Expression of Human Recombinant Granzyme A Zymogen and Its Activation by the Cysteine Proteinase Cathepsin C

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Human granzyme A is one of the serine proteinases present in the granules of cytotoxic T lymphocytes and natural killer cells. Granzymes are synthesized as inactive proenzymes with an amino-terminal prodipeptide, which is processed during transport of granzymes to the cytotoxic granules, where they are stored as active proteinas. In this study, we explored the possibility of producing recombinant granzymes. Recombinant human granzyme A zymogen was expressed in several eukaryotic cell lines (HepG2, J urkat, and COS-1) after infection with a recombinant vaccinia virus containing full-length granzyme A cDNA. Immunoblot analysis of cell lysates showed that all infected cells produced a disulfide-linked homodimer of identical molecular weight as natural granzyme A. Infected HepG2 cells produced the largest amount of this protease (approximately 160 times more than lymphokine activated killer (LAK) cells). The recombinant protein only had high mannose type oligosaccharides as did the natural protein. Although infected HepG2 and COS cells contained high granzyme A antigen levels, lysates from these cells did not show any granzyme A proteolytic activity. However, the inactive proenzyme could be converted into active granzyme A by incubation with the thiol protease cathepsin C (dipeptidyl peptidase I).

This study is the first to demonstrate expression of an active recombinant human cytotoxic lymphocyte proteinase and conversion of inactive progranzyme A into an active enzyme by cathepsin C. We suggest that a similar approach can be used for the production of other granzymes and related proteinases.
Recombinant Human Granzyme A Expression

(Stockholm, Sweden). Nonidet P-40 and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma.

Cell Culture—Human 143 tk fibroblasts and RK-13 rabbit kidney cells were maintained in Eagle's medium supplemented with 10% (v/v) fetal calf serum, streptomycin, penicillin, and nonessential amino acids. SV-40 transformed COS-1 monkey cells, the human T-helper cell line J urkat, and a human liver cell line HepG2 were grown in modified Dulbecco's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, streptomycin, penicillin, and β-mercaptoethanol (medium for J urkat cells also contained 20 units/ml interleukin-2; Chiron, Emeryville, CA). Lymphokine-activated killer (LAK) cells were prepared by culturing peripheral blood mononuclear cells (obtained from healthy donors by Percoll density gradient centrifugation) at a concentration of 0.5 × 10^6 cells/ml for 7 days with 1,000 units/ml interleukin-2 in the same medium as for J urkat cells. LAK cell lysate (20 × 10^6 cells/ml) was prepared as described below for vaccinia-infected cells.

Construction of Recombinant Vaccinia Virus for the Expression of Human Granzyme A—To obtain full-length granzyme A cDNA, including the nuleotide coding for the pre- and propeptide, specific primers containing the appropriate restriction sites were prepared based on the published granzyme A cDNA sequence (9). First strand cDNA was prepared from mRNA of LAK cells as described (23) and amplified with the polymerase chain reaction using the granzyme A primers. The amplified cDNA fragment was isolated, digested with the appropriate restriction enzymes, and ligated into the gradient control of the vaccinia virus recombinant vector p11k-ATA-18 (24) (Fig. 1). The authenticity of the cloned cDNA was confirmed by nucleotide sequence analysis (Sequenase kit; U.S. Biochemical Corp.). Isolation of plasmid DNA, conditions for digestion by restriction enzymes, and agarose gel electrophoresis were as described (25).

Granzyme A recombinant virus for 30 was prepared by homologous recombination of the granzyme A/p11k-ATA-18 plasmid with the temperature-sensitive vaccinia virus mutant ts7. Plaques were selected and purified as described (26). Briefly, subconfluent plates of human 143 tk fibroblasts were infected for 1 h with vaccinia virus ts7 (0.1 plaque-forming unit/cell). Subsequently, cells were incubated with fresh medium for 60 min at 37°C, diluted 10-fold with fresh medium, and then further incubated for 24 h at 37°C. Thereafter, cells and supernatant were harvested. The supernatant was centrifuged at 3,000 rpm to remove cell debris, incubated for 16 h at 37°C in the presence of 2 mM PMSF, and stored at 20°C. This viral stock was titrated on RK-13 cells to determine the number of plaque-forming units/ml of virus stock.

Construction of a Granzyme A Recombinant Vaccinia Virus—A recombinant vaccinia virus was constructed by homologous recombination of the granzyme A/p11k-ATA-18 plasmid with the temperature-sensitive vaccinia virus mutant ts7. Plaques were selected and purified as described above. Briefly, subconfluent plates of human 143 tk fibroblasts were infected for 1 h with vaccinia virus ts7 (0.1 plaque-forming unit/cell). Subsequently, cells were incubated with fresh medium for 60 min at 37°C, diluted 10-fold with fresh medium, and then further incubated for 24 h at 37°C. Thereafter, cells and supernatant were harvested. The supernatant was centrifuged at 3,000 rpm to remove cell debris, incubated for 16 h at 37°C in the presence of 2 mM PMSF, and stored at 20°C. This viral stock was titrated on RK-13 cells to determine the number of plaque-forming units/ml of virus stock.

Granzyme A Expression in Different Cell Lines—Granzyme A expression was analyzed by cellular lysates or permeabilized cells following 143 tk fibroblasts and RK-13 rabbit kidney cell infection with the recombinant vaccinia virus recombinant vector p11k-ATA-18 (24) (Fig. 1). The authenticly of the cloned cDNA was confirmed by nucleotide sequence analysis (Sequenase kit; U.S. Biochemical Corp.). Isolation of plasmid DNA, conditions for digestion by restriction enzymes, and agarose gel electrophoresis were as described (25).

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Affinity Purification of Granzyme A Species Using Benzimidin-Sepharose—Cell lysates or supernatant was diluted in lysis buffer to a final volume of 250–500 μl and incubated with 10 μl of benzimidin-Sepharose 4 h at room temperature on a head-over-head rotator. The Sepharose beads were then washed four times with 1 ml of lysis buffer. The supernatant of the last washing step was carefully removed, after which an equal volume of SDS-PAGE loading buffer was added. The mixture was incubated for 5 min at 100°C and centrifuged for 3 min at 13,000 × g. The supernatant was electrophoresed on SDS-polyacrylamide (12.5%, w/v) gels and subsequently analyzed by immunoblot (see below).

Immunoblotting—Cell lysates or benzimidin-Sepharose precipitates were separated on 12.5% (w/v) polyacrylamide gels. Proteins were then transferred onto nitrocellulose sheets (Schleicher and Schuell), which were then incubated for 30 min with blocking buffer (i.e., PBS containing 5% (w/v) nonfat dry milk (Prokif; Nutricia, Zoetermeer, the Netherlands) and 0.1% (w/v) Tween 20. The sheets were then incubated with monoclonal antibody GRA-8 (2.5 μg/ml in the same buffer), for 14 h at room temperature. Sheets were washed by repeated (3 times) incubation for 10 min with PBS, 0.1% (w/v) Tween 20, and then probed with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins diluted in blocking buffer for 2 h. After wash with PBS, 0.1% (w/v) Tween, and with PBS alone, sheets were developed for 2 min in chemiluminescent detection reagent (Amersham International) and exposed to 15–120 s to Kodak XAR films (Kodak, Rochester, NY).

RESULTS

Construction of a Granzyme A Recombinant Vaccinia Virus—A full-length granzyme A cDNA was cloned from activated T lymphocytes as described under "Materials and Methods." The nucleotide sequence of the clone obtained was identical to that published by Geresh et al. (7). The cDNA was inserted into the vaccinia virus recombinant vector p11k-ATA-18, under the control of the 11k late promoter (24) (Fig. 1). It encoded the complete protein including the signal peptide and the propeptide (Fig. 1). The cDNA was integrated into the viral DNA of the wild type virus by recombination as described elsewhere (24). The promoter used enables the synthesis of a large amount of foreign polypeptides in the late phase of the viral infection, initiating translation at their authentic start codon.

Characterization of rGA Produced by Different Cell Lines—J urkat, HepG2, and COS-1 cells were infected with rGA vaccinia virus. Twenty-four and 48 h after infection all cell lines produced granzyme A protein. Cell lysates as well as culture supernatants from J urkat or HepG2 cells obtained 48 h after infection appeared to contain rGA only when infected with granzyme A recombinant virus (Fig. 2, lanes 3–6 and lanes 9–12, respectively), but not after infection with a control virus (Fig. 2, lanes 7–8 and lanes 13–14, respectively). RecGrA consisted of a disulfide-linked homodimer with a relative mobility (M,) identical to the natural protein (LAK cells; Fig. 2, lanes 1 and 2).

Each granzyme A monomer has one glycosylation site to
The 11k late promoter is indicated. The arrows (in case of medium) per lane were analyzed: nonreduced samples. The following number of cells or cell equivalents 14, 0.03 Jurkat or HepG2 was found (ilar shift in mannose as well as complex-type sugar chains, yielded a sim-
ditionally, digestion of rGA with PNGase F, which cleaves high
lanes 1 derived granzyme A (Fig. 3, 29). No difference in Endo H susceptibility between LAK cell-
which an 11k protein from Jurkat and HepG2 cells migrated with the same
M, compared with deglycosylated granzyme A from LAK cells, indicating that the molecular weight of the protein backbone of the recombinant species was similar to that of natural granzyme A.
The Amount of rGA Produced by HepG2 Is Considerably Higher than That Produced by LAK Cells—In general, infected cells produced a maximal amount of rGA antigen 48 h postinfection. During infection an increase of cell lysis and a concomitantly release of rGA into the supernatant was observed (Fig. 2, lanes 11 and 12). The extent of cell lysis and subsequent granzyme A release into the supernatant varied during different infection experiments.
Compared with the number of LAK cells (Fig. 2, lanes 1 and 2), 53 times fewer HepG2 cells (lanes 9 and 10) and 10 times fewer J urkat cells (lanes 3 and 4) were analyzed, whereas the amount of HepG2 cell supernatant tested (lanes 11 and 12) corresponded to 10 times fewer cells. However, in spite of testing a lower number of infected cells, the intensity of protein bands rGA (compare, for example, lanes 2 and 10, Fig. 2), was equal to or higher than that observed with LAK cells, suggesting the latter produced less granzyme A than the infected cells. This was further assessed semiquantitatively using an immunoblot; per given amount of cells, infected Jurkat cells produced approximately 20 times, COS-1 40 times, and HepG2 cells even 160 times more granzyme A antigen (48 h postinfection) than did LAK cells.
Recombinant Granzyme A Shows No Proteolytic Activity—Enzymatic activity of the recombinant protein was determined in two ways; first, cell lysates were tested for hydrolysis of the chromogenic substrate BLT, and, second, the affinity of recombinant proteins for benzamidin-Sepharose was determined, as benzamidin only binds to proteolytically active granzyme A. In these experiments LAK, HepG2, J urkat, and COS-1 cell lysates were adjusted to contain approximately equal amount of granzyme A antigen as assessed by immunoblotting. Lysates from COS-1 or HepG2 cells showed hardly any BLT hydrolysis (the activity corresponded to less than 0.5% of that of LAK cells; Table I). In agreement herewith, rGA from HepG2 cells did not bind to benzamidin-Sepharose (Fig. 4, lane 5), whereas natural granzyme A from LAK cells did (Fig. 4, lane 2). In contrast, lysates of infected J urkat cells had significant BLT activity, corresponding to about 20% of that present in LAK cell lysates (Table I). No BLT activity was observed in lysates from J urkat cells infected with FXII.lpc recombinant virus, consistent with the observed lack of granzyme A antigen (Fig. 2, lanes 7 and 8). The BLT activity observed with infected J urkat cells was due to

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**Fig. 1.** A schematic representation of the vaccinia recombinant vector p11k-ATA-18 containing full-length granzyme A cDNA. L-TK and R-TK represent the left and right thymidine kinase locus. The 11k late promoter is indicated. The arrows on top indicate the cleavage sites for the signal and pro- (EK) peptide.

**Fig. 2.** Immunoblot analysis (using a monoclonal antibody directed against granzyme A, GrA-8) of the granzyme A content of cell lysates (L) and supernatant (m) of J urkat (lanes 3-8) and HepG2 cells (lanes 9-14) infected either with granzyme A recombinant virus (lanes 3-6 and lanes 9-12) or with a control virus (FXII, lanes 7-8 and lanes 13-14). As a positive control, natural granzyme A-containing LAK cells are analyzed in lanes 1 and 2. The even lanes contain reduced samples, and the odd lanes contain nonreduced samples. The following number of cells or cell equivalents (in case of medium) per lane were analyzed: lanes 1 and 2, 1 x 10⁶; lanes 3-8 and lanes 11 and 12, 0.16 x 10⁶; lanes 9 and 10 and lanes 13 and 14, 0.03 x 10⁶.

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**Fig. 3.** Immunoblot showing PNGase F (PF) and Endo H (EH) digestion of granzyme A in LAK cells (lanes 1 and 2), infected HepG2 (lanes 3-5) and J urkat (lanes 6-8) cells. Control samples (lane 5) were treated identically except that no enzyme was added.
Recombinant Human Granzyme A Expression

Table I

| Cell line | Virus       | BLT hydrolysis (E494 after 60 min) | Granzyme A antigen equivalent | Relative granzyme A activity % |
|-----------|-------------|----------------------------------|-------------------------------|-------------------------------|
| HepG2     | Granzyme A  | 0.049                            | 160                           | 0.38                          |
| HepG2     | FXII.lpc    | 0.001                            |                               |                               |
| HepG2     | FXII.lpc    | 0.086                            |                               |                               |
| Jurkat    | Granzyme A  | 0.146                            | 10                            | 18.3                         |
| Jurkat    | FXII.lpc    | 0.007                            |                               |                               |
| Jurkat    | FXII.lpc    | 0.023                            |                               |                               |
| COS-1     | Granzyme A  | 0.007                            | 40                            | 0.22                         |
| COS-1     | FXII.lpc    | 0.001                            |                               |                               |
| LAK       |             | 0.08                             | 1                             | 100                          |

Apparent, after processing of the prodipeptide, cathepsin C was not able to further modify active granzyme A.

The effect of cathepsin C on the proteolytic activity of rGA in HepG2 cell lysates was also assessed (Fig. 5B). This proteolytic activity increased during incubation with cathepsin C, whereas no increase of BLT activity by rGA was detected when cathepsin C was omitted. It is to be noted that cathepsin C itself, at the concentrations used, did not convert the BLT substrate (not shown). Thus, these results demonstrated that cathepsin C was able to convert recombinant granzyme A in HepG2 cell lysates into a proteolytically active enzyme.

Discussion

Here we report the expression of recombinant human granzyme A in mammalian cells by a granzyme A recombinant vaccinia virus and the ability of cathepsin C to convert rGAzymogen into an active protease. Using immunoblotting, we estimated that infected HepG2 cells produced about 160 times more granzyme A than LAK cells. These levels exceeded that produced by transient expression in COS cells using a mammalian expression vector (not shown). This is in concordance with previous studies comparing conventional transient expression systems with expression by vaccinia virus (30).

Different cell lines were infected by recombinant vaccinia virus harboring cDNA coding for full-length granzyme A including the signal and propeptide. Infected cell lines produced a disulfide-linked homodimer with the same molecular weight as natural granzyme A. In vitro translation of granzyme A mRNA, using rabbit reticulocytes and dog microsomes in the presence of oxidized glutathione, produced a granzyme A dimer unable to bind to benzamidin-Sepharose. Apparently, dimerization can occur before formation of active granzyme A. Furthermore, these in vitro translation experiments showed that dimerization of granzyme A likely takes place in the rough endoplasmic reticulum, i.e. before the protease is activated.

Experiments with PNGase F and Endo H showed that rGA only contained high mannose-type oligosaccharides, similar to natural granzyme A. Binding of the mannose 6-phosphorylated sugar to the mannose 6-phosphate receptor leads to selective transport of granzyme A to the cytotoxic granules (31, 32). The majority of the rGA produced by HepG2 or COS cells was retained in the infected cells. Immunofluorescence showed a granular staining pattern (not shown) comparable with that in LAK cells (23), suggesting recombinant protein was targeted to lysosomes. Alternatively, overproduction of recombinant protein, combined with the virus infection, may have destroyed the cellular architecture, leading to an accumulation of the protein in the cytoplasm.

\[ M. J. Bijlmakers and H. L. Ploegh, unpublished results. \]
in the endoplasmic reticulum or the Golgi apparatus.

Granzymes are synthesized as inactive precursor molecules with a short propeptide consisting of Glu-Lys in the case of Gly-Glu in human granzymes B and H and mouse granzyme B. Mast cell and neutrophil serine proteinases contain similar, short acidic propeptides. Formation of proteolytically active human elastase and cathepsin G involves a dual proteolytic processing pathway. First, the signal peptide is cleaved off, generating an inactivezymogen; second, the amino-terminal dipeptide and a carboxyl-terminal extension are removed, thereby generating active enzyme (20). Although rGA produced by vaccinia-infected cells consisted of a homodimer with similar molecular weight as natural granzyme A, it had no proteolytic activity, except for rGA produced by J urkat cells. Similar observations have been made for COS cells transfected with full-length cDNA coding for human leukocyte elastase or murine granzyme B cDNA (22, 33), whereas COS cells transfected with a mutant granzyme B cDNA lacking the propeptide did produce active granzyme B (22). Together these data suggest that HepG2 and monkey COS cells, in contrast to J urkat cells, lack the ability to process the propeptide of granzymes and related proteinases correctly.

It has been suggested that the lysosomal cysteine proteinase dipeptidyl peptidase I, previously termed cathepsin C, is the putative enzyme involved in the processing of the amino-terminal propeptides of granzymes and myeloid associated serine proteinases (21). In agreement with that, high levels of cathepsin C occur in the spleen and other lymphoid or myeloid cells (34, 35). Thus, the inability of COS and HepG2 cells infected with recombinant vaccinia virus, coding for the full-length granzyme A cDNA, to generate active rGA may have been due to low levels of cathepsin C in these cells. In contrast, J urkat cells, a human T-helper leukemia cell line, constitutively express granzyme A (9) and thus presumably contain cathepsin C. Part of the rGA produced by infected J urkat cells indeed appeared to be active, although this activity was only 20% compared with natural granzyme A. Overproduction of recombinant protein, together with suppression of host protein synthesis by the viral infection, may have disturbed complete processing of recombinant progranzyme A by J urkat cells.

Activation of serine proteases results from the ability of the α-amino group of the first isoleucin of the mature enzyme (Fig. 1), generated after processing of the propeptide, to form an ion pair with the aspartic acid of the catalytic pocket. This interaction enables the formation of a functional catalytic center (20). Apparently, after removal of propeptide by cathepsin C, rGA had proteolytic activity. No decrease in esterolytic activity or loss in affinity for benzamidin-Sepharose was observed after prolonged cathepsin C treatment of natural or activated rGA. This indicates that active granzyme A is refractory to further cathepsin C treatment. If cathepsin C-mediated N-terminal dipeptide cleavage would proceed, the protease would lose its stable conformation and, therefore, its proteolytic activity.

This study is the first to demonstrate expression of an active recombinant human cytotoxic lymphocyte proteinase. By expressing rGA as an inactive zymogen, proteolytic damage to the expression system was minimized, thereby allowing high expression levels. Furthermore, the cysteine proteinase cathepsin C was shown to be able to convert rGA zymogen into an active enzyme, implying that cathepsin C may be involved in the processing of natural granzymes. A similar strategy may be feasible for the expression of other granzymes or related proteinases.

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Fig. 5. A, activation of rGA from infected HepG2 cells by cathepsin C. Lysates of LAK cells or HepG2 cells were incubated with cathepsin C in the presence of cysteine, after which binding of granzyme A to benza
midin-Sepharose was analyzed on immunoblot (lanes 3 and 6, respectively). HepG2 cell lysate incubated with buffer alone and absorbed to benzamidin-Sepharose is shown as control (lane 7). In addition, cell lysates of LAK cells (lane 1) and HepG2 cells (lane 4), not incubated with cathepsin C, as well as their benzamidin-bound fraction (lanes 2 and 5, respectively) were analyzed. All lanes contain lysates or absorbed fractions equivalent to 200 × 10⁶ LAK or 5 × 10⁶ HepG2 cells. B, time course of activation of rGA from infected HepG2 cells by cathepsin C. The amount of BLT hydrolysis is expressed as LAK cell equivalents. HepG2 cell lysate is incubated with cathepsin C and cysteine (●), with cysteine alone (○), or without cathepsin C and cysteine (△), as described under “Materials and Methods.”

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