Published in final edited form as:

Cell Immunol. 2009 ; 255(1-2): 82–92. doi:10.1016/j.cellimm.2008.12.001.

Regulation of perforin lysis: Implications for protein disulfide isomerase proteins

David L. Tamang^{*}, Bryce N. Alves^{*}, Viki Elliott^{*}, Doug Redelman[†], Renu Wadhwa[‡], Stephanie A. Fraser[§], and Dorothy Hudig^{*}

^{*} Department of Microbiology and Immunology, University of Nevada School of Medicine, Reno, NV 89557

[†] Department of Physiology & Cell Biology, University of Nevada, School of Medicine, NV 89557

[‡] Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan

[§] Sierra Biomedical Charles River Laboratories, Reno, NV 90431

Abstract

Perforin, a membrane-permeabilizing protein, is important to T cell cytotoxic action. Perforin has potential to damage the T cell in the endoplasmic reticulum (ER), is sequestered in granules, and later is exocytosed to kill cells. In the ER and after exocytosis, calcium and pH favor perforin activity. We found a novel perforin inhibitor associated with cytotoxic T cell granules and termed it Cytotoxic Regulatory Protein 2 (CxRP2). CxRP2 blocked lysis by granule extracts, recombinant perforin and T cells. Its effects lasted for hours. CxRP2 was calcium stable and refractory to inhibitors of granzyme and cathepsin proteases. Through mass spectrometric analysis of active 50–100 kD proteins, we identified CxRP2 candidates. Protein disulfide isomerase A3 was the strongest candidate but was unavailable for testing; however, protein disulfide isomerase A1 had CxRP2 activity. Our results indicate that protein disulfide isomerases, in the ER or elsewhere, may protect T cells from their own perforin.

Keywords

T cells; cytotoxic; cytotoxicity; perforin; cathepsin B; inhibitor; protein disulfide isomerase

Introduction

Perforin (gene, *Prf1*) is an essential component of cytotoxic granules which directly contributes to T cell-mediated death via apoptosis or necrosis [1–3] by permeabilizing target cell membranes. Perforin inserts itself into the target cell's plasma membrane and facilitates endocytosis of itself and granzyme proteases. The subsequent translocation of pro-apoptotic granzymes into the cytoplasm [4–6] provides these proteases access to numerous protein substrates that promote apoptosis after cleavage [7–11]. At higher concentrations, perforin also contributes to necrosis by facilitating osmotic disruption of the target cells. Perforin is highly conserved among mammals, expressed in lymphocytes [12–14] and is essential to the T and

Address correspondence to: David L. Tamang, Ph.D., Dept. of Microbiology & Immunology MS320, School of Medicine, University of Nevada, Reno, Reno, NV 89557, eMail: david@tamang.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

NK lymphocyte-mediated cytotoxicity that controls infections by viruses and by intracellular bacteria (reviewed, [6;15;16]).

Deadly proteins are usually tightly controlled. Perforin, the cytotoxic protein of T and NK lymphocytes is no exception. For example, the pH and calcium concentration inside the cytotoxic granules of killer lymphocytes are too low for perforin to damage the cell prior to exocytosis. The pH and calcium concentrations of the endoplasmic reticulum (ER) are high enough to support lysis [17–19]. Previously, it was thought that the N-terminus of perforin blocked its activity [20] but recently it has been found that recombinant perforin with the N-terminus is lytic [21], indicating that another mechanism must protect T cells from their own perforin. Remarkably, when perforin is exocytosed it damages only the cell that is the target of the lymphocyte and fails to damage the killer lymphocyte [22–24]. How T cells achieve resistance to perforin is still an unresolved question. Furthermore, during the killing process, surrounding cells may be put at risk of bystander killing, whereby the cytotoxic proteins intended for a specific target lead to the indiscriminant death of surrounding cells. One potential way to control the bystander killing effect is to inhibit the action of perforin.

Several reports suggest that proteins associated with cytotoxic granules modulate the action of perforin during lysis, possibly by inhibiting perforin lytic potential [25–29]. Henkart, *et al.* contributed a piece to the puzzle when his group found that a granule-associated protein, cathepsin B cysteine protease (gene, *Ctsb*), directly contributed to T cell protection from perforin-mediated cytotoxic reactions [25]. Cathepsin B bound to the plasma membrane of the effector cell after degranulation and provided resistance to perforin. However, more recently, Trapani, *et al.*, using *Ctsb*^{−/−} mice, found that cathepsin B is not necessary to protect CTLs during perforin-mediated killing [30], which means that there is still an unidentified mechanism. We have termed our perforin regulatory protein “Cytotoxic Regulatory Protein 2” (CxRP2¹) in recognition of the documented activity of cathepsin B as ‘CxRP1’, the first regulatory protein described from T cells.

The present work adds to our previous studies in which we have described several perforin enhancers and regulators [26;27;29;31]. We observed that by addition of *Prf1*^{−/−} granule extracts to perforin-containing wild type lytic granule proteins attenuated lysis. Then we fractionated *Prf1*^{−/−} granule proteins to identify the inhibitory fractions and proteins. This report describes the activity of a previously unknown perforin inhibitor that is associated with cytotoxic granule extracts, is capable of ablating red blood cell lysis and that significantly reduces T cell killing of allogeneic targets. This new perforin inhibitor is active at less than 1 µg/ml and is unaffected by the cathepsin B inhibitors or by a general serine protease inhibitor DCI that inactivates granzymes. We have identified and eliminated several abundant proteins within a bioactive fraction with Mr's between 50–100 kD, found protein disulfide isomerase (PDI) A3 and demonstrated that a related protein PDI A1 has CxRP2 activity. The data in aggregate indicate that PDI proteins, in the ER and potentially in granules, can regulate perforin activity.

Methods

Animals and Housing

Wild type C57BL/6 and C57BL/6 *Prf1*^{−/−} mice were obtained from Jackson Laboratories, ME. F344 rats were obtained from the NCI, Frederick, MD. The animals used in this study were housed and maintained in the University of Nevada, Reno animal facility under veterinary

¹**Abbreviations:** CTL, cytotoxic T lymphocyte; *Prf1*, perforin gene; CatB, cathepsin B; CxRP2, cytotoxic regulating protein 2; LU, lytic unit; PDI, protein disulfide isomerase; RBCs, red blood cells.

supervision. All animal use protocols were approved by the University of Nevada IACUC Animal Use Review Committee.

Granule preparations

Cytotoxic granules [32;33] were obtained from either activated mouse T cells or rat RNK-16 cells, an NK cell-like leukemia [34]. Granules from WT and *Prfl*^{-/-} mice (Jackson Labs) were generated from activated murine C57BL/6 splenocytes. Spleens were harvested and stimulated in culture with RPMI-1640 (Sigma) media supplemented with 10% fetal bovine serum (HyClone), 2 g/L sodium bicarbonate (Fisher), 10 mM HEPES (Fisher), 1% v/v penicillin/streptomycin (Sigma), 2.5×10^{-5} M 2-mercaptoethanol (Sigma), 1000 u/ml human recombinant interleukin-2 (Tecin, Roche) and 1 µg/ml concanavalin A (Sigma). After 3 days, the cells were cultured in the same media without concanavalin A. After 10 passages (3–4 days between passages), the cell culture contained exclusively CD8⁺ T cells (as assessed by flow cytometry, not illustrated). Granule extracts were prepared from $0.1 - 1 \times 10^9$ cells. The experiments were performed with murine granules unless otherwise stated. Generally, there were between 0.1–1 mg/ml of protein in each mouse granule fraction with a final volume between 0.5 and 1.5 ml, and the lower and denser fractions have the least amounts of protein.

The RNK-16 cells were grown as ascites cells in F344 rats. The mouse T cells or the RNK-16 cells were washed with Borregaard's relaxation buffer [35] and then disrupted using a nitrogen cavitation bomb (Parr Instrument, Moline, IL) pressurized to 450 psi. The lysate was layered over 54% Percoll^R (Sigma, St. Louis, MO), which formed a gradient during a 20-min centrifugation at $45,000 \times g$ at 4°C in a Beckman Ti50.2 rotor (Fullerton, CA). The dense fractions (up to 1.068 g/cm^3) were pooled, though in some experiments the entire Percoll^R gradient was collected as six separate fractions. Nuclei were removed from the densest fractions by filtration through 3-µm Nucleopore filters (Millipore, Bedford, MA). The Percoll^R was then pelleted by a high-speed spin for 4 h at $145,000 \times g$. Granules were collected from above the Percoll^R pellet. Dry NaCl was added to increase the salt concentration to 1.15 M. The granule membranes were disrupted by three freeze-thaw cycles. The granule extracts were stored at -20°C. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL) using BSA for calibration. The WT and KO mouse granule extracts had similar granzyme A, B and chymase granzyme activities [36] per mg protein and contained identical bands by SDS-PAGE (not illustrated).

Other reagents

Hamster BiP was purchased from MBL International (Wolburn, MA). We are grateful to Drs. J. Trapani and R. Wadhwa for their kind contributions of recombinant perforin [37] and recombinant mouse mortalin [38], respectively. Bovine PDI A3 (Sigma, P3818) was available commercially.

Cytotoxicity assays

The cytotoxic activity of perforin was determined by measuring the hemoglobin released from erythrocytes and the cytotoxic activity of CTLs was determined by ⁵¹Cr release assays. Dilutions of granule extracts or recombinant perforin were incubated with 0.5% (v/v) red blood cells (RBC) from rabbits at room temperature in 0.2 ml in round-bottom microtiter plates (Becton Dickinson Labware, Lincoln Park, NJ). The assays were incubated for 4 hours at room temperature unless otherwise stated. The assay buffer contained 10 mM HEPES, 0.15 M NaCl, and 10 µg/ml BSA (A4503; Sigma), pH 7.5, with the addition of calcium with the red blood cells (1 mM during incubation) to initiate lysis [39]. The microtiter plates were spun at $250 \times g$ for 5 min. The cell-free supernatants were transferred to a second microtiter plate. The released hemoglobin was detected at 412 nm with a Molecular Devices (Palo Alto, CA) microplate reader. The percent lysis was calculated as follows: [(experimental hemolysis –

spontaneous hemolysis)/(maximal hemolysis – spontaneous hemolysis)] × 100. Saponin (Sigma), 0.015% final concentration, was added to produce maximal erythrocyte lysis. The activity of perforin was expressed as lytic units per ml. One lytic unit is the amount of perforin needed to mediate 50% lysis of the RBCs. The units of activity were calculated by linear regression of the perforin activity at several dilutions [40]. ⁵¹Cr release assays were done either as allogeneic killing (C57BL/6 [H2^b haplotype] vs. P815 cells [H2^d haplotype]) or as antibody-redirected lysis using clone 2C11 monoclonal rat anti-mouse CD3 epsilon (eBiosciences).

Lytic inhibition

Inhibitory effects on perforin hemolysis were determined by combining WT granule extracts or r-perforin with *Prf1*^{-/-} granule extract, r-mortalin, r-BiP or PDI A3 in HEPES-buffered saline without calcium, and then adding erythrocytes suspended in buffer with calcium to initiate hemolysis. The granule extracts were diluted with salt-free HEPES buffer, pH 7.5, to 0.15 M physiological salt concentrations for lytic assays. After dilution, the 0.1 ml samples in 96-well round-bottomed microtiter plates received 0.1 ml of erythrocytes suspended in buffer with calcium to initiate lysis. The lytic assays, see above, were then conducted. For inhibition of CTLs, *Prf1*^{-/-} granule extract or control Borregaard's buffer was added to the CTL assays.

CxRP2 inactivation by protease inhibitors

Perforin WT or KO granule extracts were pretreated for 15 minutes at room temperature with the following irreversible protease inhibitors: E64 (MP Biomedical, Inc.) which is a general cysteine protease inhibitor; 3,4-dichloroisocoumarin (DCI) (Sigma) a general serine protease inhibitor; and/or CA074 (Sigma) a selective inhibitor for cathepsin B. The lytic assays were conducted as described above.

Granule protein fractionation and mass spectrometry

Perforin KO and/or WT granule proteins were separated using Microcon centricones (Millipore). Granule extracts (100 µl – 500 µl) were centrifuged for 2 hours at 14,000 rpm or until the contricone filter was almost dry. The retentate was reconstituted with an equal volume of HBS (10 mM HEPES, 0.15 M NaCl) with 10 µg/ml BSA). The proteins were then further separated on 7.5% SDS PAGE (BioRad) and stained with either Coomassie blue (BioRad) or Sypro Ruby (BioRad) for imaging. The gels were processed by the UNR Mass Spectrometry Core Facility to extract the proteins, generate tryptic peptides, and determine the masses of the tryptic peptides using an ABI 4700 Proteomic Analyzer Maldi ToF-ToF instrument. The data were analyzed with Mascot software to match with proteins in the NCBI databases to identify the granule-associated proteins and best-match peptide coverage.

Depletion of CxRP2 with plate-bound RNK-16 granule proteins

Five hundred µg/ml RNK-16 granule extract or 1 mg/ml BSA were placed in 96 well plates and serially diluted in HBS with 10 µg/ml BSA. The diluted proteins were transferred to Immulon-2TM protein capture plates (Falcon), sealed air tight, and allowed to incubate for 30 min at room temperature. The wells were washed with PBS with 10 µg/ml crystalline BSA to block further binding. Perforin KO granule extracts (~12 µg/ml, or as indicated) were placed in the wells and allowed to absorb for 30 minutes at room temperature. The absorbed perforin KO granule extract was then transferred to the RBC lytic assay plate and inhibitory activity was measured as described above.

Data reproducibility and analysis

The data panels illustrated are representative of at least 2 similar experiments. Paired student's T tests were performed to determine statistical significance between different series relative to the control groups.

Results

Prf1^{-/-} granule extracts inhibit perforin-mediated lysis

Granule extracts from *Prf1*^{-/-} T cells inhibited perforin-dependent lysis of red blood cells (RBCs) mediated by WT granule extracts (Fig. 1A–C). RBC lysis was completely dependent on perforin as the *Prf1*^{-/-} granules have no lytic activity (not illustrated). When adding *Prf1*^{-/-} granule extracts, we effectively kept perforin constant while manipulating potential regulatory factors (Fig. 1A). *Prf1*^{-/-} granule extracts inhibited WT perforin in a dose dependent manner, with strong inhibition at 4 µg/ml (Fig. 1A&B). Inhibition persisted for 4 hours (Fig. 1A), when hemolysis was complete.

To analyze the effect of *Prf1*^{-/-} granule extracts more completely, we performed a WT perforin titration with varying amounts *Prf1*^{-/-} granule extracts. The matrix of WT vs. *Prf1*^{-/-} granule extracts (Fig. 1B) illustrates that inhibition of perforin by *Prf1*^{-/-} granules was dependent on the concentrations of both perforin and the non-perforin inhibitory proteins. We have observed previously with Coomassie-stained gels [26;27] that the most abundant protein in granule preparations accounts for less than 10% of the total protein. The data depicted in figure 1B show that less than 10 µg/ml granule protein, which would contain less than 1 µg/ml of any single protein, was able to block perforin lytic activity. Therefore, the protein concentration precludes calreticulin as the active agent, as much higher calreticulin concentrations are required for perforin inhibition [26;27]. We designated the new regulatory protein CxRP2.

Next, we investigated the ability of *Prf1*^{-/-} granule extracts to inhibit recombinant perforin [37]. CxRP2 also inhibited r-perforin, though more *Prf1*^{-/-} granule extract was needed than was required to block lysis by the perforin in wild type (WT) cytotoxic granule extracts. Twenty-three µg/ml of *Prf1*^{-/-} granules inhibited r-perforin (Fig. 1C) more than three-fold, based on lytic units reduced from ~ 26,000 LU/ml to ~ 8,000 LU/ml. This requirement for more CxRP2 could be a reflection of the presence of endogenous CxRP2 regulatory protein within WT granule extracts and of the absence of CxRP2 from the recombinant perforin preparation.

Since *Prf1*^{-/-} granule extracts could block perforin-mediated lysis of red blood cells, we next tested whether *Prf1*^{-/-} granule extracts inhibited nucleated cell lysis that is mediated by CTLs. We considered this question important because this form of lysis is more physiologically relevant. However, it takes 30–50 fold higher concentrations of granule extracts to lyse nucleated cells than to lyse RBCs. We performed an allogeneic lytic assay between C57BL/6 (H-2^b haplotype) WT effectors and P815 targets (H-2^d haplotype) and found that the *Prf1*^{-/-} granules blocked the allogeneic killing (Fig. 1D). Previous experiments show that *Prf1*^{-/-} T cells demonstrate less than 1% allogeneic killing under these E:T conditions (not illustrated) which indicates that most of the killing by the intact wild type T cells of figure 1D is mediated by cytotoxic granules. At 42 µg/ml *Prf1*^{-/-} granule extract, lysis was attenuated a little more than two-fold, based on lytic units of activity. These data demonstrated that the inhibitory activity in *Prf1*^{-/-} granules could inhibit cell-mediated cytotoxicity as well as block cell lysis by isolated cytotoxic granules.

CxRP2 co-purifies with the dense cytotoxic granules after cell fractionation

After nitrogen cavitation, the disrupted cell lysate was separated by Percoll^R gradient and separated into six fractions in which the denser three contained the cytotoxic granules. The six Percoll^R fractions were secondarily centrifuged to pellet the Percoll^R. The granules and vesicles were collected from just above the Percoll while cytosolic proteins were in the supernatants after centrifugation. The soluble proteins of each supernatant fraction lacked CxRP2 activity (Fig. 2A). The pelleted membranes, vesicles and granules were disrupted by freeze thawing

and extracted after addition of high salt to dissociate proteins from granule proteoglycans. CxRP2 activity peaked in the vesicles associated with the dense Percoll^R fraction 2 (Fig. 2B), as did tryptase activity (mediated by Grzs A and K) (Fig. 2C). The tryptase activity in the lighter fractions 5 and 6 of 2C is probably due to furins and other cellular tryptases.

CxRP2 retains its activity after Ca⁺⁺ exposure and is heat-sensitive

Perforin has a calcium-binding domain and the lytic activity of granule extracts is exquisitely sensitive to calcium inactivation [21;28;41]. Indeed, if one exposes granule extracts to calcium before addition of the targets, the lytic capability is completely ablated within seconds. We considered whether CxRP2 shared similar calcium sensitivity and tested the possibility by pretreating the WT and *Prf1*^{-/-} granule extracts with 2 mM calcium for 15 minutes before adding them to a lytic reaction. We found that lytic activity of the WT granule extracts was inactivated by Ca⁺⁺ pretreatment as expected (not illustrated) while CxRP2 activity was insensitive to calcium exposure (Fig. 3A). However, CxRP2 is heat sensitive (Fig. 3B), losing substantial activity after 30 minutes at 70–75 °C, which suggests that the activity is independent of sulfated glycans that would be unaffected by heating and that have been previously demonstrated to reduce perforin lytic activity [42].

CxRP2 activity persists after treatment with irreversible inhibitors of cysteine- and serine-dependent proteases, which suggests that CxRP2 is distinct from known granule-associated proteases

T lymphocyte cytotoxic granules have potent and unique protease activities mediated by the granzyme serine proteases and by cysteine proteases including dipeptidyl peptidase I and cathepsin B. We were curious if CxRP2 had protease activity that may contribute to the inhibition of perforin-mediated lysis. Because CxRP2 bioactivity is closely associated with granzyme serine protease activity in the Percoll^R separation fractions, we considered these granule proteases as candidates for CxRP2. Furthermore, we wanted to ascertain the role cathepsin B plays in CxRP2 activity, since cathepsin B cleaves perforin [25;30]. Broad-spectrum cysteine (E64) and serine (DCI) protease inhibitors were used to block protease activities in the *Prf1*^{-/-} granules. We pretreated *Prf1*^{-/-} granule extract with 14 μM E64 for 10 minutes at 37°C before using it in an RBC lytic assay. We found that after blocking cysteine protease activity perforin inhibitory activity remained (Fig. 4A). DCI pretreatment (1 mM at 37°C for 15 min) also failed to affect CxRP2 (Fig 4B). To assess cathepsin B as a candidate for CxRP2, we pretreated *Prf1*^{-/-} granules with CA074, a synthetic inhibitor selective for cathepsin B (Fig. 4C). Our data show that CxRP2 is unaffected by CA074 and therefore CxRP2 is unlikely to be cathepsin B. Concurrent controls, not illustrated, indicated that granzymes and cathepsin B would have been inhibited by these treatments.

CxRP2 biological activity can be depleted by binding to cytotoxic RNK-16 granule proteins

We decided to test if CxRP2 could be depleted from *Pfn*^{-/-} granules. Our thinking was that CxRP2 may directly interact with components of the granules and could be selectively depleted with immobilized cytotoxic granule proteins. WT rat RNK-16 granule proteins were bound to ImmulonTM protein binding plates (Fig. 5) and the plates were used to deplete CxRP2 from perforin KO granule extracts. Plate-bound RNK-16 depletion resulted in a reduction of CxRP2 activity relative to the controls with BSA bound to the plates. These data suggest to us that perforin inhibition might be due to a direct interaction between CxRP2 and perforin.

CxRP2 bioactivity is associated with proteins that are between 50–100 kDa

In order to estimate the size of the inhibitory material, we used centriconesTM with molecular weight filters of 50 and 100 kDa to fractionate undiluted *Prf1*^{-/-} granule proteins. We chose to use this method because the quantity of protein we are able to extract from murine granule

preparations is limited, and we were unable to get satisfactory resolution using Superdex 200 gel filtration chromatography. Furthermore, dilution and subsequent concentration of the granule proteins results in a progressive reduction of CxRP2 activity. CxRP2 activity was stable after *Prf1*^{-/-} granule extracts were separated by passage through centricone membranes with a 100 kDa cutoff (Fig. 6A circles). *Prf1*^{-/-} granule extracts were also separated with 50 kDa centricones and the CxRP2 inhibitory activity was found in the larger than 50 kDa fraction (Fig. 6B, squares). Taken together, these data suggest that CxRP2 has a molecular weight between 50–100 kDa.

Next, we separated the *Prf1*^{-/-} granule extract in series, with the proteins less than 100 kDa from the 100 kDa centricone being retained by a second 50 kDa centricone, resulting in a 50–100 kDa fraction with CxRP2 inhibitory activity (Fig. 6C). We identified several proteins in this 50–100 kDa active fraction, after separating the proteins by SDS-PAGE. The major proteins were stained with Coomassie blue and digested with trypsin for peptide characterization by mass spectrometry. These proteins are listed in Table I.

Mortalin/GRP75 and BiP/Hspa5 are present in the CxRP2 fraction but failed to down regulate perforin lysis

The proteins listed in Table I represent the major protein constituents of the active fraction that were detected by Coomassie blue staining. Protein abundance was sufficient for MS analyses. The low molecular weight protein bands contained granzymes and granule proteins (Table I). While the granzymes in question have molecular weights less than 50 kDa, they would be retained by 50–100 kDa fractionation for several reasons. Some granzymes would be retained because they form homodimers and others because they are highly glycosylated. Furthermore, some of the most abundant proteins (*e.g.*, Grz B) may remain in the >50 kDa protein fraction because the protein mixture was separated without repeated washes that would have allowed better depletion of the lower Mr proteins. Repeated washes inactivated CxRP2. Because CxRP2 was refractory to several broad-spectrum protease inhibitors (Fig. 4), including DCI, we view granzymes as unlikely CxRP2 candidates and focused on the other proteins as candidates for CxRP2. We identified citrate synthase in our granule extract. Citrate synthase is a protein associated with the mitochondria (a dense organelle) and may be a residual contaminate from mitochondria.

We identified three granule-associated proteins in the 50 – 100 kDa molecular weight range that were evaluated for CxRP2 activity. Heat shock 70 kDa protein 5 (hspa5), is a glucose-regulated protein (also called immunoglobulin heavy chain-binding protein or BiP) that is thought to be involved in protein transport in the ER [43]. Hspa9a, known as mortalin, is of particular interest. Hspa9a can interact with p53 and can lead to growth arrest of certain cancer cells [44]. Further, Hspa9a binds to complement proteins C8 and C9, which are membrane attack proteins in the same protein family as perforin [45;46], and anti-mortalin antibodies increased complement-mediated lysis [47;48]. The authors' interpretation was that the antibodies neutralized mortalin released from the target cells and thereby increased complement activity. Given the combination of these observations, it seemed reasonable to hypothesize that T lymphocytes might utilize mortalin to regulate perforin. "Q99LF6, a protein similar to an ER-60 protease" has similarity to glucose regulated proteins. The entry is also listed as Protein Disulfide Isomerase Precursor A3, which is hypothesized to catalyze rearrangement of disulfide bonds based on theoretical homology modeling. We fully appreciate that these candidate CxRP2 proteins could be ER contaminants instead of cytotoxic granule proteins and still could possess CxRP2 activity.

To examine BiP and mortalin as candidates for CxRP2, we used recombinant proteins in RBC lytic assays. While control perforin KO granules ablated all lytic activity at protein concentrations as low as 2.5 µg/ml, r-BiP (8 µg/ml, 1.1×10^{-7} M) and r-mortalin (1 µg/ml, 1.3

$\times 10^{-8}$ M) were without effect and lysis was similar to the untreated WT perforin control (not illustrated). These results suggest that the proteins, BiP and mortalin, that we identified in the 50–100 kDa fraction lack CxRP2 bioactivity.

Protein Disulfide Isomerase has CxRP2 bioactivity

The protein with accession number Q99LF6, “similar to ER-60 protease” is murine protein disulfide-isomerase A3 and has 4 of 5 domains in common with PDI. PDI A3 is predicted to have disulfide oxidase and reductase activities similar to protein disulfide isomerase (PDI). While murine PDI A3 is unavailable, we decided to examine bovine PDI A1. (There is sequence homology of ~40% between mouse PDI A3 and bovine PDI A1). We found that purified PDIA1 markedly reduced perforin-mediated lysis by WT granule extracts at 1 μ g/ml (Fig. 7A). Higher concentration of PDI A1 (5 μ g/ml) could block 100% lysis (Fig. 7B). PDI A1, in combination with *Prf1*^{-/-} granule proteins, blocked lysis better than PDI A1 alone or the *Prf1*^{-/-} granule proteins alone (Fig. 7C). In the lowest 3 concentrations of *Prf1*^{-/-} granule proteins had negligible inhibitory activity but when combined with a constant 2.5 μ g/ml of PDI A1, there was an additive response of the *Prf1*^{-/-} granule proteins. The low amounts of *Prf1*^{-/-} granule proteins (e.g., 0.2 μ g/ml) that increased the activity of 2.5 μ g/ml of PDI A1 suggest that more than one protein may be inhibitory in the *Prf1*^{-/-} granules.

Discussion

The question of how T cells can synthesize and employ perforin without harming themselves has been of interest to immunologists for 30 years. Our report provides insight into two potential levels of protection. One level of protection is needed after the synthesis stage when perforin is transported into the ER. Another level is during the killing process itself.

This study describes a novel perforin inhibitory activity found within cytotoxic granule extracts and implicates PDI A3. Our data suggest the T cell might utilize protective agents during the cytotoxic process. We consider the possibility that PDI A3 is CxRP2. PDI A3, also known as ERp57, is found in several other locations as well as the ER (reviewed, [49]) and, in the case of cytotoxicity, might be in granules. PDI A3 is a chaperone protein common to many cell types and has biological and biochemical properties that suggest it could be regulatory in both the ER and after exocytosis. PDI A3 was found to be secreted and on cell surfaces when cells were genetically engineered to over-express ERp57 [50]. Both PDI A1 and PDI A3 bind proteins through two domains, the a and b'. High affinity binding via the b' domain occurs even without thioreductase activity which requires glutathione redox reagents for optimal activity *in vitro*. Our experimental conditions lacked redox reagents; therefore, it is likely that binding to perforin, rather than refolding of perforin into inactive isomers, affects perforin lytic activity under our experimental conditions. Thus both PDI A1 and PDI A3 would be likely to affect perforin, consistent with our observations that granule fractions containing PDI A3 and purified PDI A1 have CxRP2 activity. Interestingly, calreticulin, another ER protein, has also been found associated with cytotoxic T granules and may also interact with PDI [28;51]. Calreticulin as a lectin helps glycosylated proteins interact with PDI A3 [52;53]. The availability of calreticulin may explain why granule extracts (with associated calreticulin) were easier to inhibit than r-perforin.

We would like to venture a hypothetical model for PDI A3 action in regulating lysis. We suggest that PDI A3 is associated with the inner membrane of the cytotoxic granules. Upon exocytosis, PDI A3 is exposed on the outer T cell plasma membrane, effectively forming a protective “armor plate” at the immunological synapse. The attachment might be via the QDEL C terminus that binds it to ER membranes [54], though additional evidence is necessary to conclude the QDEL binding proteins are present on the inner membrane of the cytotoxic granules. This model is consistent with the observations of Henkart, *et. al*, [25] in which the

authors reported that T cells gained protection from cytotoxic granule proteins after they were induced to secrete their own cytotoxic arsenal. Henkart attributed the effect to cathepsin B, which was later shown to be unnecessary for regulation of perforin [30].

There are other proteins available *in situ* that can inhibit the action of perforin, but we believe that CxRP2 is distinct from these proteins for several reasons. For example, we demonstrated that calreticulin can block perforin lysis [26–28], but only at much higher concentrations (~30 µg/ml) than are needed for CxRP2. Furthermore, it is noteworthy that calreticulin (~60 kDa) is in such low concentrations within the granule extracts that it was undetectable in the 50–100 kDa proteins that were later identified by mass spectrometry. Both PDI A1 and CxRP2 act independently of the high Mr sulfated proteoglycan (which would exceed the 100 kDa cutoff), another protein that can suppress perforin activity. Furthermore, two serum proteins block perforin, vitronectin/protein S [55] and apolipoprotein III [56]. *In vivo*, the inhibitory activity of these serum proteins may enhance the inhibitory activity of CxRP2.

The presence of mortalin and BiP in our granule extracts may be taken as a warning for potential contamination of the granules with ER proteins. Isolating pure membrane-bound organelles like cytotoxic granules is problematic, particularly when one is employing ultra-sensitive mass spectrometric techniques. Nonetheless, we believe CxRP2 is in granules because the inhibitory activity follows the granule enzyme activity in the Percoll^R cellular fractions. Even if CxRP2 is an ER contaminant protein, its inhibitory activity may explain why perforin fails to disrupt the ER. In the ER, calcium levels are near 300–700 µM [17–19] and the pH is near 7 [17], conditions where perforin could pose a potential threat to the cell. While proteolytic processing to remove the C terminus from perforin to activate it [57] seems unnecessary since intact r-perforin is lytic, the mechanism of self-protection is still unresolved.

Our discovery that PDI A1 possesses CxRP2 activity provides another component to a puzzle that has persisted for 30 years. Further study of proteins in the PDI family may provide key information regarding how perforin is processed and mobilized in killer lymphocytes and insight into how we might use such proteins to modulate granule-mediated cellular damage.

Acknowledgements

We thank Dr. Kathleen Schegg and Ms. Rebekah Woolsey at the University of Nevada, Reno Proteomics Core for their insights regarding mass spectrometry analysis. We gratefully acknowledge that this work was supported by the grants NIH RO1CA38942 (DH), NIH T32CA09563 (DT and BNA), NIH INBRE P20RR016463 (for mass spectrometry), the Fraternal Order of the Eagles Research Award (DT), and a University of Nevada, Reno Graduate Student Association Student Research Grant Award (DT).

Reference List

1. Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM, Hengartner H. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* 1994;369:31–37. [PubMed: 8164737]
2. Kagi D, Vignaux F, Ledermann B, Burki K, Depraetere V, Nagata S, Hengartner H, Golstein P. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 1994;265:528–530. [PubMed: 7518614]
3. Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. *Nat Rev Immunol* 2006;6:940–952. [PubMed: 17124515]
4. Froelich CJ, Orth K, Turbov J, Seth P, Gottlieb R, Babior B, Shah GM, Bleackley RC, Dixit VM, Hanna W. New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. *J Biol Chem* 1996;271:29073–29079. [PubMed: 8910561]

5. Pinkoski MJ, Hobman M, Heibin JA, Tomaselli K, Li F, Seth P, Froelich CJ, Bleackley RC. Entry and trafficking of granzyme B in target cells during granzyme B-perforin-mediated apoptosis. *Blood* 1998;92:1044–1054. [PubMed: 9680374]
6. Pipkin ME, Lieberman J. Delivering the kiss of death: progress on understanding how perforin works. *Curr Opin Immunol* 2007;19:301–308. [PubMed: 17433871]
7. Bredemeyer AJ, Townsend RR, Ley TJ. Use of protease proteomics to discover granzyme B substrates. *Immunol Res* 2005;32:143–153. [PubMed: 16106065]
8. Lieberman J, Fan Z. Nuclear war: the granzyme A-bomb. *Curr Opin Immunol* 2003;15:553–559. [PubMed: 14499264]
9. Lord SJ, Rajotte RV, Korbutt GS, Bleackley RC. Granzyme B: a natural born killer. *Immunol Rev* 2003;193:31–8. [PubMed: 12752668]
10. Trapani JA, Sutton VR. Granzyme B: pro-apoptotic, antiviral and antitumor functions. *Curr Opin Immunol* 2003;15:533–543. [PubMed: 14499262]
11. Waterhouse NJ, Trapani JA. H is for helper: granzyme H helps granzyme B kill adenovirus-infected cells. *Trends Immunol* 2007;28:373–375. [PubMed: 17766182]
12. Masson D, Tschopp J. Isolation of a lytic, pore-forming protein (perforin) from cytolytic T-lymphocytes. *J Biol Chem* 1985;260:9069–9072. [PubMed: 3874868]
13. Podack ER, Konigsberg PJ. Cytolytic T cell granules. Isolation, structural, biochemical, and functional characterization. *J Exp Med* 1984;160:695–710. [PubMed: 6332169]
14. Podack ER, Young JD, Cohn ZA. Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. *Proc Natl Acad Sci USA* 1985;82:8629–8633. [PubMed: 2417226]
15. Voskoboinik I, Trapani JA. Addressing the mysteries of perforin function. *Immunol Cell Biol* 2006;84:66–71. [PubMed: 16405653]
16. Bolitho P, Voskoboinik I, Trapani JA, Smyth MJ. Apoptosis induced by the lymphocyte effector molecule perforin. *Curr Opin Immunol* 2007;19:339–347. [PubMed: 17442557]
17. Kim JH, Johannes L, Goud B, Antony C, Lingwood CA, Daneman R, Grinstein S. Noninvasive measurement of the pH of the endoplasmic reticulum at rest and during calcium release. *Proc Natl Acad Sci USA* 1998;95:2997–3002. [PubMed: 9501204]
18. Montero M, Brini M, Marsault R, Alvarez J, Sitia R, Pozzan T, Rizzuto R. Monitoring dynamic changes in free Ca²⁺ concentration in the endoplasmic reticulum of intact cells. *EMBO J* 1995;14:5467–5475. [PubMed: 8521803]
19. Robert V, De GF, Massimino ML, Cantini M, Pozzan T. Direct monitoring of the calcium concentration in the sarcoplasmic and endoplasmic reticulum of skeletal muscle myotubes. *J Biol Chem* 1998;273:30372–30378. [PubMed: 9804801]
20. Uellner R, Zvelebil MJ, Hopkins J, Jones J, MacDougall LK, Morgan BP, Podack E, Waterfield MD, Griffiths GM. Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. *EMBO J* 1997;16:7287–7296. [PubMed: 9405358]
21. Voskoboinik I, Thia MC, Fletcher J, Ciccone A, Browne K, Smyth MJ, Trapani JA. Calcium-dependent plasma membrane binding and cell lysis by perforin are mediated through its C2 domain: A critical role for aspartate residues 429, 435, 483, and 485 but not 491. *J Biol Chem* 2005;280:8426–8434. [PubMed: 15576364]
22. Blakely A, Gorman K, Ostergaard H, Svoboda K, Liu CC, Young JD, Clark WR. Resistance of cloned cytotoxic T lymphocytes to cell-mediated cytotoxicity. *J Exp Med* 1987;166:1070–1083. [PubMed: 3498787]
23. Verret CR, Firmenich AA, Kranz DM, Eisen HN. Resistance of cytotoxic T lymphocytes to the lytic effects of their toxic granules. *J Exp Med* 1987;166:1536–1547. [PubMed: 2445890]
24. Shinkai Y, Ishikawa H, Hattori M, Okumura K. Resistance of mouse cytolytic cells to pore-forming protein-mediated cytolysis. *Eur J Immunol* 1988;18:29–33. [PubMed: 3345794]
25. Balaji KN, Schaschke N, Machleidt W, Catalfamo M, Henkart PA. Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. *J Exp Med* 2002;196:493–503. [PubMed: 12186841]
26. Fraser SA, Karimi R, Michalak M, Hudig D. Perforin lytic activity is controlled by calreticulin. *J Immunol* 2000;164:4150–4155. [PubMed: 10754310]

27. Fraser SA, Michalak M, Welch WH, Hudig D. Calreticulin, a component of the endoplasmic reticulum and of cytotoxic lymphocyte granules, regulates perforin-mediated lysis in the hemolytic model system. *Biochem Cell Biol* 1998;76:881–887. [PubMed: 10353724]
28. Andrin C, Pinkoski MJ, Burns K, Atkinson EA, Krahenbuhl O, Hudig D, Fraser SA, Winkler U, Tschopp J, Opas M, Bleackley RC, Michalak M. Interaction between a Ca²⁺-binding protein calreticulin and perforin, a component of the cytotoxic T-cell granules. *Biochemistry* 1998;37:10386–10394. [PubMed: 9671507]
29. Winkler U, Fraser SA, Hudig D. Perforin-enhancing protein, a low molecular weight protein of cytotoxic lymphocyte granules, enhances perforin lysis. *Biochem Biophys Res Commun* 1997;236:34–39. [PubMed: 9223421]
30. Baran K, Ciccone A, Peters C, Yagita H, Bird PI, Villadangos JA, Trapani JA. Cytotoxic T lymphocytes from cathepsin B-deficient mice survive normally in vitro and in vivo after encountering and killing target cells. *J Biol Chem* 2006;281:30485–91. [PubMed: 16914553]
31. Woodard SL, Fraser SA, Winkler U, Jackson DS, Kam CM, Powers JC, Hudig D. Purification and characterization of lymphocyte chymase I, a granzyme implicated in perforin-mediated lysis. *J Immunol* 1998;160:4988–4993. [PubMed: 9590247]
32. Millard PJ, Henkart MP, Reynolds CW, Henkart PA. Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors. *J Immunol* 1984;132:3197–3204. [PubMed: 6373925]
33. Winkler U, Pickett TM, Hudig D. Fractionation of perforin and granzymes by immobilized metal affinity chromatography (IMAC). *J Immunol Methods* 1996;191:11–20. [PubMed: 8642196]
34. Ward JM, Reynolds CW. Large granular lymphocyte leukemia. A heterogeneous lymphocytic leukemia in F344 rats. *Am J Pathol* 1983;111:1–10. [PubMed: 6837719]
35. Borregaard N, Heiple JM, Simons ER, Clark RA. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J Cell Biol* 1983;97:52–61. [PubMed: 6408102]
36. Winkler U, Allison NJ, Woodard SL, Gault RA, Ewoldt GR, Kam CM, Abuelyaman A, Powers JC, Hudig D. Characterization, application and potential uses of biotin-tagged inhibitors for lymphocyte serine proteases (granzymes). *Mol Immunol* 1996;33:615–623. [PubMed: 8760273]
37. Voskoboinik I, Thia MC, De BA, Browne K, Cretney E, Jackson JT, Darcy PK, Jane SM, Smyth MJ, Trapani JA. The functional basis for hemophagocytic lymphohistiocytosis in a patient with co-inherited missense mutations in the perforin (PFN1) gene. *J Exp Med* 2004;200:811–816. [PubMed: 15365097]
38. Takano S, Wadhwa R, Mitsui Y, Kaul SC. Identification and characterization of molecular interactions between glucose-regulated proteins (GRPs) mortalin/GRP75/peptide-binding protein 74 (PBP74) and GRP94. *Biochem J* 2001;357:393–398. [PubMed: 11439088]
39. Hudig D, Callewaert DM, Redelman D, Allison NJ, Krump M, Tardieu B. Lysis by RNK-16 cytotoxic lymphocyte granules. Rate assays and conditions to study control of cytolysis. *J Immunol Methods* 1988;115:169–177. [PubMed: 3198921]
40. Pross HF, Maroun JA. The standardization of NK cell assays for use in studies of biological response modifiers. *J Immunol Methods* 1984;68:235–249. [PubMed: 6200539]
41. Henkart PA, Millard PJ, Reynolds CW, Henkart MP. Cytolytic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors. *J Exp Med* 1984;160:75–93. [PubMed: 6736872]
42. Young JD, Damiano A, DiNome MA, Leong LG, Cohn ZA. Dissociation of membrane binding and lytic activities of the lymphocyte pore-forming protein (perforin). *J Exp Med* 1987;165:1371–1382. [PubMed: 3494808]
43. Hendershot LM, Valentine VA, Lee AS, Morris SW, Shapiro DN. Localization of the Gene Encoding Human BiP/GRP78, the Endoplasmic Reticulum Cognate of the HSP70 Family, to Chromosome 9q34. *Genomics* 1994;20:281–284. [PubMed: 8020977]
44. Kaul SC, Aida S, Yaguchi T, Kaur K, Wadhwa R. Activation of wild type p53 function by its mortalin-binding, cytoplasmically localizing carboxyl terminus peptides. *J Biol Chem* 2005;280:39373–39379. [PubMed: 16176931]
45. Shinkai Y, Takio K, Okumura K. Homology of perforin to the ninth component of complement (C9). *Nature* 1988;334:525–527. [PubMed: 3261391]

46. Young JD, Cohn ZA, Podack ER. The ninth component of complement and the pore-forming protein (perforin 1) from cytotoxic T cells: structural, immunological, and functional similarities. *Science* 1986;233:184–190. [PubMed: 2425429]
47. Pilzer D, Gasser O, Moskovich O, Schifferli JA, Fishelson Z. Emission of membrane vesicles: roles in complement resistance, immunity and cancer. *Springer Semin Immunopathol* 2005;27:375–387. [PubMed: 16189651]
48. Pilzer D, Fishelson Z. Mortalin/GRP75 promotes release of membrane vesicles from immune attacked cells and protection from complement-mediated lysis. *Int Immunol* 2005;17:1239–1248. [PubMed: 16091382]
49. Turano C, Coppari S, Altieri F, Ferraro A. Proteins of the PDI family: unpredicted non-ER locations and functions. *J Cell Physiol* 2002;193:154–163. [PubMed: 12384992]
50. Hirano N, Shibasaki F, Sakai R, Tanaka T, Nishida J, Yazaki Y, Takenawa T, Hirai H. Molecular cloning of the human glucose-regulated protein ERp57/GRP58, a thiol-dependent reductase. Identification of its secretory form and inducible expression by the oncogenic transformation. *Eur J Biochem* 1995;234:336–342. [PubMed: 8529662]
51. Baksh S, Burns K, Andrin C, Michalak M. Interaction of calreticulin with protein disulfide isomerase. *J Biol Chem* 1995;270:31338–31344. [PubMed: 8537405]
52. Oliver JD, Roderick HL, Llewellyn DH, High S. ERp57 functions as a subunit of specific complexes formed with the ER lectins calreticulin and calnexin. *Mol Biol Cell* 1999;10:2573–2582. [PubMed: 10436013]
53. Zapun A, Darby NJ, Tessier DC, Michalak M, Bergeron JJ, Thomas DY. Enhanced catalysis of ribonuclease B folding by the interaction of calnexin or calreticulin with ERp57. *J Biol Chem* 1998;273:6009–6012. [PubMed: 9497314]
54. Andres DA, Rhodes JD, Meisel RL, Dixon JE. Characterization of the carboxyl-terminal sequences responsible for protein retention in the endoplasmic reticulum. *J Biol Chem* 1991;266:14277–14282. [PubMed: 1650354]
55. Tschopp J, Masson D, Schafer S, Peitsch M, Preissner KT. The heparin binding domain of S-protein/vitronectin binds to complement components C7, C8, and C9 and perforin from cytolytic T-cells and inhibits their lytic activities. *Biochemistry* 1988;27:4103–4109. [PubMed: 2458130]
56. Matsuda K, Ishiura S, Koizumi H, Kawasaki H, Arahata K, Sugita H. Apolipoprotein B is a major perforin inhibitor protein in human serum. *Mol Immunol* 1991;28:1211–1216. [PubMed: 1961197]
57. Uellner R, Zvelebil MJ, Hopkins J, Jones J, MacDougall LK, Morgan BP, Podack E, Waterfield MD, Griffiths GM. Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. *EMBO J* 1997;16:7287–7296. [PubMed: 9405358]

Figure 1. *Pfn1*^{-/-} granule extracts inhibited perforin-mediated RBC lysis and CTL-mediated killing
 * Indicates a p-value < 0.05 relative to the paired controls. **(A) Inhibition of perforin lysis was stable.** *Pfn1*^{-/-} granule extract was diluted to produce the final concentrations indicated on the X axis and mixed with WT granule extract, at a fixed concentration of 1.5 μg WT protein/ml in every well. RBCs with calcium were added and the lytic reaction (hemoglobin release) was monitored at 0.5, 2 and 4 hours. *Pfn1*^{-/-} granule extract inhibited RBC lysis at 30 minutes and inhibition persisted at 4 hours when lysis was complete. The control values without *Pfn1*^{-/-} granule extracts are indicated by the crosses. **(B) Inhibition of lysis increased with the concentration of *Pfn1*^{-/-} granule extract.** A matrix was constructed by diluting WT granule extract indicated on the X axis and then adding a series of *Pfn1*^{-/-} granule extract

dilutions indicated by each line. The highest concentration of WT granule extract was 3 ug/ml. RBCs were added with calcium and the reaction was allowed to proceed for 4 hours. The data indicate that ~2-fold inhibition was achieved at 4 ug/ml KO extract and ~4-fold inhibition was achieved at 8 ug/ml KO extract. The inhibition was estimated on the basis of lytic units, comparing the amounts of control and inhibited WT extract needed to produce 50% lysis. This figure is representative of over 4 experiments. **(C) Pfn1^{-/-} granule extract inhibited lysis by r-perforin.** RBC lytic assays using recombinant perforin was conducted as in part A. The Pfn1^{-/-} extract at a concentration of 23 ug/ml inhibited lysis by r-Pfn by greater than 3-fold as determined by lytic units. **(D) Pfn1^{-/-} granule extract inhibited lysis by CTLs.** P815 target cells (from a DBA/2 H-2^d haplotype mouse) were labeled with ⁵¹Cr and incubated with ConA-activated effector T lymphocytes from C57BL/6 mice of the H-2^b haplotype at the indicated ratios. The Pfn1^{-/-} granule extract, at 42 ug/ml, resulted in inhibition of the allogeneic killing. Taken together, these data indicate there is an inhibitor of perforin-mediated lysis within the Pfn1^{-/-} granule extract.

Figure 2. Perforin inhibitory activity is localized in the dense granule fractions

Perforin KO granule extracts were prepared and each Percoll fraction and supernatant was assayed for CxRP2 inhibitory and tryptase activity. Hemolytic assays were performed as in figure 1 for 4 hrs (fraction dilutions of 1/400), with combinations of WT and *pfn1*^{-/-} granule extracts. **(A–B) CxRP2 activity is localized to the dense granule fractions.** **(A)** Supernatants from the Percoll fractions (containing cytosolic and organelle-free proteins) were assayed for perforin inhibitory activity. None of the supernatants was able to inhibit perforin, suggesting CxRP2 activity is mediated by a protein from the organelles. **(B)** CxRP2 activity is present in the heavy granule fractions, suggesting co-localization with granule proteins. The heaviest fraction is G1 and the lightest G6. Final protein concentrations of the fractions were 2.5 $\mu\text{g/ml}$

or lower, with the most effective fractions G1 and G2 actually containing the lowest amounts of protein. The * indicates significance between the individual series and the no-addition control at the indicated points. This figure is representative of three preparations of perforin KO granule extracts. **(C) Tryptase enzyme and CxRP2 activity co-localize.** Tryptase activity associated with granzymes A and B is elevated in the fraction G2 that contains the highest CxRP2 activity.

Figure 3. Perforin inhibitory activity is retained after Ca^{++} exposure and is heat-sensitive Hemolytic assays were performed for 4 hrs as in figure 1, with combinations of WT and *Pfn1*^{-/-} granule extracts. **(A) Ca^{++} exposure.** The WT and *pfn1*^{-/-} granule extracts were pretreated with or without 2 mM calcium at 37°C for 15 minutes. The WT extract lost all lytic activity as expected (not illustrated). Perforin lytic activity was inhibited by the *pfn1*^{-/-} granule extract (at a constant concentration of 4 µg/ml) regardless of its calcium pretreatment, indicating that the bioactivity of the inhibitory factor is insensitive to calcium inactivation. **(B) The perforin inhibitory activity was reduced after heat exposure.** The *pfn1*^{-/-} granule extract was held at 70–75°C for 30 minutes. The control *Pfn1*^{-/-} granule extract was held at 4°C for 30 minutes. *Pfn1*^{-/-} granules were serially diluted at the indicated concentrations. The black bar represents the lysis achieved with the WT granule concentration (2.3 µg/ml) present at all data points. The heat treated *pfn1*^{-/-} granule extract lost their inhibitory activity, becoming 3 fold less effective than the unheated KO granules (p-values below 0.005 at the indicated points). These data suggest the inhibitory factor is heat sensitive at these temperatures, but is calcium insensitive.

Figure 4. CxRP2 activity is stable after treatment with cysteine and serine protease inhibitors
Broad-spectrum serine and cysteine protease inhibitors, E-64 (10 uM) and DCI (1 mM), were used to determine if CxRP inhibitory activity could be blocked by ablating protease activity. **(A) CxRP2 activity was independent of cysteine protease activity.** E64 was introduced to the *pfn1*^{-/-} granule extract (12 ug/ml) and incubated at 37C for 5 minutes before RBCs and WT granules were added. The lytic reaction was for 4 hours. Inhibition of WT granule RBC lysis was similar with or without the presence of E-64. E-64 itself did not modify lytic activity of the WT granules, nor did it abolish the *pfn1*^{-/-} granule inhibition of WT granule lytic activity, suggesting the inhibitory factor is not a cysteine protease. **(B) CxRP2 activity is independent of serine protease activity.** The *pfn1*^{-/-} granule extract (12 ug/ml) was treated with 1 mM DCI for 20 minutes at room temperature and remained able to block perforin lysis, with or without DCI treatment, suggesting the inhibitory factor is independent of serine proteases. **(C) CxRP2 activity was refractory to a specific inhibitor of cathepsin B.** *Pfn1*^{-/-} granule extract (8 ug/ml) was pretreated with the cathepsin B specific inhibitor CA074, and run simultaneously with 2 uM E64. CxRP2 activity was unaffected by CA074 pretreatment, suggesting cathepsin B is not the active agent.

Figure 5. CxRP2 activity is partially depleted by immobilized RNK-16 granule proteins
PfnI^{-/-} granule extracts were incubated with RNK-16 rat granule extract or with BSA bound to microtiter plates and then transferred to another microtiter plate for evaluation. Control *PfnI*^{-/-} granule extract was used without pre-incubation with microtiter plates. These three *PfnI*^{-/-} granule extracts were 4 µg/ml. Control lysis was by WT mouse granules without additions. Negligible inhibitory activity was depleted by BSA and significantly more inhibitory activity was depleted by the RNK-16 proteins. This figure is representative of three experiments.

Figure 6. CxRP2 bioactivity is mediated by a protein between 50–100 kDa

Pfn1^{-/-} granule extract proteins were fractionated by molecular weight using centricones™ with the indicated sizing filters, and inhibitory activity was assessed by blocking perforin-mediated lysis of RBCs. **(A) Granule proteins of less than 100 kDa demonstrated CxRP2 bioactivity.** Similar fractionation with a 100 kDa filter revealed that CxRP2 inhibitory activity resided in *pfn1*^{-/-} proteins less than 100 kDa. In A, B & C, the fractions were reconstituted to their original volume so that it is appropriate to compare each fraction with the starting material, which was the basis for the statistical comparisons. **(B) Granule proteins of greater than 50 kDa demonstrated CxRP2 bioactivity.** *Pfn1*^{-/-} granule proteins greater than 50 kDa suppressed perforin activity, while fractions below 50 kDa were less effective. **(C) Granule proteins between 50–100 kDa demonstrated CxRP2 bioactivity.** *Pfn1*^{-/-} granule extract was separated by two centricones. The flow-through from the 100 kDa filter (less than 100 kDa fraction) was secondarily fractionated with a 50 kDa filter. Inhibitory activity was observed in the 50–100 kDa range. P-values were < 0.05 between series for the indicated points.

Figure 7. Protein disulfide isomerase has CxRP2 bioactivity

PDI was used to inhibit perforin-mediated lysis of red blood cells. For (A) and (B), * indicates a p-value < 0.05 relative to the paired WT control values. **(A) PDI blocked perforin lysis.** Perforin WT granule extracts were kept at a fixed concentration of 0.3 μg/ml protein and PDI was titrated. Increasing concentration of PDI resulted in reduced red blood cell lysis. **(B) PDI blocked maximal perforin lysis.** WT granule extracts were titrated with or without 4.8 μg/ml perforin KO granule extracts or 5 μg/ml PDI. **(C) PDI augmented the CxRP2 activity of concentrations perforin KO granule extracts that lacked inhibitory activity on their own.** WT granule extracts (0.3 μg/ml protein) were inhibited by either a straight titration of perforin KO granule extract or a titration of perforin KO granule extract combined with a constant concentration of 2.5 μg/ml PDI. The lowest three dilutions of perforin KO extract indicate that very low amounts of granule proteins are effective when combined with PDI. The

P values for differences of PDI + KO from the KO alone controls were less than 0.05 at the indicated points.

Table 1
Identification of Proteins in the 50–100 kDa CxRP2 Fraction

Protein Name	Access Number	Protein Score	Total Ion Score	Total Ion C. L. %	Pep. Count	Protein Score C.I. %	Species	Coverage	Protein MW
Heat shock 70kD protein 5 (BiP)	Q9DC41	510	250	100	29	100	Mus musculus	53%	72378.4
Hspa9a protein (Mortalin)	Q7TSZ0	107	72	100	9	100	Mus musculus	28%	73415.6
Similar to ER-60 protease (Protein Disulfide Isomerase A3)	Q99LF6	493	169	100	32	100	Mus musculus	66%	56642.7
Citrate Synthase	Q9CZU6	159	106	100.00	10	100	Mus musculus	36%	21759.5
cytotoxic T-lymphocyte protease (Gzm G)	S06176	93	63	100	5	100	Mus musculus	38%	21759.5
preprogranzyme G (Gzm G)	AAB19191	293	138	100	16	100	Mus musculus	54%	27335.6
Granzyme B(G, H) precursor (Gzm B)	P04187	194	116	100	10	100	Mus musculus	51%	27452.4