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CHARACTERIZATION OF RECOMBINANT MOUSE PERFORIN EXPRESSED IN INSECT CELLS USING THE BACULOVIRUS SYSTEM

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Summary Perforin, a potent pore-forming protein, plays an important role in killer lymphocyte-mediated cytolysis. The studies on perforin, although already extensive, have been hampered by the limited amount of perforin naturally available from killer lymphocytes. In the present study, a full-length mouse perforin was expressed in insect cells using recombinant baculovirus. Recombinant perforin appeared to be functional in terms of lysing erythrocytes and nucleated target cells. These results suggest that the recombinant DNA approach developed in the present study may offer a new way for preparing sufficient amounts of engineered perforin for use in the studies aimed at dissecting the functional domains of the perforin molecule.

Perforin, a cytolytic pore-forming protein, is produced by killer lymphocytes and is normally stored in the cytoplasmic granules of these cells (1-3). According to the "granule exocytosis-osmotic lysis" model of lymphocyte-mediated cell killing, perforin is secreted vectorially from the effector lymphocyte towards the target cell following the conjugation between these two cells. In the presence of calcium, the secreted perforin lyses a variety of target cells by binding, inserting, and polymerizing into transmembrane channels in target membranes (4-7). Despite the fact that the nucleotide sequence and the deduced molecular structure of perforin have been available for some time (8-10), very little is known about its precise functional domains. Our previous studies using synthetic peptides encompassing different portions of the perforin molecule have provided some clues (11,12). However, a detailed understanding of the individual functional domains (e.g., membrane-binding, self-polymerization, pore-formation) of

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perforin will still depend on studies using the protein molecules. A major obstacle for performing extensive biochemical and structural/functional analyses of perforin is that this protein is produced by killer lymphocytes in only modest amounts. To circumvent this problem, we decided to prepare large quantities of perforin by employing recombinant DNA technology. In the present study, we expressed mouse perforin in insect cells using the baculovirus expression system. Results on the biochemical and functional characterization of the recombinant perforin produced are reported.

Materials and Methods

Cell Culture. *Spodoptera frugiperda* (*Sf*9) cells were maintained at 27°C as either monolayer or suspension culture in Grace's medium (Invitrogen Co., San Diego, CA) containing 10% fetal bovine serum (FBS; Hyclone Lab., Logan, UT) and 100 μg/ml gentamicin (Sigma Co., St. Louis, MO). Mouse tumor cell lines P815 (mastocytoma) and YAC-1 (lymphoma) were maintained in α-modified minimum essential medium (αMEM) supplemented with FBS (5%) and penicillin/streptomycin (50 IU/50 μg per ml).

Construction of Recombinant Transfer Vectors and Baculovirus. The mouse perforin cDNA was prepared by reverse transcriptase-polymerase chain reaction (RT-PCR) using poly(A)$^+$-RNA derived from cloned murine cytotoxic T cells (CTL) R8 as the template. Two oligonucleotides, based on the published cDNA sequence (10), were designated to amplify a fragment which contained sequence encoding amino acid residues +1 through +534 (full length) of mouse perforin. The amplified fragment, after its sequence was verified by nucleotide sequencing, was subcloned downstream to the polyhedrin promoter in a baculovirus transfer vector pVL1393 (Invitrogen Co., San Diego, CA). The recombinant transfer vector was co-transfected with the wild-type baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcMNPV), into *Sf*9 cells. The recombinant baculovirus clones were selected and then plaque-purified according to the established protocol (13).

Immunoblotting and Immunofluorescence Staining. Expression of perforin in *Sf*9 cells infected with the recombinant virus was detected by immunoblotting analysis and immunofluorescence microscopy using anti-perforin antibodies. *Sf*9 cells were infected with the wild-type AcMNPV or the recombinant clone at an m.o.i. (multiplicity of infection) of 5. At the indicated post infection (p.i.) time points, the cells were harvested, washed in phosphate-buffered saline (PBS), and lysed in PBS containing 0.5% Nonidet-P 40 (NP-40) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were electrophoresed on 10% SDS-polyacrylamide gels, under reducing or non-reducing conditions, and transferred onto Immobilon P membranes (Millipore Co., Bedford, MA). A monospecific rabbit antiserum against perforin and [^{125}I]-protein A (New England Nuclear, Boston, MA) were used, respectively, as the primary and secondary reagent for detecting the immunoreactive protein species. For subcellular localization of recombinant perforin, *Sf*9 cells infected with either AcMNPV or recombinant clone were harvested 48 h p.i., sedimented onto microscopic slides, fixed with 2% paraformaldehyde and permeabilized with acetone. Cells were incubated with either preimmune serum or anti-perforin anti-serum followed by the fluorescence labelling using FITC-conjugated goat-anti-rabbit IgG (Tago). The stained cells were examined using a Nikon fluoromicroscope.

Purification of Recombinant Perforin. *Sf*9 cells were infected with *pfp*-1 at an m.o.i. of 5 and cultured in a spinner flask at 27° C for 72 hours. The cells were harvested, washed, and lysed by freezing and thawing. Perforin present in the cell lysate was purified using column chromatography techniques based on a published protocol (14). The homogeneity of the purified recombinant perforin was demonstrated by SDS-PAGE and silver staining (data not shown).

Hemolysis and [^{51}Cr]-release Cytotoxicity Assays. Lysis of anucleated cells (erythrocytes)

mediated by recombinant perforin was measured using a hemolysis assay. Briefly, sheep erythrocytes were washed and resuspended to 10^8 cells/ml in PBS. The erythrocyte suspension was aliquoted into wells of 96-well plates, at 200 μl/well. Various amounts (in volumes ranging from 5 to 20 μl) of recombinant perforin or control protein (BSA or perforin-negative *Sf*9 cell lysate) were added to individual wells in duplicate. The reaction mixture in each well was then supplemented with 2 mM Ca^{++}. The plates were incubated at 37°C for 0.5 to 2 h as indicated. At the end of the incubation, the plates were centrifuged at 800 x g to sediment unlysed erythrocytes. Aliquots of 100 μl supernatants from individual wells were transferred to fresh plates, followed by absorbance reading using a microplate reader (MR700, Dynatech) equipped with a 410 nm filter. The percentage of hemolysis was determined against a standard curve. To investigate the pH dependence of the hemolytic activity of perforin, the hemolysis assay was carried out with sheep erythrocytes resuspended in isotonic phosphate buffer with pH ranging from 4.5 to 9.0. Lysis of nucleated tumor cells, P815 or YAC-1, mediated by highly purified recombinant perforin was measured by a standard [^{51}Cr]-release assay using the procedure previously reported (15).

Results and Discussion

Expression of Recombinant Perforin in *Sf*9 Cells. The original mouse perforin cDNA clones obtained in our laboratory contain either the nearly full-length (residues +97 through +1800) or the 5' one-fourth (residues -194 through +445) sequence (10), which, in combination, provide the full-length nucleotide sequence. The lack of a full-length cDNA fragment for the purpose of expressing recombinant perforin prompted us to generate such a fragment by employing the RT-PCR technique (see **Materials and Methods**). The full-length cDNA produced using this approach contains sequences encoding both the leader peptide and the entire perforin molecule. This cDNA fragment was expressed in *Sf*9 cells using recombinant baculovirus (the construct was designated *pfp*-1). To verify the production of recombinant perforin, the culture supernatants and cell lysates derived from uninfected *Sf*9 cells and those infected with the wild-type AcMNPV or *pfp*-1 were analyzed by immunoblotting using a monospecific polyclonal antiserum against perforin. As shown in Fig. 1A, only the lysate but not the culture supernatant of *pfp*-1-infected *Sf*9 cells contained a polypeptide species with an apparent molecular mass (M_r) of 65 kD which was recognized by the anti-perforin antiserum. This polypeptide species, although slightly smaller than the native mouse perforin (70 kD), may represent an immature, yet partially glycosylated, form of the full-length perforin. The discrepancy in molecular mass between the recombinant perforin (hereafter referred to as rPFP-1) and the native perforin probably results from the differential post-translational modifications occurring in insect cells versus mammalian cells. A time-course analysis revealed that the expression of rPFP-1 in *Sf*9 cells began as early as 24 h p.i. and reached the maximum at around 72 - 96 h p.i. (Fig. 1B). rPFP-1 was retained in the cytoplasm but not the nuclei of the infected cells, as evidenced by the association of the 65 kD immunoreactive species with the cytoplasmic organelles but not the nuclear fraction of infected cells (data not shown). Mouse perforin

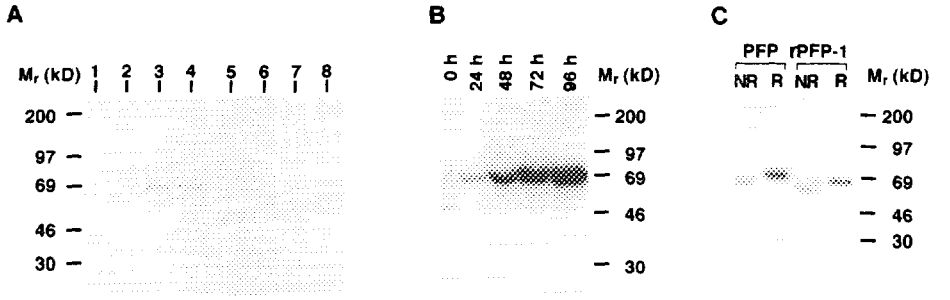

Figure 1. Detection of recombinant perforin (rPFP-1) produced in *Sf*9 cells by Immunoblotting. (A) The presence of rPFP-1 in the whole cell lysate (lanes 1-4) and the culture supernatant (lanes 5-8) of uninfected (lanes 1 and 5), AcMNPV-infected (lanes 2 and 6), *pfp*-1-infected (lanes 3, 4, 7, and 8) *Sf*9 cells were tested using the anti-perforin antiserum. (B) Time course of rPFP-1 expression: the cell lysates derived from *Sf*9 cells infected with *pfp*-1 for the indicated periods of time were analyzed. (C) rPFP-1 and the native perforin (PFP) were electrophoresed under reduced (R) or non-reduced (NR) conditions and detected using the anti-perforin antiserum.

possesses some intramolecular disulfide bonds and exhibits different apparent molecular masses upon SDS-polyacrylamide gel electrophoresis under reducing or non-reducing conditions (3). rPFP-1 also appeared to migrate faster on the SDS-polyacrylamide gel under non-reducing conditions than under reducing conditions (Fig. 1C). In addition to the biochemical similarities, rPFP-1 appeared to resemble closely the native mouse perforin in their functional properties (see below).

Subcellular Localization of Recombinant Perforin Expressed in *Sf*9 Cells. The intracellular location of perforin in *pfp*-1-infected *Sf*9 cells were further investigated. When indirect immunofluorescence staining was performed, a diffuse fluorescence pattern was observed in the cytoplasm of the cells infected with *pfp*-1 for 48 h and labelled with an antiserum specific for perforin (Fig. 2). In contrast, uninfected cells and cells infected with wild-type AcMNPV were not stained by the anti-perforin antiserum (data not shown). In the control experiment, the preimmune serum stained none of the three populations of cells (Fig. 2; data shown only for *pfp*-1-infected cells). Due to the fact that baculovirus-infected cells have a huge nucleus and scanty cytoplasm, it is difficult to locate rPFP-1 to specific subcellular compartment(s) with the resolution capacity of light microscopy. Immunoelectron microscopy is being performed to further investigate this issue. Preliminary results obtained showed that rPFP-1 appeared to be retained in the rough endoplasmic reticulum (ER) and did not undergo further intracellular transport/packaging as does perforin in killer lymphocytes (unpublished data). These results are consistent with those obtained from the immunoblotting study described above. Taken together, the data indicate that rPFP-1 was not properly processed and could not be secreted, despite the presence of the leader sequence in the *pfp*-1 construct. Some post-translational modifications

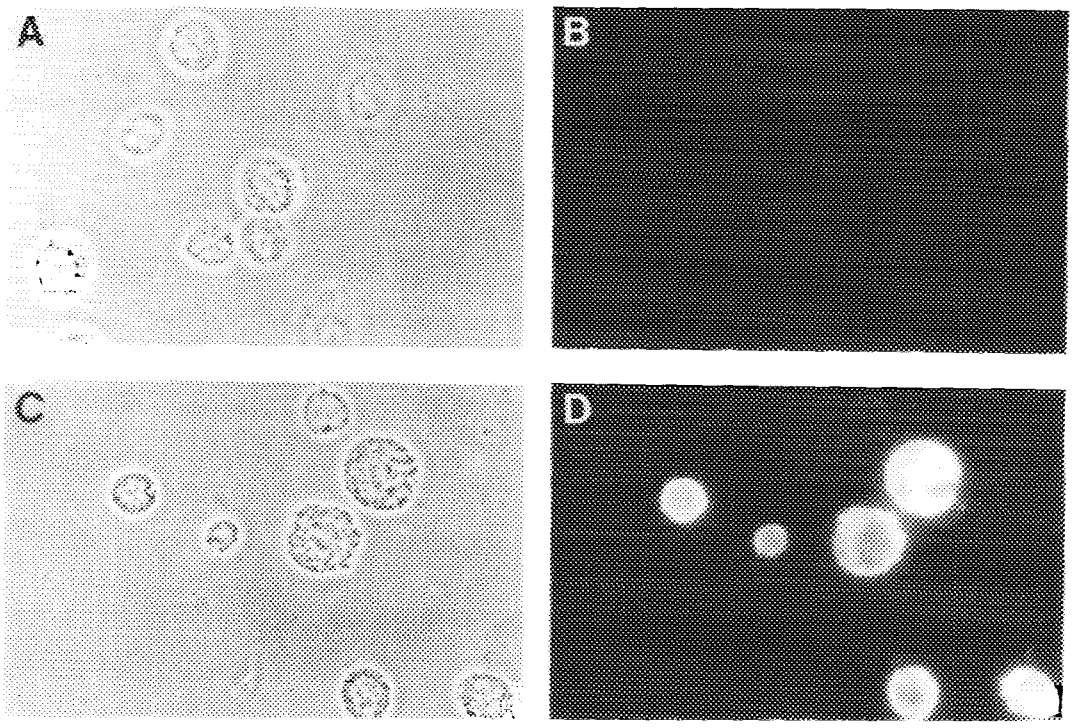

Figure 2. Immunofluorescence staining of *pfp*-1-infected *Sf*9 cells. *Sf*9 cells were infected with *pfp*-1 for 48 h, sedimented on the microscopic slides, fixed and permeabilized. The cells were stained with either preimmune serum (panels A and B) or anti-perforin antiserum (panels C and D) followed by the labeling using FITC-conjugated secondary antibody. Panels A and C are the phase contrast photographs corresponding to panels B and D, respectively (360X).

(e.g., glycosylation), nevertheless, may still occur for rPFP-1, as suggested by its molecular mass of 65 kD (molecular mass deduced for the polypeptide backbone being 60kD) (Fig. 1). The mechanism underlying the blockage of the post-translational processing and transport of rPFP-1 in *Sf*9 cells is currently being investigated.

Functional Characterization of Recombinant Perforin rPFP-1. Recombinant rPFP-1 was purified using conventional column chromatography techniques. rPFP-1 displayed functional properties similar to those of the native mouse perforin based on several parameters. Previous studies have shown that mouse perforin is capable of lysing erythrocytes and nucleated target cells in a Ca^{++}- and temperature-dependent manner (3). The hemolytic activity of rPFP-1 showed comparable characteristics in these aspects. rPFP-1 lysed sheep erythrocytes only in the presence of Ca^{++} (Fig. 3A), and at 37°C but not at 4°C (data not shown). The optimal pH range for the hemolytic activity of rPFP-1 was determined to be between 7 and 8 (Fig. 3B), which is consistent with our previous observation made for the native perforin (3,16). Heparin has previously been shown to be able to inhibit the hemolytic activity of perforin (16). Similarly, rPFP-1-mediated

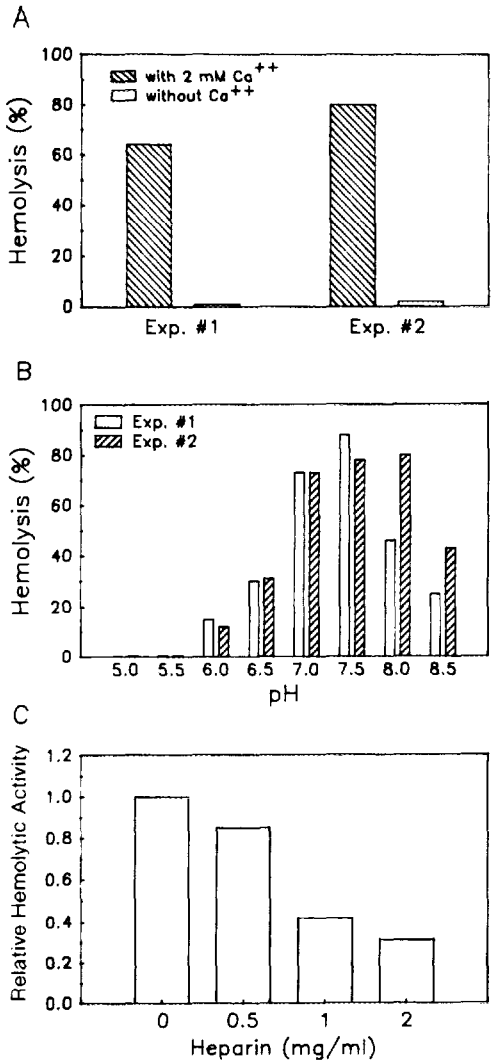

Figure 3. Lysis of sheep erythrocytes by rPFP-1. (A) Ca^{++} dependence of the hemolytic activity of rPFP-1. (B) pH dependence of rPFP-1-mediated hemolysis. (C) Inhibition of rPFP-1-mediated hemolysis by heparin.

hemolysis was also found to be blocked by heparin at 1 or 2 mg/ml (Fig. 3C). Moreover, rPFP-1 was also able to lyse nucleated mouse tumor cells P815 and YAC-1 (Fig. 4) as does the native perforin. It should, however, be pointed out that rPFP-1 appeared to be less efficient than the native perforin. Based on these results, we conclude that rPFP-1 is qualitatively indistinguishable from the native mouse perforin in terms of cytolytic activities. The decreased efficacy may be, at least in part, due to the faulty post-translational processing (e.g., glycosylation) of rPFP-1 within *Sf*9 cells.

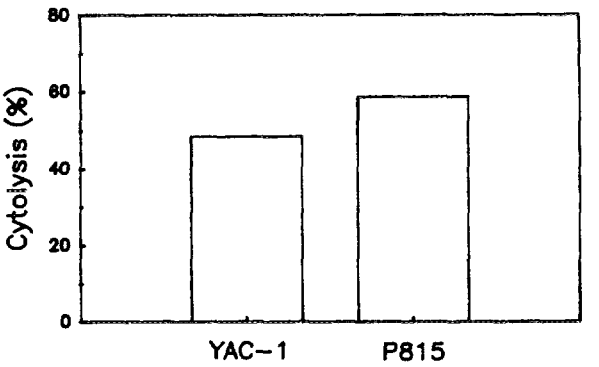

Figure 4. Lysis of nucleated murine tumor cells P815 and YAC-1 by rPFP-1. P815 or YAC-1 cells were labeled with [^{51}Cr], washed, and resuspended in PBS containing 0.5% BSA. 10^4 labeled cells were dispensed into individual wells of a 96-well microplate which contained purified rPFP-1 (20 hemolytic units). The reaction mixture in each well was then supplemented with 2 mM Ca^{++}. The incubation was carried out at 37°C for 6 h. rPFP-1 samples were tested in triplicate. The result shown is representative of three different experiments.

Perspectives. The results presented above show, for the first time, the production of recombinant perforin which exhibits the functional activities similar to the native perforin. The strategy employed in this study may be taken further to synthesize various mutated forms of perforin by way of deletional or site-directed mutagenesis. The different forms of perforin thus generated would be useful in the studies aimed at dissecting the structural/functional relationships of different parts of the perforin molecule. Nucleotide sequence comparison have revealed significant homology between the putative membrane-spanning domains of the complement components C6 through C9 and the central one-third of perforin, which contains predicted α-helix and β-sheet structures (8,10). The N-terminal region (an approximately 40-amino acid residue stretch), unique for perforin, has been shown to be involved in the lytic activity of perforin (11,12). These regions are among the ones to be investigated using the approach developed in the present study. We have already obtained preliminary results showing that the N-terminal truncated perforin lost significantly the lytic efficiency (unpublished data), which is consistent with our previous study using the N-terminal peptides (11,12).

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References

1. Podack, E.R., Young, J.D.-E, and Cohn, Z.A. (1985) Proc. Natl. Acad. Sci. USA 82, 8629-8633.
2. Masson, D. and Tschopp, J. (1985) J. Biol. Chem. 260, 9069-9072.
3. Young, J.D.-E, Hengartner, H., Podack, E.R., and Cohn, Z.A. (1986) Cell 44, 849-859.
4. Henkart, P.A. (1985) Annu. Rev. Immunol. 3, 31-58.
5. Young, J.D.-E and Cohn, Z.A. (1986) Cell 46, 641-642.
6. Tschopp, J. and Nabholz, M. (1990) Annu. Rev. Immunol. 8, 279-302.
7. Podack, E.R., Hengartner, H., and Lichtenheld, M.G. (1991) Annu. Rev. Immunol. 9, 129-157.
8. Shinkai, Y., Takio, K., and Okumura, K. (1988) Nature 334, 525-527.
9. Lowrey, D.M., Aebischer, T., Olsen, K., Lichtenheld, M., Rupp, F., Hengartner, H., and Podack, E.R. (1989) Proc. Natl. Acad. Sci. USA 86, 247-251.
10. Kwon, B.S., Wakulchik, M., Liu, C.-C., Persechini, P.M., Trapani, J., Haq, A.K., Kim, Y., and Young, J.D.-E (1989) Biochem. Biophys. Res. Commun. 158, 1-10.
11. Ojcius, D.M., Persechini, P.M., Zheng, L.M., Notaroberto, P.C., Adeodato, S.C., and Young, J.D.-E (1991) Proc. Natl. Acad. Sci. USA 88, 4621-4625.
12. Persechini, P.M., Ojcius, D.M., Adeodato, S.C., Notaroberto, P.C., Daniel, C.B., and Young, J.D.-E (1992) Biochemistry 31, 5017-5021.
13. Summers, M.D. and Smith, G.E. (1987) A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Argricultural Experiment Station Bulletin No. 1555.
14. Persechini, P.M. and Young, J.D.-E (1988) Biochem. Biophys. Res. Commun. 156, 740-745.
15. Brunner, K.T., Mauel, J., Cerottini, J.-C., and Chapius, B. (1968) Immunology 14, 181-185.
16. Young, J.D.-E, Damiano, A., DiNome, M.A., Leong, L.G., and Cohn, Z.A. (1987) J. Exp. Med. 165, 1371-1382.