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Perforin-Enhancing Protein, a Low Molecular Weight Protein of Cytotoxic Lymphocyte Granules, Enhances Perforin Lysis

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Perforin is a 68 kD protein found in the granules of cytotoxic lymphocytes and is used by lymphocytes to form lethal pores in the membranes of the cells they kill. We and others have found that when perforin is purified, its lytic activity is markedly reduced. ELISAs indicated that our final recovery of perforin protein was excellent. We decided to determine if depletion of other granule proteins contributed to the loss of lytic activity. We isolated perforin to the point where lytic activity was diminished and added back granule proteins that had no lytic activity or detectable (antigenic) perforin. Perforin was isolated by Cu^{2+}-immobilized metal affinity chromatography (IMAC) followed by phenyl-Superose hydrophobic interaction chromatography (HIC). Its lytic activity was enhanced by a low molecular weight (<15 kD) protein, perforin enhancing protein (PEPr). We have isolated PEPr by two methods, HIC and MonoQ. Nonlytic PEPr restored perforin to close to its original lytic activity. A protein similar if not identical to PEPr was also detectable as an ^{125}I-labeled protein associated with lytic perforin. We propose that PEPr acts in conjunction with perforin to form lethal pores and suggest that PEPr may be the rat equivalent of the human cytotoxic lymphocyte protein, granulysin.

Natural killer and cytotoxic T lymphocytes are important for the body's defense against tumor growth, viral infections and tissue transplantation (1). Cytotoxic lymphocytes have two lytic mechanisms which involve either Fas-Fas ligand interaction or perforin-mediated lysis (2-4). In perforin-dependent cytotoxicity, granules are released from cytotoxic lymphocytes prior to the lysis of target cells (5). The exact mechanism of this lysis is not completely understood but involves membrane pores (6). Granules isolated from cytotoxic lymphocytes will produce these pores (7,8) and contain the pore-forming protein perforin (9-12), as well as a number of different serine-dependent proteases termed granzymes (13-16), and proteoglycan (17). Perforin is essential for lethal pore formation; mice lacking the gene for perforin lack both NK- and T cell-mediated cytotoxicity (4,18,19). The following report illustrates the depletion of PEPr during perforin isolation. It also shows reconstitution of lytic activity by the addition of PEPr and provides a practical method for the isolation of PEPr.

MATERIALS AND METHODS

Granule preparation. The granules (20) were obtained from RNK-16 NK- like leukemia cells (21) grown as an ascites line in F344 rats obtained from the National Cancer Institute (USA). The ascites cells were washed with Borregard's relaxation buffer (22) and then ruptured using a nitrogen cavitation bomb (Parr instrument Co., Moline, IL) pressurized to 450 psi. The lysate was layered over 54% Percoll (Sigma Chemical Co., St. Louis, MO) which formed a gradient during a 20 minute centrifugation at 45,000 x g in a Beckman Ti50.2 rotor at 4°C. The dense fractions (up to 1.068 $g \cdot cm^{-3}$) were pooled. Nuclei were removed by filtration through a 3 μm Nucleopore filter (Millipore, Bedford, MA) (23). Percoll was then removed by a high speed spin, 4 hours at 145,000 x g. Granules were collected from above the Percoll pellet, disrupted by three freeze-thaw cycles after adding NaCl to bring the salt concentration up to 1 M (22) and stored at −20°C. Protein concentrations were determined by a BCA assay (Pierce, Rockford, IL) using bovine serum albumin (BSA) for calibration.

Protease assays. Protease activities were measured using colorimetric assays with peptide thiobenzyl ester substrates (20). After cleavage these substrates release the thiobenzyl group which is detected using Ellman's reagent (24), dithiobis-(2-nitrobenzoic acid). The product absorbs at 412 nm. A Thermomax microplate reader

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Abbreviations: IMAC, immobilized metal-affinity chromatography; NK, natural killer cells; RBC, rabbit red blood cells; IP, intraperitoneal; CTL, cytotoxic lymphocyte; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ADCC, antibody-dependent cell-mediated cytoxicity; MHC, major histocompatibility complex; KLH, keyhole limpet hemocyanin.

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(Molecular Devices, Palo Alto, CA) in the kinetics mode was used to measure the rates of substrate hydrolysis.

Cytotoxicity assays. Cytolytic activity was determined by the hemoglobin released from lysed red blood cells (RBC) (23,25). Dilutions of isolated perforin were incubated with 0.5% (v/v) RBC at room temperature for the times indicated in 0.2 ml in round bottom microtiter plates (Falcon 3910, Becton Dickinson Labware, Lincoln Park, NJ). The assay buffer contained 10 mM HEPES, 0.15 M NaCl, and 10 μg/ml bovine serum albumin (Sigma A4503), pH 7.5 with addition of calcium to 1 mM during incubation to start lysis (7). The reaction was halted by acidification with pH 6.0 2-[N-morpholino]ethane-sulfonic acid (MES, Sigma M-8250) (25). The microtiter plates were spun at 1500 x g for 10 minutes, the cell-free supernatants transferred to a second microtiter plate, and the released hemoglobin detected with a microplate reader at 412 nm. The percent lysis was calculated as [(% expt'l hemolysis - % spontaneous hemolysis)/(% maximal hemolysis - % spontaneous hemolysis)] $\times$ 100. Addition of 0.01% saponin (Sigma) produced maximal RBC lysis. The activity of perforin was expressed as lytic units per mg total protein. One lytic unit is the amount of perforin needed to mediate lysis of 50% of the red cells. The units of activity were calculated by linear regression of the perforin activity at several dilutions and the lines had r values $>$0.95.

Proteoglycan assay. We used a colorimetric assay for sulfated glycosaminoglycans (26). Samples (25 μl) were mixed with 200 μl of the color reagent (4.6 μM 1,9-dimethyl-methylene blue (Aldrich Chemical Co., Milwaukee, WI. Prod. # 34,108-8) in 40 mM NaCl (Fisher Scientific, Pittsburgh, PA) and 40 mM glycine (Fisher), pH 3.0 in microtiter wells. Assays were read immediately at 525 nm. Chondroitin sulfate A (Sigma) was used for calibration.

SDS–PAGE of proteins. For SDS-PAGE the samples were electrophoresed (27) using precast 12% gels (Bio-Rad Laboratories, Hercules, CA). The proteins were visualized with either a Coomassie blue stain (28), silver stain (28) or detected by radioactivity with a Bio-Rad phosphoimager. Images were obtained using either a Vmax scanner (Envisions Solutions Technology, Burlingame, CA) and Adobe Photoshop (Adobe Systems, Mountain View, CA) capturing software or using a Bio-Rad phosphoimager with its software. Labels were added to the images using Corel Draw software (Corel, Ottowa, Ontario).

Perforin and PEPr isolation. Columns were attached to a FPLC work station fitted with LC-500 pumps (Pharmacia, Piscataway, NJ) and controlled by the FPLCmanager software program. *Perforin isolation:* Perforin was prepared by IMAC (29) followed by HIC. Prior to the IMAC column, EGTA was removed by Bio-Gel P-6DG chromatography (Bio-Rad Laboratories, Richmond, CA) (1.6 cm $\times$ 20 cm) to exchange the granule proteins into IMAC starting buffer which contained 20 mM HEPES (Fisher), 10% Betaine (Sigma B-2629), 0.1% NaN_3 (Fisher) and 1 M NaCl (Fisher) pH 7.5. These granule proteins were then loaded onto an IMAC (Poros MC/P 5mm/50mm, PerSeptive Biosystems, Cambridge, MA) column that was precharged with copper and eluted with a gradient of 0 to 0.2 M imidazole. Perforin eluted at $\sim$0.06 M imidazole. After the addition of dry NaCl to make the perforin sample 2 M NaCl, it was loaded onto a phenyl-Superose HIC column (Pharmacia, HR 5/5). The loaded column was washed with starting HIC buffer containing 2 M NaCl, 10% betaine, 20 mM Tris (Fisher), 1 mM EGTA (Sigma) and 0.1 % NaN_3, pH 7.2. The bound proteins were eluted with a NaCl gradient decreasing to 0 M NaCl using NaCl-free HIC buffer. Perforin was detected by cytotoxicity assays and by enzyme-linked immunosorbent assays (ELISA) using affinity-purified anti-perforin peptide antibodies and alkaline phosphatase-linked goat anti-rabbit IgG antibodies (Sigma). **PEPr isolation:** PEPr was isolated by HIC in the perforin preparation just described (Fig 1A) or by MonoQ (Fig. 1B). The proteoglycan and proteins that did not bind to the IMAC column were further separated using a MonoQ (Pharmacia HR5/5) anion exchange column. The unbound IMAC fraction (1 M NaCl) was diluted to 0.5 M NaCl in 20 mM Tris, 0.1 mM EGTA pH 7.0 and loaded. Proteins were eluted with a gradient from 0.5 to 1 M NaCl. For some experiments the unfractionated granule proteins or the IMAC-perforin fraction were ^{125}-I radiolabeled (Pierce, Rockford, IL; Iodogen Protocol).

PEPr supplementation of lysis. Dilutions of isolated perforin were placed into round bottom microtiter plates (Falcon). To this a 0.5% (v/v) RBC dilution containing 2 mM calcium was added. This was immediately followed by the addition of a single concentration of PEPr or a control. The samples were incubated at room temperature for varying time points and the reaction was halted by acidification (25).

The chymase granzyme inhibition. The HIC hydrophobic pool was treated with 1 mM Z-Gly-Leu-Phe-chloromethylketone (Enzyme Systems Products, Dublin, CA) (30) for 30 min. at room temperature. Before dialysis to remove excess inhibitor, 1 mg of bovine serum albumin was added to the sample. The samples were dialyzed overnight with three buffer changes. The dialysis buffer contained 0.5 M NaCl (Fisher), 20 mM Tris (Fisher) and 1 mM EGTA (Sigma) pH 7.5. The inhibition of the chymase activity was confirmed before the sample was used in a supplementation experiment. HIC perforin was similarly treated, diluted and immediately assayed for lysis.

RESULTS

Perforin loses lytic activity when purified. As we isolated perforin, it lost lytic activity at each step despite excellent recovery of the perforin protein. Perforin was isolated (Fig. 1A) by a combination of two steps previously used to purify perforin: Cu^{2+}-IMAC (29) and then HIC (31). At this point perforin was enriched but not pure (Fig. 2, lane 3) and routinely had $\sim$20-25% of its original lytic activity. The recovery of perforin protein was $>$90% when quantified by ELISA. MonoQ anion exchange chromatography (32) was used as a third step to purify perforin. However, the pure perforin was nonlytic and our efforts to restore its lytic activity were unsuccessful. We turned to an alternative approach to determine if other granule proteins could contribute to the pore-forming activity of perforin.

Perforin regains lytic activity by the addition of PEPr. A $\sim$15 kD granule protein has now been isolated (Fig. 1 and Fig 2 lanes 4 & 6) that can restore lytic activity to perforin. This 15 kD protein has been named perforin enhancing protein (PEPr). PEPr is nonlytic by itself. Low M_r proteins are found in the original granule extracts (Fig. 2, lane 1) and associated with lytic IMAC perforin (Fig. 2, lane 2). The low M_r proteins are also detectable in HIC-purified perforin when proteins are radio-iodinated (Fig 2., lane 3). We first found PEPr as the major component of a nonlytic hydrophobic fraction that restored lysis.

HIC isolation of PEPr. Perforin lytic activity is dependent upon chymase granzyme activity (20). Most granzymes are depleted from perforin by Cu^{2+}-IMAC (29). We thought that further depletion of chymase from perforin by HIC might reduce its lytic activity and therefore tested the effects of restoration of the HIC chymase fraction (indicated by triangles in Fig. 1A).

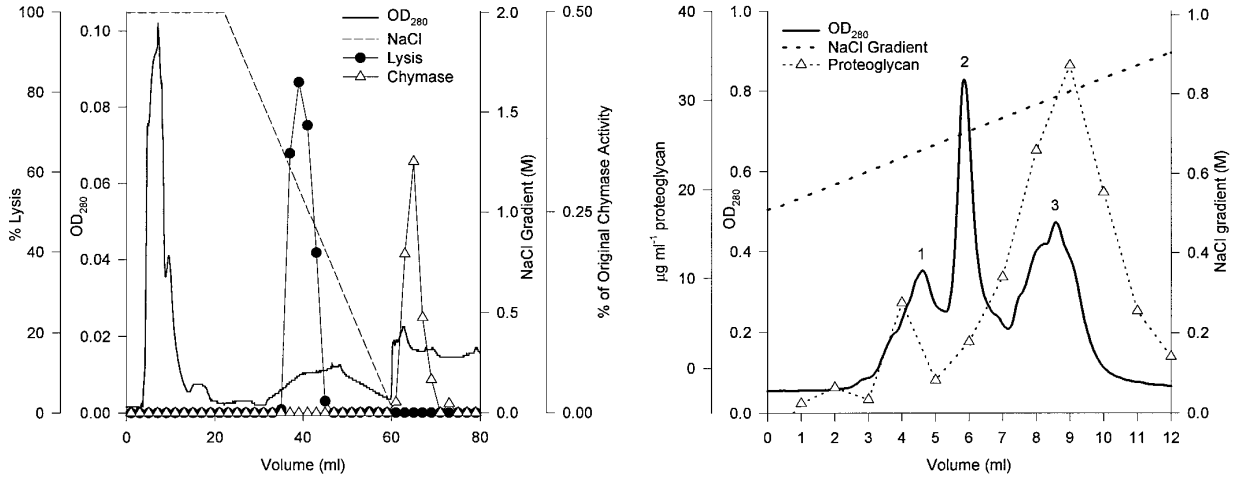

FIG. 1. Isolation of Perforin PEPr by HIC (A) and of PEPr by MonoQ (B). A. HIC. Perforin was first separated by Cu^{2+}-IMAC and then further enriched by phenyl-Superose HIC (illustrated). Lytic activity is indicated by the filled circles expressed as the percentage red cells lysed by a 1/100 dilution of the fractions. The salt gradient is indicated by the dashed line. The hydrophobic chymase fraction, indicated by the triangles, also contains PEPr. The proteins from the perforin fraction and the chymase fraction are illustrated in Fig 2., lanes 3 and 4, respectively. B. MonoQ. Proteins that passed through the Cu^{2+}-IMAC column, the first step of perforin isolation, were further separated by MonoQ. By supplementation experiments PEPr was found in peak 1. About 98% of the granule proteoglycan was loaded onto this anion exchange column. Most eluted in peak 3.

The fraction enhanced lysis (Fig. 3A). However, the enhancing activity was unchanged when the chymase activity was irreversibly inactivated by chymase inhibitors (which were dialyzed away). Examination of radioiodinated proteins in the hydrophobic "chymase" fraction revealed that the chymases were in low abundance: bands were absent at the >25 kD M_r's of granzymes. Only enzyme activity indicated the presence of the chymase(s). The predominant protein was ~15 kD (Fig. 2, lane 4). This fraction (circles Fig 1A, 60-70 ml volume) was completely nonlytic by itself but could double the lytic activity of HIC-perforin and thereby restore perforin to its lytic activity prior to HIC. Addition of excess PEPr has never been observed to increase perforin lytic activity above the original, unfractionated activity. The increase was in the final number of red cells damaged (lysed) as well as the rate of lysis. After ~2 hours the lytic damage by unsupplemented

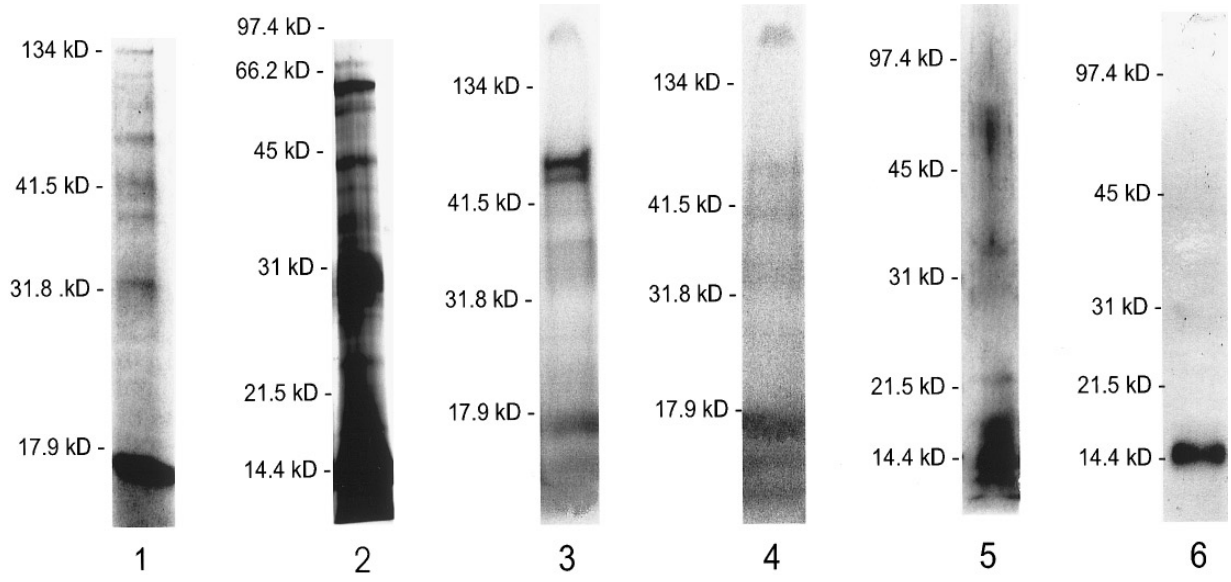

FIG. 2. SDS PAGE of Perforin and PEPr. Lane 1 illustrates Coomassie blue-stained proteins of the granule extract (10 μg). Lanes 2 and 3 contain silver-stained IMAC and ^{125}I-labeled HIC perforin preparations, respectively. Lane 4 contains ^{125}I-labeled proteins of the PEPr HIC fraction. Lanes 5 and 6 contain silver-stained IMAC (unbound fraction) and MonoQ PEPr fractions (peak 1), respectively.

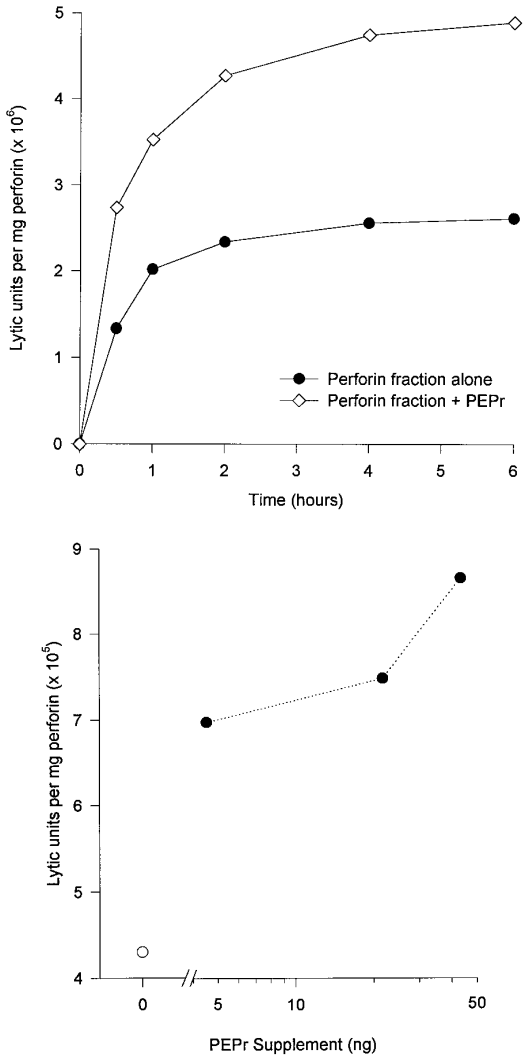

FIG. 3. Enhancement of perforin lysis by PEPr that was isolated by HIC. A. PEPr increases final lytic activity. HIC-perforin was supplemented with 87 ng of hydrophobic PEPr per well. The PEPr was pooled from 64-68 ml volumes of Fig 1A. Lysis was stopped at the times indicated on the abscissa. PEPr alone was not lytic. B. Dose-dependent increases in lysis by PEPr supplements. PEPr was added to perforin at the concentrations indicated. The reaction was allowed to proceed for 20 minutes.

perforin was complete (Fig 3A). The ~15 kD hydrophobic fraction caused an initial acceleration of lysis that also reached a maximum after 2-3 hours of incubation (Fig 3A). The effects of the 15 kD protein were dose-dependent (Fig. 3B) and occurred when 87 ng of perforin was added per reaction ($\sim 2.8 \times 10^{-8}$ M). BCA protein quantification indicated a recovery of 35 μg PEPr protein from 3.6 mg of starting granules in a volume of >8 ml. Efforts to further characterize PEPr from this HIC fraction were complicated by losses of protein during concentration.

MonoQ isolation of PEPr. We developed a second method for the purification of PEPr after determining that substantial ~15 kDa granule protein was depleted from perforin during the first step of purification and that this protein had the same bioactivity. The 15 kD protein was unbound by Cu^{2+}-IMAC (Fig. 2, lane 5). Its enhancing activity towards perforin was variable and appeared consistently only after the ~15 kD protein was separated by MonoQ anion exchange chromatography (Fig. 1B). Most proteoglycan was found in the OD_{280} peak 3 when PEPr was fractionated but small amounts eluted prior to the first OD_{280} peak (triangles, Fig. 1B). PEPr activity is found in peak 1 (Fig. 4), with some activity in peak 2 and no detectable activity in peak 3 (not illustrated). PEPr prepared by MonoQ was also nonlytic by itself. It had a steep dose titration for enhancement. In the experiment illustrated, addition of 80 ng of peak 1 doubled the lytic activity of perforin while 40 ng had little effect (Fig. 4). The PEPr peak 1 contains proteins with molecular weights of 15.6, 13.8, 7.7 and 7.2 kD (Fig 2, lane 6). Recovery of the peak 1 proteins was ~24 μg protein from 10 mg of protein in the starting granule extract.

DISCUSSION

Isolation and characterization of PEPr. To the best of our knowledge, this is the first report of a cytotoxic granule protein that enhances lysis by perforin. The protein, PEPr, increases the number of red cells that are lysed by fixed amounts of perforin. PEPr appears

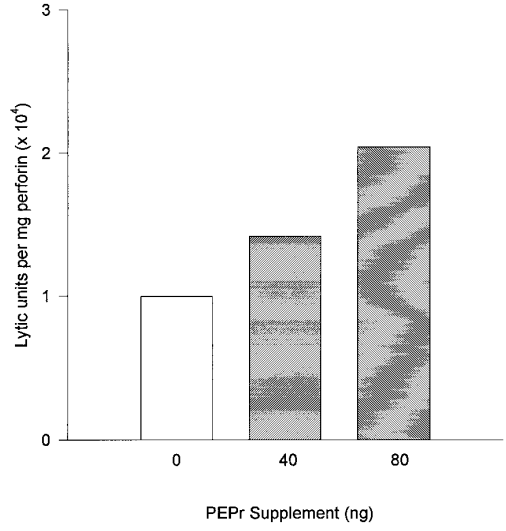

FIG. 4. Enhancement of perforin lysis by PEPr isolated by MonoQ. Perforin was diluted and assayed alone for 20 min for the calibration of lytic units. Enhancement of lysis was measured when the indicated amounts of PEPr isolated by MonoQ (Fig. 1B, peak 1) were added to constant amounts of perforin. The increased lysis of the supplemented perforin was converted into relative lytic units of activity from the perforin calibration curve. MonoQ peak 2 (Fig 1B) also had some enhancing activity while the proteoglycan peak 3 did not (not illustrated).

to be a hydrophobic protein based on its HIC elution at ~0 M salt. The bioactive PEPr proteins isolated by HIC (a) or MonoQ (b) have M_r's of ~15 kD and are effective at similar concentrations. A protein with similar M_r (c) co-purifies with lytically active perforin. It is likely (but still unproven) that the 15 kD proteins indicated by the letters in parentheses are one protein. We feel it is premature to conclude that PEPr is a cationic protein even though it binds to the anionic resin MonoQ. Very large amounts of proteoglycan are bound to the MonoQ column and may bind PEPr in a piggyback manner. Thus PEPr may be dissociating from proteoglycan and not from the MonoQ cationic resin. Several properties suggest that PEPr might be the rat equivalent of a human cytotoxic lymphocyte protein termed granulysin (33), including granule subcellular localization, M_r and hydrophobicity. Granulysin is a ***s***phingolipid ***a***ctivator ***p***rotein ***li***ke ***p***rotein (***SAPLIP***) (33) which at >50 μg/ml can disrupt membranes of mammalian cells and bacterial protoplasts. Granulysin is naturally processed into 15 and 9 kDa forms (33) similar to the proteins we have isolated. We are currently amassing PEPr isolated by MonoQ to obtain sufficient protein for microsequencing.

Potential interaction of PEPr with perforin. Based on its hydrophobic properties and co-purification with perforin, we suggest that PEPr may bind to membranes and also associate there with perforin. We propose that the assembly promotes entry of perforin into membranes. cDNAs for mouse, rat and human perforin have been sequenced (9,10,34,35). The encoded perforins lack a continuous hydrophobic region of >20 amino acids necessary to span a lipid membrane. They have a region of ~370 amino acids that has 17-21% overall amino acid sequence identity with the complement proteins C6, C7, C8 alpha and beta and C9 and the greatest identity with C8 alpha. Only C9 of the complement proteins ultimately spans lipid membranes to fully open channels. Furthermore, C9 must be associated with a complex of proteolytically activated C5b associated with C6, C7, and C8 before it can form pores. It has been suggested that regions of both C9 (AA310-325) (36) and perforin (AA190-207) form amphipathic alpha helices (10,37-39) to facilitate their entry into membranes, with hydrophilic residues facing into channels and hydrophobic residues facing membrane lipids. Prior to this report, it has been assumed that perforin could assemble independently of other proteins to form pores. The >80% losses of lytic activity associated with different methods of perforin isolation (31,40) were attributed to protein losses and/or denaturation. Our report indicates that some lytic activity is lost without perforin denaturation and is recoverable upon addition of the endogenous protein PEPr. Lytic recombinant perforin has been reported (41,42) which suggests that PEPr may not be absolutely essential for *in vitro* lysis. *In vivo*, the presence of perforin inhibitory molecules, including vitronectin (43) and lipoproteins (44), may make PEPr-mediated enhancement of lysis critically relevant. The data presented here indicate that cytotoxic granules contain at least one protein in addition to perforin and chymase granzymes (20,45) which participate in lethal pore formation.

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REFERENCES

1. Timonen, T., Ortaldo, J. R., and Herberman, R. B. (1981) *J. Exp. Med.* **153,** 569–582.
2. Kagi, D., Vignaux, F., Ledermann, B., Burki, K., Depraetere, V., Nagata, S., Hengartner, H., and Golstein, P. (1994) *Science* **265,** 528–530.
3. Markham, P. N., Coon, J. S., Chong, A. S., and Gebel, H. M. (1993) *Transplant. Proc.* **25,** 96–97.
4. Walsh, C. M., Matloubian, M., Liu, C. C., Ueda, R., Kurahara, C. G., Christensen, J. L., Huang, M. T., Young, J. D., Ahmed, R., and Clark, W. R. (1994) *Proc. Natl. Acad. Sci. (USA)* **91,** 10854–10858.
5. Yannelli, J. R., Sullivan, J. A., Mandell, G. L., and Engelhard, V. H. (1986) *J. Immunol.* **136,** 377–382.
6. Dennert, G., and Podack, E. R. (1983) *J. Exp. Med.* **157,** 1483–1495.
7. Millard, P. J., Henkart, M. P., Reynolds, C. W., and Henkart, P. A. (1984) *J. Immunol.* **132,** 3197–3204.
8. Podack, E. R., and Konigsberg, P. J. (1984) *J. Exp. Med.* **160,** 695–710.
9. Lichtenheld, M. G., Olsen, K. J., Lu, P., Lowrey, D. M., Hameed, A., Hengartner, H., and Podack, E. R. (1988) *Nature* **335,** 448–451.
10. Ishikawa, H., Shinkai, Y., Yagita, H., Yue, C. C., Henkart, P. A., Sawada, S., Young, H. A., Reynolds, C. W., and Okumura, K. (1989) *J. Immunol.* **143,** 3069–3073.
11. 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.
12. Podack, E. R., Lowrey, D. M., Lichtenheld, M., Olsen, K. J., Aebischer, T., Binder, D., Rupp, F., and Hengartner, H. (1988) *Immunol. Rev.* **103,** 203–211.
13. Hameed, A., Lowrey, D. M., Lichtenheld, M., and Podack, E. R. (1988) *J. Immunol.* **141,** 3142–3147.
14. Hudig, D., Gregg, N. J., Kam, C., and Powers, J. C. (1987) *Biochem. Biophys. Res. Commun.* **149,** 882–888.
15. Bleackley, R. C., Lobe, C. G., Duggan, B., Ehrman, N., Fregeau, C., Meier, M., Letellier, M., Havele, C., Shaw, J., and Paetkau, V. (1988) *Immunol. Rev.* **103,** 5–19.
16. Masson, D., and Tschopp, J. (1987) *Cell* **49,** 679–685.
17. MacDermott, R. P., Schmidt, R. E., Caulfield, J. P., Hein, A., Bartley, G. T., Ritz, J., Schlossman, S. F., Austen, K. F., and Stevens, R. L. (1985) *J. Exp. Med.* **162,** 1771.
18. Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B.,

Olsen, K. J., Podack, E. R., Zinkernagel, R. M., and Hengartner, H. (1994) *Nature* **369,** 31–37.

19. Lowin, B., Beermann, F., Schmidt, A., and Tschopp, J. (1994) *Proc. Natl. Acad. Sci. (USA)* **91,** 11571–11575.
20. Woodard, S. L., Jackson, D. S., Abuelyaman, A. S., Powers, J. C., Winkler, U., and Hudig, D. (1994) *J. Immunol.* **153,** 5016–5025.
21. Reynolds, C. W., Bere, E. W., Jr., and Ward, J. M. (1984) *J. Immunol.* **132,** 534–540.
22. Borregard, N., Heiple, J. M., Simons, E. R., and Clark, R. A. (1983) *J. Cell Biol.* **97,** 52–61.
23. Henkart, P. A., Millard, P. J., Reynolds, C. W., and Henkart, M. P. (1984) *J. Exp. Med.* **160,** 75–93.
24. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77.
25. Hudig, D., Callewaert, D. M., Redelman, D., Allison, N. J., Krump, M., and Tardieu, B. (1988) *J. Immunol. Methods* **115,** 169–177.
26. Farndale, R. W., Buttle, D. J., and Barrett, A. J. (1986) *Biochem. Biophys. Acta* **883,** 173–177.
27. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
28. Bollag, D. M., and Edelstein, S. J. (1991) *Protein Methods, Wiley-Liss, New York.*
29. Winkler, U., Pickett, T. M., and Hudig, D. (1996) *J. Imm. Methods* **191,** 11–20.
30. Zunino, S. J., Allison, N. J., Kam, C., Powers, J. C., and Hudig, D. (1988) *Biochim. Biophys. Acta* **967,** 331–340.
31. Shi, L., Kraut, R. P., Aebersold, R., and Greenberg, A. H. (1992) *J. Exp. Med.* **175,** 553–566.
32. Jones, J., Hallett, M. B., and Morgan, B. P. (1990) *Biochem. J.* **267,** 303–307.
33. Sarin, A., Nakajima, H., and Henkart, P. A. (1995) *J. Immunol.* **154,** 5806–5812.
34. Kwon, B. S., Wakulchik, M., Liu, C., Persechini, P. M., Trapani, J. A., Haq, A. K., Kim, Y., and Young, J. D. (1989) *Biochem. Biophys. Res. Commun.* **158,** 1–10.
35. Shinkai, Y., Takio, K., and Okumura, K. (1988) *Nature* **334,** 525–527.
36. Stanley, K. K., and Herz, J. (1987) *EMBO J.* **6,** 1951–1957.
37. Peitsch, M. C., Amiguet, P., Guy, R., Brunner, J., Maizel, J. V., Jr., and Tschopp, J. (1990) *Mol. Immunol.* **27,** 589–602.
38. Tschopp, J., Masson, D., and Stanley, K. K. (1986) *Nature* **322,** 831–834.
39. Persechini, P. M., Ojeius, D. M., Adeodato, S. C., Notaroberto, P. C., Daniel, C. B., and Young, J. D. (1992) *Biochemistry* **31,** 5017–5021.
40. Siekierka, J. J., Hung, S. H., Poe, M., Lin, C. S., and Sigal, N. H. (1989) *Nature* **341,** 755–757.
41. Liu, C. C., Persechini, P. M., and Young, J. D. E. (1996) *J. Immunol.* **156,** 3292–3300.
42. Liu, C. C., Persechini, P. M., and Young, J. D. (1994) *Biochem. Biophys. Res. Commun.* **201,** 318–325.
43. Tschopp, J., Masson, D., Schafer, S., Peitsch, M., and Preissner, K. T. (1988) *Biochemistry* **27,** 4103–4109.
44. Matsuda, K., Ishiura, S., Koizumi, H., Kawasaki, H., Arahata, K., and Sugita, H. (1991) *Mol. Immunol.* **28,** 1211–1216.
45. Hudig, D., Ewoldt, G. R., and Woodard, S. L. (1993) *Curr. Opin. Immunol.* **5,** 90–96.