Binding of Granzyme B in the Nucleus of Target Cells

RECOGNITION OF AN 80-KILODALTON PROTEIN*

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Granzyme B (cytotoxic cell proteinase 1) is a serine proteinase that has been implicated in cytotoxic T lymphocyte-induced apoptosis. In order to understand how granzyme B is involved in mechanisms of target cell destruction, characterization and identification of substrates are required. We have developed an in situ binding assay using permeabilized cells and recombinant granzyme B that allows us to visualize potential substrates after immunostaining with anti-granzyme B antiserum.

Confocal laser scanning microscopy and immunoelectron microscopy analyses demonstrate that granzyme B recognizes a nuclear substrate. The labeling pattern observed corresponds with regions of positive staining with uranyl acetate which binds to heterochromatin in the nucleus. Positive labeling of target cells with granzyme B is dependent on the presence of a catalytically active proteinase, since an inactive proenzyme form of granzyme B fails to give rise to any binding in the target cells. Far-Western blotting and immunoprecipitation of subcellular fractions of target cells have shown that the putative substrate of catalytically active granzyme B is an 80-kDa nuclear protein. Minor cytosolic bands of 50 and 94 kDa are also observed. A cytoplasmic band of 69 kDa is detected by both active and zymogen forms of granzyme B.

Apoptosis induced in target cells by cytotoxic T lymphocytes is believed to occur after CTL-target conjugation which is facilitated by engagement of the T-cell receptor and its accessory molecules (1). This cell-cell contact induces the exocytosis, from the CTL, of granules which contain the potential cytolytic effector molecules. These include the pore-forming protein perforin (cytolysin) and a family of serine proteases collectively known as granzymes. The series of events that occur after degranulation, which lead to target cell death, remain to be elucidated.

Perforin is believed to create pores in the target cell membrane which may disrupt osmoregulation and facilitate transfer of the granzymes. Once the granzymes enter a target cell, it is hypothesized that cleavage of their respective substrates results in cell death (2). Hallmarks of CTL-induced cell death include many of the signs of apoptosis: DNA fragmentation, chromatin condensation, membrane blebbing, and cell shrinkage (3).

Granzyme B has been implicated in a number of these processes, most notably with the fragmentation of DNA. Granzyme B is the prototypic member of this family of proteases (4) and is most abundantly expressed in response to a diverse array of CTL stimuli: α-CD3, ConA, phorbol 12-myristate 13-acetate, and alllogeneic stimulation (5). Expression of granzyme B correlates with lytic activity of CTL in vitro (5) and with the activity of infiltrating T lymphocytes in allografts in vivo (6). Granzyme B has also been isolated as the activity known as fragmentin (7). This activity is necessary for degradation of DNA in target cells by CTL. A role for granzyme B in the degradation of target cell DNA is further supported by the granzyme B homozygous null mutant mice (8). These mice have a severely depressed ability to cause rapid DNA fragmentation in target cells.

Granzyme B has an uncommon substrate specificity of aspartic acid at P1 (9, 10). This preference is also observed in ced3 (11) and interleukin 1α-converting enzyme (12). These molecules play a pivotal role in apoptosis in Caenorhabditis elegans and higher eukaryotes, respectively. Physiological substrates of this group of enzymes with the rare substrate specificity of Asp at P1 will undoubtedly provide insights into the respective apoptotic processes in which they are involved. In this study, we present evidence to demonstrate that catalytically active granzyme B will recognize a nuclear protein that co-localizes with heterochromatin in target cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The cell lines EL4, P815, L1210, L1210-FAS, a stably transfected variant of L1210 expressing the FAS antigen (13), YAC-1, and J urkat were grown in RPMI 1640 with 5% fetal calf serum, 2-mercaptoethanol in 10 mM Hepes, pH 7.2. COS M5 cells were cultured in Dulbecco’s modified Eagles’ medium. All cells were incubated at 37 °C in 5% CO2.

Catalytically active granzyme B and inactive proenzyme cloned in the vector pAX142 were expressed by transient transfection of COS M5 cells as described previously (14). Enzymatic activity of mature granzyme B was assayed by cleavage of the synthetic substrate tert-butyloxycarbonyl-Ala-Ala-Asp-thiobenzyl ester in the presence of the chromogenic indicator 5,5'-dithiobis(2-nitrobenzoic acid) as described (14).

Cellular Extracts—Whole cells lysates were harvested in 10 mM NaCl, 15 mM KCl, and 10 mM Tris-HCl, pH 7.4, by physical disruption by Dounce homogenization. Nuclear extracts were prepared as per Schreiber et al. (15).

Far-Western and Immunoblotting—Denaturing polyacrylamide gel electrophoresis and electrophoretic transfer of proteins to either nitrocellulose (MSI, Westboro, MA) or polyvinylidene difluoride (Immobilon-P, Millipore, Bedford, MA) was performed by standard methods (16).
Granzyme B Substrates in Target Cells

Nonspecific protein interactions were blocked in milk powder (3% w/v in Tris-buffered saline and 0.1% Tween 20 (TBS-T)) for 30 min at room temperature. COS M5 lysates were used at a final concentration of 100 μg/ml (total protein). Affinity-purified rabbit anti-cytotoxic cell protease antisera 7572 (α-CCP), which recognizes a conserved octapeptide in the lymphoid/myeloid granule serine proteinases, was used at a final concentration of 2.5 μg/ml. Horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Life Sciences, Oakville, ON) was used at a dilution of 1:1,500. The ECL™ (Amersham) detection system was used.

Immunoprecipitation—Cellular lysates or subcellular fractions were fixed in 1% formalin for 30 min prior to immunoprecipitation. Sepharose CL-4B protein A resin (Pharmacia Biotech, Baie D’Urfe, PQ) was incubated sequentially with rabbit antisera 5722 (5 μg/ml), COS M5 lysate containing active or inactive granzyme B (100 μg/ml), and finally prefixed EL4 protein sample. The resin was washed extensively in TBS-T, and the protein disassociated following the addition of gel sample buffer containing 2-mercaptoethanol and heating at 100°C for 10 min (16).

Confocal Laser Scanning Microscopy—Target cells were adhered to poly-L-lysine-coated coverslips, fixed in formaldehyde, and labeled essentially as noted above for far-Western blots with the exception of the addition of saponin (0.1% w/v) to permeabilize the cells. In these experiments, the detection of labeled proteins was through the use of Texas Red™-conjugated goat anti-rabbit Ig (Jackson Immunoresearch). Cells were counterstained with FITC-conjugated ConA. Confocal images were obtained with a Leica CLSM™ using a 100×/1.32 N.A. oil immersion objective and analyzed with the accompanying CLSM™ and the Silicon Graphics Imageview software. Images are the result of 64-scan line averaging.

Proteinase-linked Immunoelectron Microscopy—EL4 target cells were collected by centrifugation, washed with 0.2 M sodium phosphate (pH 7.2), and fixed in 1% glutaraldehyde. The cells were then washed with phosphate-buffered sucrose (6.84% w/v), postfixed in OsO4 (0.2 M sodium phosphate, 1.7% sucrose), washed with sucrose-free buffer, dehydrated in ethanol, and embedded in Epon 812. Sections 80 to 120 nm thick were mounted on nickel grids for immunocytochemistry studies. Grids were labeled as above for immunofluorescence studies with additional incubations in 10% H2O2 for 10 min and saturated NaHIO4 (Sigma) in combination with goat anti-human-conjugated FITC (Jackson Immunoresearch). Labeled cells were viewed with a Leica CLSM™ using a 100×/1.32 N.A. oil immersion objective and analyzed with the accompanying CLSM™ and the Silicon Graphics Imageview software. Images are the result of 64-scan line averaging.

FIG. 1. Far-Western blot of nuclear extracts labeled with granzyme B and zymogen. Electrophoresis of nuclear extracts (50 μg/lane) labeled sequentially with active or zymogen, α-CCP, and donkey anti-rabbit IgG-horseradish peroxidase. Detection is a result of chemiluminescence with ECL. A single band of approximately 80-kDa is detected in all cell lines tested. Blots were probed first with zymogen, stripped, and rescreened with active granzyme B.

FIG. 2. Far-Western blot of EL4 subcellular fractions labeled with granzyme B or zymogen. Each lane contains 50 μg of the indicated protein lysate. Detection is by chemiluminescence as in Fig. 1. A nuclear band of 80 kDa is detected with active granzyme B, as are faint cytosolic bands of 50 and 94 kDa. This signal is dependent on the addition of COS M5 lysate containing either of these granzyme B species. Screening with α-CCP antiserum alone does not give rise to any 69-kDa bands (13). Granzyme B and zymogen both recognize a cytoplasmic protein of 69 kDa.

FIG. 3. Proteinase-linked immunoprecipitation of [35S]Met-labeled EL4 protein lysates. Nuclear and cytoplasmic fractions were incubated sequentially in 1% formaldehyde and COS M5 lysate containing active granzyme B followed by precipitation with α-CCP and Sepharose CL-4B-conjugated protein A. Following immunoprecipitation, individual proteins were purified by elution from SDS-polyacrylamide gel electrophoresis. A nuclear protein of 80 kDa is precipitated (lane A) as well as cytoplasmic proteins of 94, 69, and 50 kDa (lanes B–D, respectively). The 69-kDa band is also precipitable with inactive granzyme B zymogen.

Granzyme B Recognizes Proteins in the Nucleus and Cytoplasm of Target Cells—Granzyme B was expressed in COS M5 by transient transfection. Enzymatic activity was assayed by the ability to cleave the synthetic substrate Boc-Ala-Ala-Asp-thiobenzyl ester. The proenzyme form of granzyme B was expressed in the same manner and does not possess catalytic activity.

Granzyme B was used in a far-Western blot to screen for potential substrates. Protein blots of whole cell lysates and subcellular fractions of target cell protein were analyzed in this manner. Fig. 1 shows that granzyme B binds to a nuclear protein of Mₚ = 80,000 in all cell lines examined. This protein is not recognized by inactive granzyme B zymogen, which differs from the mature protease by an additional dipeptide (Gly-Glu) at the N terminus. Minor cytosolic proteins of Mₚ = 50,000 and 94,000 are also detectable (Fig. 2). A cytoplasmic protein of Mₚ = 69,000 is recognized by both active and zymogen forms of granzyme B (Fig. 2).

These observations are supported by proteinase-linked immunoprecipitation of target cell proteins. EL4 proteins were fixed in 1% formaldehyde prior to incubation with granzyme B and precipitated with α-CCP antiserum and protein A-Sepharose. Fig. 3 shows an 80-kDa nuclear protein and 50-, 69-, and 94-kDa cytoplasmic proteins following purification by proteinase-linked immunoprecipitation. Each band was subsequently iso-
labels the endoplasmic reticulum and provides a reference of cellular morphology.

In all cell lines examined, approximately 50 to 80% of cells displayed uptake of label and all cells that labeled demonstrate similar labeling patterns. Fig. 4 shows representative cells labeled with granzyme B or zymogen. P815 target cells also show a nuclear labeling with granzyme B. However, this pattern appears to be heavily concentrated in the nucleolus (Fig. 4A). Endogenous serine proteinases can be detected with the α-CCP antiserum in a cytoplasmic granular distribution, but this labeling pattern is not shown in order to highlight the proteinase-specific nucleolar labeling of P815 as a target cell. Permeabilization of cells with 0.1% saponin was necessary to observe any signal arising from the addition of granzyme B or α-CCP.

Granzyme B gives rise to a staining patterns in EL4, L1210 (Fig. 4B), and Jurkat (Fig. 5) that is granular throughout the nucleoplasm. This staining is consistent with nucleolar patterns observed in multinucleolar cell types and lines (17–19). Labeling of these cells with granzyme B is observed throughout the cytoplasm, but cannot be attributed to a specific subcytoplasmic localization. Closer scrutiny of localization of granzyme B binding proteins in EL4 cells is described in immunoelectron microscopic studies below.

Double label of Jurkat cells with the nucleolar specific antiserum ANA-N and granzyme B shows a co-localization pattern. In these experiments, granzyme B binding appears red and the nucleolus is labeled green. Where the two patterns overlap, the label appears yellow. Fig. 5 shows that murine granzyme B gives rise to a nucleolar labeling pattern in the human J urkat target cell line. The J urkat nuclei appear green when labeled with the inactive zymogen, due to a positive green signal from the ANA-N and a lack of a red signal from granzyme Bzymogen.

Interaction between Granzyme B and Potential Substrates Requires an Open Catalytic Site—Nonspecific electrostatic interactions between proteinase and the proteins detected are ruled out by the absence of binding by the inactive proenzyme. This proenzyme differs from the active proteinase by only an additional two amino acid residues (Gly-Glu) at the N terminus. Removal of this dipeptide is necessary for the mature protein to yield enzymatic activity (14).

The importance of the negative control utilizing the zymogen granzyme includes other relevant negative controls for successful application of this system. Positive signals caused by interaction of the antisera binding directly to antigens in the target cells are ruled out, as well as any signals that may have arisen from the interaction of any of the secondary labeled antibodies to target cell proteins.

We first performed the far-Western blots with either active enzyme or zymogen. This was followed by stripping of the blots and rescreening with zymogen or active enzyme, respectively. This removed the possibility that the stripping process adversely affected the ability of either active enzyme or zymogen...
to bind proteins in this far-Western blot arrangement.

An additional measure to demonstrate that the positive signals attributed to binding of catalytically active granzyme B also represents a specific interaction between the α-CCP antiserum, and the granzyme B from the COS lysate is the observation that preincubation of this antiserum with a bovine serum albumin-conjugated octapeptide (the peptide that served as the original immunogen) successfully competes out binding of the antiserum to granzyme B COS lysate-labeled target cells.

Nuclear Granzyme B Substrate(s) Co-localizes with Heterochromatin—EL4 target cells were fixed, embedded, and sectioned onto nickel grids. Sections were labeled according to the scheme outlined above for immunofluorescence analyses. The detection method utilized a gold-conjugated anti-rabbit IgG to detect the α-CCP antiserum. Fig. 6 shows representative cells labeled with granzyme B orzymogen. Granzyme B gives rise to a nuclear staining that uniformly co-localizes with the heterochromatin. Heterochromatin binds greater amounts of the counterstain uranyl acetate and stains darker than euchromatin.

Cytoplasmic labeling by granzyme B observed by CLSM is not observed by electron microscopy. The glutaraldehyde fixative used for electron microscopy masks the binding of granzyme B to any potential cytoplasmic substrates. This masking effect was also observed when glutaraldehyde-fixed cells were viewed by confocal microscopy.

DISCUSSION

Far-Western blot analysis of EL4 subcellular fractions demonstrate that catalytically active granzyme B binds to a nuclear protein with an apparent molecular weight of 80,000 and cytosolic proteins of 50,000 and 94,000 that are not detected by inactivezymogen. This suggests that the interaction of granzyme B with these proteins is dependent on an active catalytic site within the proteinase facilitated through an open specific-
CTL and target cell and ultimate destruction of the target cell is as low as 6 min (27), it is quite likely that the effects of the CTL are mediated through a pathway that utilizes existing components within the target. Included in this observation is the probability that substrates of granzyme B are widely and constitutively expressed in all cell types that are sensitive to destruction by CTL. This hypothesis is supported by the observation of an 80-kDa granzyme B-reactive protein in a diverse panel of cell lines. The binding of granzyme B to these proteins may represent a proteolytic cleavage event that results in either the release of a destructive activity or renders some vital sequestered motif accessible to pre-existing cellular degradative processes.

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