Isolation of a Lytic, Pore-forming Protein (Perforin) from Cytolytic T-Lymphocytes*

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Cytolytic granules from a T-cell line with specific cytolytic activity were isolated. Granules were solubilized and fractionated on a TSK 4000 gel filtration column. Lytic activity was eluted as a single retarded peak. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate indicated that the lytic fractions contained a single protein (perforin) with an apparent molecular weight of approximately 66 kDa. It separated well from the other proteins present in the granules. Isolated perforin polymerized and inserted into lipid bilayers in the presence of Ca++, forming tubular structures with inner diameters varying from 6 to 16 nm. Lipid insertion of perforin was demonstrated using a membrane-restricted, photoactivatable probe.

The lytic properties of perforin suggest an important role of this particular protein during cytolytic T-cell-mediated target cell lysis.

Cells attacked and lysed by cytotoxic T-lymphocytes carry tubular structures (polyperforins) on their surface (1, 2). Polyperforins are derived from cytoplasmic granules present in large granular lymphocytes, NK cells, or cytolytic T-lymphocytes (CTL), since isolated granules are cytotoxic in the presence of Ca++, and form polyperforins (3, 4). A solubilized partially purified cytolytic fraction of these granules induces marker release from vesicles (5). Moreover, antibodies raised against polyperforin recognize granules and vice versa. SDS-PAGE analysis of isolated granules revealed the presence of three major proteins with approximate molecular weights of 27, 29, and 66 kDa (3, 4). Here we report the isolation and characterization of a 66-kDa protein (perforin) from granules with lytic properties and the propensity to form polyperforins.

MATERIALS AND METHODS

Cell Lines—B6.1, an H-2Dd specific CTL cell line was grown in Dulbecco's modified Eagle's medium, supplemented with additional amino acids (6), 10 mM HEPES, 5 x 10^{-4} M-mercaptoethanol, and 5% (v/v) fetal calf serum). Since the growth of this cell line is dependent on interleukin-2 (T-cell growth factor), the medium was supplemented with 20% supernatant of activated rat spleen cells (7).

Isolation of Granules—Cytolytic granules from B6.1 cells were isolated as described elsewhere (8). In brief, 500 million B6.1 cells were harvested by centrifugation, washed, and dissolved in phosphate-buffered saline, once in PIPES (100 mM KCl, 3.5 mM NaCl, 3.5 mM MgCl2, 1 mM ATP, 1.25 mM EGTA, 10 mM PIPES, pH 6.8) and suspended in 8 ml of PIPES. Cells were disrupted by N2 cavitation and, after the removal of nuclei and aggregates, the cell lysate was layered on top of a Percoll step gradient (15.9 ml of 39% (w/v) Percoll and 12.1 ml of 90% Percoll). The tube was centrifuged in a Beckman JA 20 rotor (19,000 rpm, 30 min at 4 °C). Fractions of 500 μl were collected and tested for hemolytic activity.

Assay for Hemolytic Activity of Granules—2-μl aliquots of perforin fractions were diluted in 100 μl of TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 0.1 mM BSA and 1 mM EDTA. 300 μl of sheep erythrocytes (10^{6} cells/ml) in Veronal (5 mM)-buffered saline (0.15 M NaCl); pH 7.4, containing 5 mM Ca²⁺, 0.3 mM Mg²⁺, and 0.1% (w/v) gelatin were added and the mixture was incubated in special plastic cuvettes (Gilford Instruments, Oberlin, OH). After 15-min incubation at 37 °C, intact erythrocytes were spun (5 min, 1500 rpm) to the bottom of the cuvettes and the hemoglobin release was quantitated at 412 nm by means of a Gilford 1 absorption reader (Model PR-50).

Isolation of Perforin—Isolated perforin was made 2 M in NaCl in order to disrupt the granule membrane (8). The solution was spun at 48,000 rpm for 4 h in a Beckman rotor. The pellet, consisting of Percoll and membranes of granules, was discarded and the hemolytically active supernatant was concentrated to 5 ml. The solution was applied to a TSK-4000 column (60 cm, 7.5 mm, LKB) attached to a fast protein liquid chromatography system (Pharmacia). The column was eluted with 0.1 M ammonium acetate, 50 mM NaH2PO4, buffer, pH 7.4, and negatively stained with 2% uranyl formate (Eastman Kodak). Photographs were taken at a direct magnification ×35,500 in a Philips EM 400 electron microscope.

Electron Microscopy—Purified perforin (10 μg) was added to 50 μg of small unilamellar vesicles (10) prepared from egg lecithin (Avanti Lipid, Birmingham, AL). The mixture was incubated for 20 min at 37 °C in TBS containing 5 mM Ca²⁺. Another solution of perforin (5 μg) was added to 10° sheep red blood cells in 500 μl of TBS containing 5 mM Ca²⁺. Red blood cell membranes were then washed and isolated exactly as described in Ref. 4. Samples were adsorbed to carbon-coated grids that had been rendered hydrophilic by 0.1% (w/v) boric acid (Sigma). The grids were washed twice with 100 mM NH4 acetate, 50 mM NH2HCO3 buffer, pH 7.4, and negatively stained with 2% uranyl formate (Eastman Kodak). Photographs were taken at a direct magnification ×35,500 in a Philips EM 400 electron microscope.

RESULTS

Isolated granules from the CTL cell line B6.1 were shown to be hemolytic. When analyzed by SDS-PAGE, a set of distinct proteins were found to be present in these granules.
We used granules from this cell line to identify the molecule(s) exhibiting the lytic activity. Granules were isolated from approximately 500 million cells by means of a Percoll density gradient. Hemolytically active granules could be purified to homogeneity. The granule solution was subsequently disrupted in 2 M NaCl (8) and their content was separated from the surrounding membranes by high speed centrifugation. Contaminating Percoll was eliminated during the same step.

Proteins from the granules were then subjected to gel filtration. A high pressure liquid chromatography column (TSK-4000), equilibrated in a buffer containing 1 M NaCl (pH 4.5), was used. Fig. 1 shows the elution profile of the TSK-4000 column. The hemolytic activity coincided with the total volume of the column. The fractions containing lytic activity, pH 4.5, was used. Fig. 1 shows the elution profile of the TSK-4000 disrupted in 2 step.

Contaminating Percoll was eliminated during the same step. Granules were isolated analyzed by SDS-PAGE (Fig. 2, panel c). For comparison, purified granules and granules that had been solubilized in 2 M NaCl are also shown (Fig. 2, lanes a and b). After high speed centrifugation of the disrupted granules, the supernatant contained the same set of proteins as intact granules: 3 predominant bands with apparent molecular weights of 24,000, 27,000, and 60,000 under nonreducing conditions were observed. Pool 1 of the TSK-4000 column corresponding to fractions eluting at the void volume of the column was devoid of any detectable protein (panel C, lane 1). A pool corresponding to proteins with apparent molecular weights of 30,000 to 200,000 (Pool 2) contained three main proteins with molecular weights of 60,000, 27,000, and 24,000 (panel C, lane 2). Under reducing conditions, the 60-kDa band completely disappeared and a new blurred band with a molecular mass of 30–40 kDa was apparent. The mobility of the 27-kDa species changed to 29 kDa and that of 24-kDa species to 27 kDa. A third pool containing the hemolytically active fractions again showed the 60-kDa protein and the 24-kDa proteins (panel C, lane 3). Whereas the 24-kDa protein migrated under reducing conditions as did the corresponding protein in pool 2, i.e. at a position of 27 kDa, the 60-kDa protein behaved quite differently: this protein now displayed a similar apparent molecular mass as bovine serum albumin. Since pool 3 still contained two proteins, it was further purified by passing it over a TSK-3000 column. A similar elution profile as for the TSK-4000 column was obtained (data not shown). The hemolytic activity was pooled. As shown in Fig. 2, lane d, the hemolytic activity was associated with a single protein (perforin) migrating at 66- and 60 kDa under reducing and nonreducing conditions, respectively.

Table I summarizes the purification procedure of perforin. Upon solubilization of isolated granules in 2 M NaCl, an approximately 2–3 times increase of specific activity was observed. 20–150 μg of perforin were usually obtained starting out from 500 million cytotoxic T-cells after the TSK columns. Approximately 5 ng of purified perforin was required to lyse 1.5 million red blood cells.

Since analysis of proteins by SDS-PAGE or other standard biochemical methods of protein analysis such as end group analysis would not detect a small impurity (<5% of total protein) evoking the lytic activity, labeling of the lipid inserted protein using a membrane-restricted probe ([125I]TID) was
performed. This probe exclusively attaches to protein in contact with lipid membrane (11). Insertion of purified perforin into small unilamellar lipid vesicles was achieved by polymerizing perforin in the presence of 5 mM Ca\(^{2+}\). After addition of (\([^{125}I\])TID), the probe was bound to a protein corresponding to perforin (Fig. 2, lane e).

Electron micrographs (Fig. 3, lanes a–c) of this vesicle/perforin mixture showed the presence of tubules with dimensions similar to those reported for polyperforins. Moreover, perforin oligomers which did not assume the typical ring structure could be discerned very frequently. Using the sheep erythrocyte as targets, again polyperforins with different diameters (8 to 15 nm) were visualized (Fig. 3, lanes d–f). Small ring-like structures with diameters of approximately 6 nm, called polyperforin 2 in an earlier report, (2) were also consistently seen. No tubules were observed without addition of Ca\(^{2+}\) (data not shown).

**TABLE 1**

| Purification of perforin | Volume | Protein | Total activity | Specific activity |
|--------------------------|--------|---------|----------------|------------------|
|                         | ml     | mg      | units          | units/mg         |
| Isolated granules\(^*\) | 4.5    | 6.59    | 91,304         | 13,855           |
| Solubilized granules\(^*\) | 4.2 | 6.15    | 174,112        | 28,311           |
| Purified perforin         | 29.4   | 0.15    | 30,940         | 206,270          |

\(^*\) One lytic unit corresponds to the lysis of 1.5 million sheep red blood cells.

\(^*\) Purified by means of a Percoll gradient.

\(^*\) Solubilized in 2 M NaCl.

**DISCUSSION**

Recent studies in the mechanism of lymphocyte effector cell-mediated cytolysis indicated that the pore-forming polyperforins present on target cells after their destruction may represent the cell-damaging structure (1, 2). Precursors of polyperforins were localized in the cytoplasmic granules of the effector cells (3–5). The importance of granules in the lytic event was corroborated by studies showing their accumulation close to the contact site of the cytolytic cell and its target during cytolysis (4). Moreover, isolated granules were shown to lyse different cells, including erythrocytes, without specificity. On the basis of these experiments, a similar mechanism of lysis was proposed as that observed in the complement cascade (12): upon exocytosis of the granules and upon contact with calcium ions, perforin changes its conformation, polymerizes into tubule-like structures (polyperforins), and inserts into the membrane. To this date, the precursor protein perforin has not yet been identified.

By following the hemolytic activity, we have fractionated granules and identify a single protein (perforin) as the factor in the granules responsible for red blood cell lysis. Purified granules were first disrupted in 2 M NaCl and then passed over a TSK-4000 column. Perforin eluted almost at the total volume of the TSK-4000 gel filtration column, a behavior which does not reflect its molecular weight as determined by SDS-PAGE. It is known that the TSK type of gel filtration column often separates proteins not solely according to the size of molecule (13). Interactions, which are not fully understood, of certain proteins with the gel material may separate proteins in an unpredictable manner. C9, the pore-forming protein of the complement cascade, exhibited the same prop-
properties as observed for perforin. This may suggest a similarity of C9 and perforin. Other results suggest certain common features of these two proteins: the mobility of C9 and perforin in SDS-PAGE is highly dependent on the integrity of the disulfide bonds. In the case of C9, this behavior is due to a domain very rich in cysteine residues (14). Their molecular weight is similar; both mouse perforin and human C9 migrate on SDS-PAGE in the vicinity of BSA (66,000). Moreover, both proteins form tubular structures in the presence of metal ions (15).

The granule proteins with an approximate molecular weight of 60 kDa in the nonreduced form were separated into two distinct proteins: a hemolytically inactive (we call it G1) form and an active form (perforin). On the basis of their migration properties in SDS-PAGE, the two proteins could not be distinguished in the unreduced form. In the reduced form, however, one protein (G1) migrated as a broad band of 30-40 kDa, whereas the other one (perforin) remained a single polypeptide of approximately 66 kDa. The role of G1 is not known; G1 may represent an inactive, cleaved form of perforin or may be a protein unrelated to perforin.

Perforin assembled to polyperforin and inserted into erythrocytes in the presence of Ca++. Tubules with the dimension described for polyperforin 1 and 2 could be discerned. The inner diameter of polyperforin 1 was not consistent, but varied from 8 to 16 nm. The role of the two types of tubules is presently not known. Polyperforin 1 and 2 were shown to be generated by NK cells and CTL. In noncytolytic cell lines, both types of polyperforins were absent. In a cell line with inducible cytolytic activity, polyperforin 1 and 2 were found in cytotytic cells, whereas noncytolytic cells formed polyperforin 2. Our results suggest that perforin can form polyperforin 1 exhibiting the shape of a closed tubule, to intermediates thereof and to polyperforin 2.

Perforin also inserted into small unilamellar lipid vesicles. Blumenthal et al. (5) showed that lipid vesicles could be lysed by a noncharacterized, soluble fraction of NK-cell derived granules called “cytolysin.” In our hands, insertion of perforin into these small unilamellar vesicles led very often to noncircular aggregates, and not to tubules. Since polyperforins and small unilamellar vesicles exhibit a similar diameter, tubule formation may be more difficult to achieve.

Perforin-lipid vesicles conjugates, formed in the presence of 5 mM Ca++, were labeled by the membrane-restricted probe [125I]TID corroborating the notion that polyperforins are embedded in the lipid bilayer. [125I]TID labeling of perforin almost certainly excludes the possibility that the tubular structures on the membranes are derived from a minor contamination of purified perforin.

Further studies have to show what role of 27-kDa, 29-kDa, and other proteins or enzymes present in the granule play in the lytic process mediated by CTL. Our study suggests that neither of these proteins nor the integrity of the granules are required for the lytic process. One individual protein (perforin) appears to be sufficient to account for the pore formation.

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