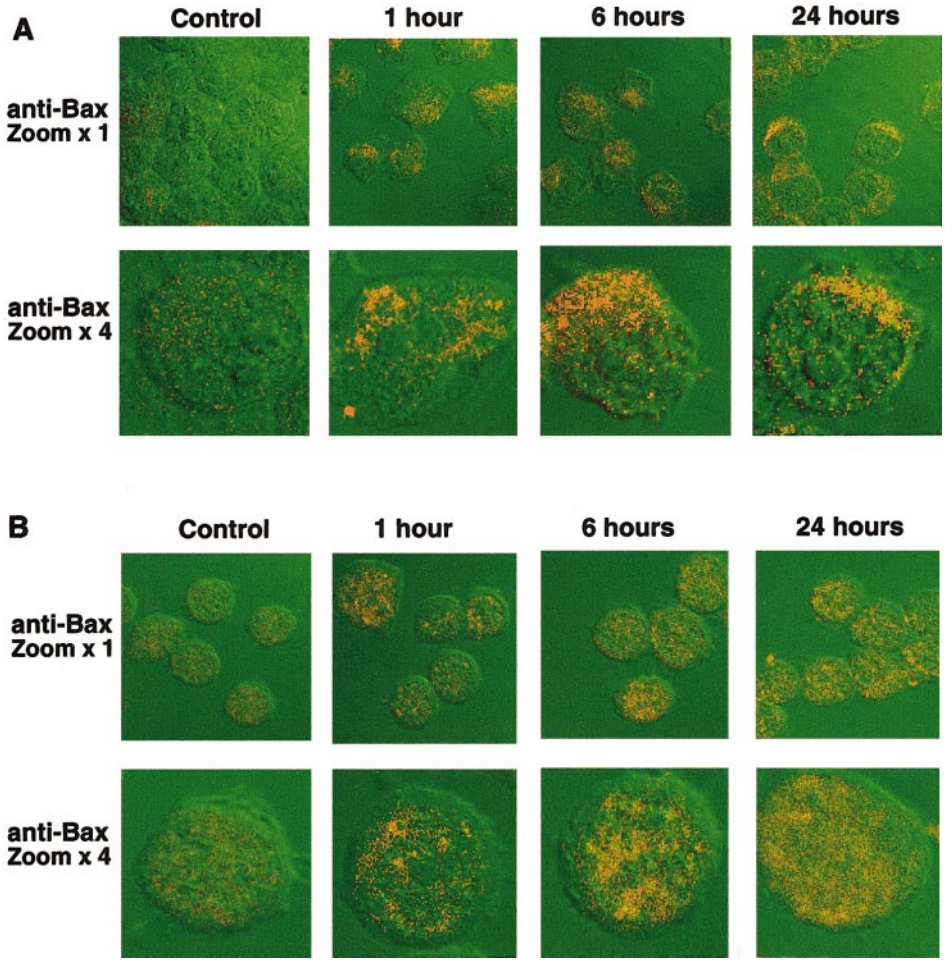

FIG. 3. Chimeric proteins are directed to the site of their endogenous counterpart. Localization of Bax in GnRH-Bax-treated HeLa (A) and Colo205 (B) cells. Cells were treated with GnRH-Bax for various periods of time and visualized by confocal microscopy, as described under Materials and Methods. Anti-Bax antibodies were visualized with FITC (orange).

enable us to deliver endogenous proteins into the cells, without having to transfect them, and rely on expression of the protein. Moreover, overexpression of such proteins may alter their subsequent localization. Furthermore, antibodies against both the targeting moiety and the killing moiety can easily monitor the chimeric proteins.

The use of chimeric proteins as a biological tool is not confined to specific targeting moieties or to their size. In the present study we used both large targeting domains (Fcϵ and IL2) and a very short targeting moiety (GnRH). Moreover, it has been shown that a short sequence from the HIV-TAT protein can serve as a targeting moiety and cause the internalization of many proteins in a nonspecific manner (33, 34). Therefore, our chimeric proteins, and similar ones, could promote the study of endogenous proteins both *in vitro* and *in vivo.*

Obviously, the intracellular location of the chimeric protein has greater implications than scientific research per se. All the chimeric proteins used in the present study were constructed for the treatment of various human diseases. Thus, monitoring the location of the proteins inside the target cell is crucial to a better understanding of their ability to induce cell death. This provides both a basis for designing better treatment regimens for humans as well as construction of improved molecules.

In conclusion, chimeric proteins composed of at least one endogenous protein can be used as an alternative/additional method for studying intracellular proteins and following their fate during cellular events such as apoptosis. However, not only apoptosis or other death-related pathways can be detected by using chimeric proteins. As the chimeras have the ability to direct any cellular protein to a target cell, chimeric proteins can assist us in studying a particular intracellular protein and its involvement in cellular event(s).

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