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Production of Recombinant Perforin Protein in Baculoviral Expression System

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We produced recombinant human perforin proteins (rs-perforin), using baculovirus expression system. rs-Perforin produced in baculovirus expression system remained within the cytoplasm of the producing cell, and was not secreted into the medium. We substituted the signal sequence of perforin with that of L2G25C, a cDNA of well-secreted MIP-1β protein, hoping to help facilitate secretion of rs-perforin from the host cells. Still the protein remained mostly within the cell cytoplasm. Immunocytochemistry of perforin-producing cells revealed cytoplasm fully laden with perforin protein. It seems to be that recombinant perforin, with its inherent membrane targeting behavior, readily integrate into the membranous part of organells in the cytoplasm soon after being produced and, despite the presence of an efficient signal peptide, could not be secreted from the cell.

Introduction

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells can lyse target cells directly, by a mechanism that requires transient but close contact between the killer cell and its target. The mechanism has been unclear though, and a number of models have been proposed. Based upon phenomenological studies, one model suggests that the CTL and NK cells release cytolytic granule contents after a specific interaction with target cells (Henkart, 1985). However, we still are not sure of what is the lytic component originating from the granule, and, correlatively, what is the initial lesion of the target cell. A first approach to identify molecules involved in cytotoxicity has been to look for molecules themselves endowed with cytotoxic activity, and present either in the supernates of cytotoxic cells, or in subcellular fractions of these. This approach, among others, contributed to the identification, and later the cloning, of perforin (Shinkai et al., 1988; Lichtenheld et al., 1988; Kwon et al., 1989). Perforin is a 60 kDa molecule present in granules in the cytoplasm of many cytotoxic T and NK cells (Podack et al., 1985; Young et al., 1986); it acts on membranes as a calcium-dependent channel-former (Ishiura et al., 1990); most interestingly, it shows significant homology to the molecule C9 of the complement cascade (Zalman et al., 1986; Zalman et al., 1987). Despite the unambiguous evidence for the strong cytotoxic activity of isolated perforin, there are, however, objections as to perforin being the sole mediator of cytotoxicity by T cells: for instance, CTL-mediated lysis of some target cells can occur in a condition in which no exocytosis of granule constituents is demonstrable (Ostergaard et al., 1987; Trenn et al., 1987), some cytotoxic T cells seem to express very little perforin (Berke and Rosen, 1988; Berke and Rosen, 1987), and some cytotoxic T cells do not require extracellular calcium while perforin polymerization does (Young et al., 1987; Trenn et al., 1987). Also, the controversy stems, in part, from the difficulty of measuring perforin activity in, and its release from, lymphocytes and of measuring perforin transfer from the killer to the target cell.

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Recently, *in vitro* expression systems using the cloned cDNA have become very practical and have provided invaluable reagents for the various biological assay systems. If we are able to produce recombinant perforin molecule and observe its independent cytolytic ability, it should help greatly in resolving the difficulties as to a major role for perforin in T cell-mediated cytotoxicity. We used baculoviral expression system to express perforin using cloned cDNA in an attempt to elucidate the molecular mechanism of target cell killing by perforin and other granule products of CTL and NK cells. We report here the results indicating that the expressed recombinant perforin molecule were targeted to and integrated with cell membrane after they were produced.

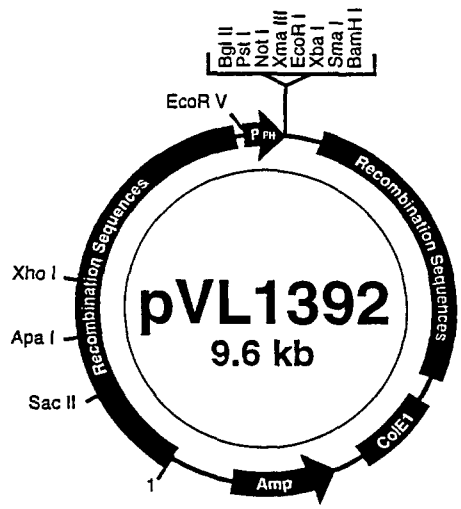

Fig. 1. pVL1392 transfer plasmid vector.

Materials and Methods

Cells

Insect cells, High-Five (Invitrogen) and sf9 (kindly provided by Dr. Max D. Summers), were maintained as a monolayer in TNM-FH (TNM-FH; Grace medium [GIBCO] supplemented with 3.3 g/L Yeastolate [DIFCO] and 3.3 g/L Lactalbumin hydrolysate [DIFCO]) medium containing 10% fetal calf serum (FCS; Bioproducts for Science, Inc.) at 27℃. COS cells were routinely maintained in RPMI-1640 (GIBCO) containing 10% FCS.

Construction of expression vectors

Human perforin cDNA, HP-10, was cloned previously based on homology to the mouse sequence. For transient expression of recombinant perforin in COS cells, whole sequence of HP-10 was inserted into the pXM vector (Yang et al., 1986). For the baculoviral expression system, HP-10 was cloned into transfer plasmid pVL1392 (Invitrogen; Fig. 1) after the signal sequence was substituted with that of L2G25C, one of the well-secreted recombinant form of MIP-1β (Oh et al., 1991), to help facilitate secretion of recombinant perforin from cell (Fig. 2).

Transfection of sf-9 cell

Transfer plasmid pVL1392 containing recombinant HP10 was cotransfected with *Autographa californica* nuclear polyhedrosis virus (AcMNPV) DNA into sf9 cells, using a modification of calcium phosphate precipitation technique (Miller et al., 1986). AcMNPV-HP10 recombinant virus was picked by visual screening and plaque-purified three times. The plaque-purified virus was analyzed for production of recombinant secretory(rs)-perforin by immunoblot analysis, using the primary antibody, P1-8 (kindly provided by Dr. Ko Okumura), a purified monoclonal rat IgG specific for murine perforin. It was previously shown to be able to react with human form of perforin (Nakata et al., 1992). Plaque-purified, perforin-positive virus strains were further amplified in sf9 cells. Culture supernatant containing amplified recombinant virus were kept at 4℃ until use.

Production and purification of recombinant proteins

Sf9 cells were infected with the recombinant virus at 10 multiplicity of infection(MOI). The cells were transferred to Excell 400 (JRH Scientific) serum-free medium after infection. At the 3rd day post-infection, The culture supernatant was collected and the rs-perforin was purified from the con-

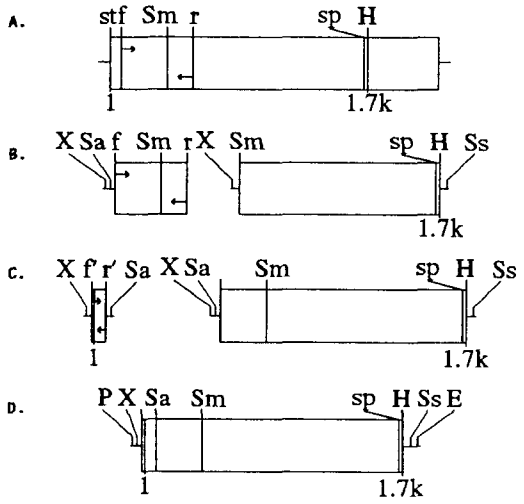

Fig. 2. Construction of baculoviral expression vectors. Human perforin cDNA clone, HP10 (A), was modified in 5′ leader sequence. To do that 456-bp 5′ part, representing N-terminal part of the mature perforin protein, was amplified with a forward primer(f) that include extra *Xba*I(X) and *Sal*I(Sa) restriction sites and reverse primer(r) that is complementary to internal sequence of HP10. The 3′ part(*Sma*I[Sm]-*Hin*dIII[H]) was cloned separately in pGEM7Zf(+) (B). After 5′ PCR-amplified part was ligated to 3′ part using internal *Sma*I site, the leader sequence of L2G25C, both 5′- and 3′-end modified with PCR to include *Xba*I and *Sal*I sites (primers f′ and r′), was added in front of leader-less HP10 (C). The whole construct was transferred to another plasmid, pUC18, using *Xba*I and *Sst*I(Ss) to obtain the necessary *Pst*I(P) and *Eco*RI(E) sites (D) before finally being put into transfer plasmid vector pVL1392 (Fig. 1) for baculoviral expression. st, start codon; sp, stop codon.

centrated and dialyzed culture supernatant by HPLC size exclusion column. In later experiment, High-five cells were used for higher production and serum-free TNM-FH medium containing gentamicin (10 mg/L) were used instead of Excell 400. Cells were harvested after 1, 2, and 3 days post-infection.

Recombinant perforin was also transiently expressed in COS cells using the pXM-HP10 vector.

Electrophoresis and Immunoblot Analysis

Cell pellet and culture supernatant were separated by centrifugation and subjected to discontinuous electrophoresis on 10% polyacrylamide gel. Protein bands in gel were visualized by Coomassie brilliant blue staining or electroblotted to Immobilon-PVDF membrane for immunoblot analysis using P1-8 as a primary anti-perforin antibody.

Immunoenzymatic Staining

For intracellular localization of perforin, recombinant virus-infected cells were subjected to conventional paraffin embedding and sectioning procedures, after being packed by centrifugation and fixed in formaldehyde and paraforaldehyde mixture. On other occasions, cell suspensions were fixed in 3.7% formaldehyde in PBS (pH 7.6) for 10 minutes, after being spun onto glass slides and air dried. Slides were fixed again in acetone, 4 min. at −20℃, followed by methanol for 1 min., at −20℃. After fixing the slides were washed three times by soaking in TBS, pH 7.6 for 5 minutes each. Normal rabbit serum diluted 1:50 in TBS, was added as blocking serum and incubated for 30 minutes. After blotting off excess blocking serum, the primary antibody P1-8 was added at 1:500 in TBS for 1 hour. After washing 3X in TBS, biotin conjugated rabbit anti-rat IgG (1:500 in TBS) was added for 30 minutes. The slides were then washed 2X in TBS, and avidin conjugated alkaline phosphatase was added for 30 minutes. Following a final wash, 2X in TBS, the substrate Fast Red was added for 30 minutes. Slides were washed in water, and counter stained with hematoxylin. After drying, cover slips were added with GVA mounting media.

sRBC hemolysis assay

Microassay method was used to detect hemolytic activity of rs-perforin (Young et al., 1986).

Results

Cross-species Specificity of the Anti-Perforin Monoclonal P1-8

The monoclonal antibody P1-8 used in the experiments was raised against recombinant murine perforin, and has previously been used to stain per-

Fig. 3. Immunohistochemical staining of COS cells that were transfected with human perforin cDNA, HP-10, in pXM vector. Cytocentrifuged, fixed, and blocked cells were sequentially incubated with anti-perforin antibody p1-8, biotinylated rabbit anti-rat immunoglobulin (Zymed), and streptavidin-alkaline phosphatase conjugate (Zymed). The slides were developed with Fast-Red (AP-Red kit; Zymed). The nuclei were counter-stained with Meyer's hematoxylin and mounted with GVA-mount (Zymed). The cells which stained dark in the photograph are perforin-positive.

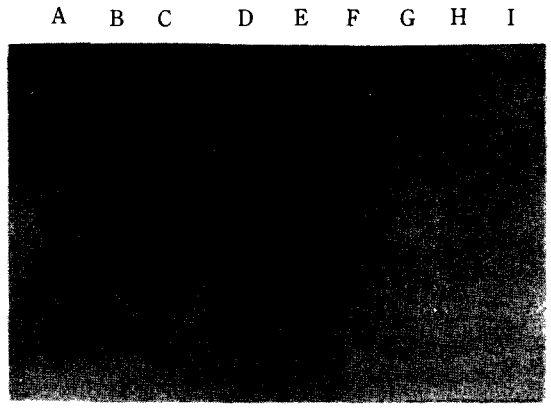

Fig. 4. Immunoblot analysis of human rs-perforin using antibody to mouse perforin, P1-8.
A, B: Uninfected sf9 cell culture supernatant
C: Fusion protein of IL-2 and HP10 from bacterial expression system, as a positive control
D, E, F: Recombinant virus-infected sf9 cell culture supernatant, 3-, 4-, and 5-day post-infection; positively stained for rs-perforin
G, H, I: Wild type virus-infected sf9 cell culture supernatant, 3-, 4-, and 5-day post-infection

forin in human peripheral blood mononu- clear cells (Nakata et al., 1992). In order to confirm the specificity of this antibody in human cells, we tested its ability to recognize recombinant human perforin expressed in COS cells (Fig. 3). Recombinant human perforin was specifically recognized by P1-8 on western immunoblot analysis (Fig. 4). P1-8 was not cross-reactive in immunocytochemical staining to any antigens of the Jurkat human T cell line, confirming its specificity for perforin (data not shown).

Localization of Perforin on the Cell Membrane

Immunoenzymatic staining of whole or cryosections of pXM-HP10-transformed COS and AcMNPV-HP10-infected High-Five cells shows positive perforin staining. They all remain within the cell, mostly in cytoplasm (Fig. 3 & 5). More intense staining for rs-perforin on the membranous parts of sectioned preparation was particularly noted.

sRBC hemolysis assay

rs-Perforin purified from the culture supernatant by HPLC size exclusion column was used for hemolytic assay. We could not detect measurable hemolytic activity of the preparation.

Discussion

Baculovirus expression system is an insect virus host-vector system for high-level expression of foreign genes (Miller et al., 1986). We tried to produce recombinant human perforin proteins (rs-perforin), using baculovirus expression system. At first, we used the whole sequence of HP-10, containing some 5′ flanking region, signal sequence, stop codon, and some 3′ flanking region. A pilot experiment using transient expression in COS cells indicated expressed perforin was not secreted well into the medium. To facilitate secretion, we substituted the signal sequence of perforin with that of L2G25C, a cDNA of well-secreted MIP-1β protein. Previously we were able to efficiently express MIP-1β protein in baculoviral system and harvest it from the culture supernatant (Oh et al., 1991). However, the rs-perforin produced in baculovirus expression sys-

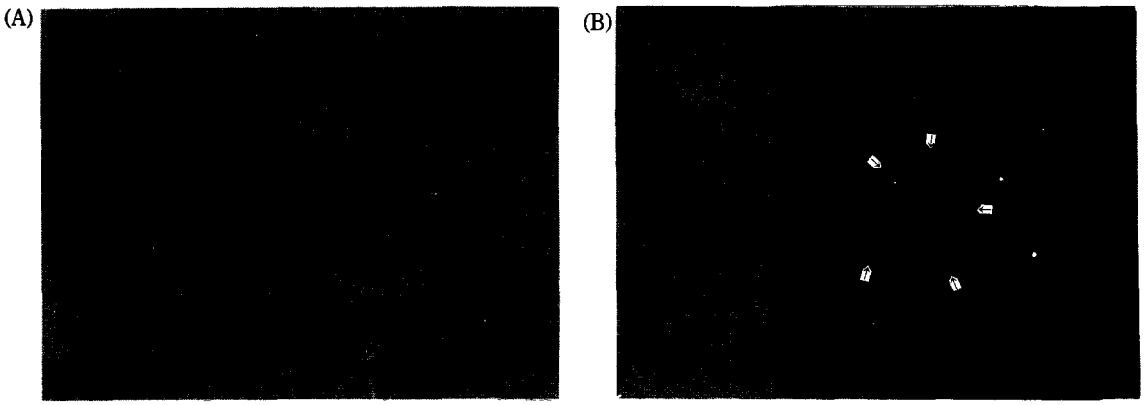

Fig. 5. Immunohistochemical stain of formalin-fixed, paraffin-embedded sections of normal (A) and AcMNPV-HP-10-infected (B) High-Five cells. 3 days post-infection. Perforin is localized within the cytoplasm, presumably associated with membranous component of the cells. Note circular and peripheral appearance of perforin-positive areas (arrows) 1000 X magnifications.

tem, either with its own signal sequence or with MIP-1β's, largely remained in cytoplasm and we could obtain very little from the culture medium. By immunocytochemistry of recombinant virus-infected cells, though, we were able to observe cytoplasm fully laden with perforin protein and confirm the high efficiency of baculoviral expression. Morphologically, all of the perforin-positive cells showed far advanced cytopathy that detailed study was not possible. However, it is strongly indicated that, despite the presence of an efficient signal peptide, most of the recombinant perforin were, with its inherent membrane targeting behavior, integrated into membranous part of the cell and, thus, prevented from being secreted.

Naturally, perforin is stored in the cytoplasmic granules of the CTL and NK cells after being produced. Recently, Shiver and Henkart (1991) transfected the noncytotoxic rat basophilic leukemia (RBL) cell line with perforin expression vectors to test the granule exocytosis model for lymphocyte cytotoxicity. They demonstrated that the expressed perforin protein was correctly targeted to secretory granules and RBL transfectants became to show a potent and calcium-dependent cytolytic activity against IgE-coated RBCs. Thus, granule-targeting may be an important mechanisms for the physiological control of perforin function, which is obviously not exsist in our cells. Membrane-targeting behavior of rs-perforin seems to be natural regarding the pore-forming function of native perforin. However, it would be more desirable for perforin study if we could obtain soluble form of the protein. In order to circumvent membrane-targeting behavior of rs-perforin during expression inside cell, we need to know more about the domain structure of perforin molecule. Currently we are expecting to make monoclonal antibodies against various domains of perforin molecule using the purified membrane-bound rs-perforin. We were not able to see the biologic function of rs-perforin. However, we provided rs-perforin purified from the culture supernatant by HPLC size exclusion column to other researchers (C.C. Liu in Rockefeller University) and they saw a minimal cytolytic activity in the preparation (personal communication). This is an encouraging result and we are planning to purify perforin protein from the cell and test if we could see cytolytic activity.

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