Interaction between a Ca^{2+}-Binding Protein Calreticulin and Perforin, a Component of the Cytotoxic T-Cell Granules†

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ABSTRACT: Calreticulin is a component of cytotoxic T-lymphocyte and NK lymphocyte granules. We report here that granule-associated calreticulin terminates with the KDEL endoplasmic reticulum retrieval amino acid sequence and somehow escapes the KDEL retrieval system. In perforin knock-out mice calreticulin is still targeted into the granules. Thus, calreticulin will traffic without perforin to cytotoxic granules. In the granules, calreticulin and perforin are associated as documented by (i) copurification of calreticulin with perforin but not with granzymes and (ii) immunoprecipitation of a calreticulin−perforin complex using specific antibodies. By using calreticulin affinity chromatography and protein ligand blotting we show that perforin binds to calreticulin in the absence of Ca^{2+} and the two proteins dissociate upon exposure to 0.1 mM or higher Ca^{2+} concentration. Perforin interacts strongly with the P-domain of calreticulin (the domain which has high Ca^{2+}-binding affinity and chaperone function) as revealed by direct protein−protein interaction, ligand blotting, and the yeast two-hybrid techniques. Our results suggest that calreticulin may act as Ca^{2+}-regulated chaperone for perforin. This action will serve to protect the CTL during biogenesis of granules and may also serve to regulate perforin lytic action after release.

Cytotoxic T lymphocyte (CTL) and natural killer (NK) cell-mediated killing of virus-infected and tumor cells is an important function of the immune system. One significant pathway of target cell destruction involves the dense cytoplasmic granules (*1*, *2*). In addition to perforin, cytolytic granules contain several macromolecules including specific proteases (granzymes) and proteoglycans (*3*−*8*). When a CTL interacts with a target cell there is a directed exocytosis of the granule contents toward the target (*9*). It is believed that perforin facilitates the entry of granzymes into the cytoplasm of the cell under attack. The granzymes then induce apoptosis in part due to the cleavage and activation of a cysteine protease caspase 3 (aka CPP32, Yama, apopain) (*10*). Dupuis et al. (*11*) discovered that CTL granules also contain calreticulin, a Ca^{2+}-binding, multifunctional protein of the ER membranes (*12*). Furthermore, Burns et al. (*13*) showed that expression of calreticulin is induced upon stimulation of CTL, suggesting that the protein may play a role in CTL-dependent killing.

Calreticulin is a resident ER membrane protein which belongs to the family of KDEL proteins (*12*). Calreticulin contains an N-terminal signal sequence and a C-terminal KDEL ER retrieval sequence (*14*). It is not surprising, therefore, that the most frequently reported localization of calreticulin is within the ER (including nuclear envelope). The protein binds Ca^{2+} with high capacity (*15*) and plays an important role in control of intracellular Ca^{2+} homeostasis (*16*−*20*). Calreticulin also modulates steroid-sensitive gene expression (*21*−*23*) and cell adhesion (*20*, *24*−*26*). The protein has a chaperone activity: it is similar to calnexin and binds specifically to partially trimmed, monoglucosylated, N-linked oligosaccharides (*27*−*32*). The protein may be divided into three structural and functional domains on the basis of analysis of its amino acid sequence: the N-terminal N-domain, the proline-rich P-domain, and the acidic, C-terminal C-domain (*14*). The P-domain is perhaps the most interesting region of calreticulin. It has a lectin-like, chaperone activity (*31*−*34*), binds Ca^{2+} with high affinity (*15*), and interacts in a Ca^{2+}-dependent manner with protein disulfide isomerase (PDI) (*35*) and perhaps with other ER lumenal proteins (*36*).

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In this paper we report that calreticulin is a component of the CTL granules and that the protein interacts with perforin in a Ca^{2+}-regulated manner in vitro and in vivo. The granule form of calreticulin contains the KDEL ER retrieval sequence, and the protein is targeted to the granules even in perforin null mice. Our results suggest that calreticulin could have a dual function in T-cells: it may act as a chaperone for perforin to protect CTL during activation and then may perform a key control function for lytic activity during killing of target cells.

EXPERIMENTAL PROCEDURES

Materials. [^{35}S]Methionine was from Amersham. Pipes, NP40, Triton X-100, formaldehyde, and poly(ethylene glycol) 8000 were from Sigma. Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim, BRL, and Bio/Can Scientific (Mississauga, Ontario). Nitrocellulose membrane filters were from Schleicher and Schuell. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) reagents and molecular weight markers were from Bio-Rad. Mono Q FPLC column and Glutathione−Sepharose 4B were from Pharmacia. Peroxidase-conjugated rabbit anti-goat and goat anti-rabbit IgGs were from Bio-Rad and Boehringer Mannheim, respectively. The ECL detection kit for Western blotting was obtained from Amersham. FITC- and Texas Red-conjugated secondary antibodies were purchased from Bio/Can Scientific or ICN ImmunoBiologicals (Montreal, Quebec). Vinol 205S was obtained from St. Lawrence Chemical (Toronto, Ontario), and 1,4-diazobicyclo-(2,2,2)-octane was from Polysciences. *p*-Phenylenediamine was from Fisher Scientific. The matchmaker two-hybrid system was purchased from Clontech Laboratories. Plasmid purification kits were purchased from QIAGEN Inc. (Chatsworth, CA). Isopropyl β-D-thiogalactopyranoside was obtained from Boehringer Mannheim. Vent polymerase was from New England Biolabs. T7 coupled TNT Reticulocyte system was from Promega (Madison, WI). Glutathione−agarose was obtained from Molecular Probes, Inc. All chemicals were of the highest grade available.

Expression and Purification of Native and Recombinant Proteins. Glutathione *S*-transferase (GST) fusion proteins, which included the full-length mature calreticulin and three calreticulin domains, N-terminal domain (N-domain), amino acid residues 1−182; proline-rich domain (P-domain), amino acid residues 182−290; and C-terminal domain (C-domain), amino acid residues 330−401, were expressed in *Escherichia coli* and purified as described by Baksh and Michalak (*15*). Native calreticulin and cardiac calsequestrin were purified by the ammonium sulfate precipitation procedures as described earlier (*37*, *38*).

Preparation of Granules and Purification of Perforin. Granules were prepared from cells of the cytotoxic NK-like cell line RNK-16 (*60*, *61*) using Borregard's buffer and Percoll (Pharmacia Fine Chemical, Piscaway, NJ) gradient fractionation (*40*). Perforin and granzymes were isolated using immobilized metal affinity chromatography (IMAC) with Cu^{2+} bound to Poros MC resin (PerSeptive Biosciences, Cambridge, MA) (*40*). Perforin was further enriched by hydrophobic interaction chromatography using a phenyl−Sepharose HR 5/5 column (Pharmacia). Co^{2+} IMAC chromatography was also used to separate perforin. With Co^{2+} instead of Cu^{2+}, granzymes are not bound to the IMAC, optimizing the separation of perforin from granzymes (*40*). The perforin fraction was identified by hemolytic activity and by Western blots using affinity-purified antibodies generated to a peptide representing amino acid residues 464−475 of perforin (*40*).

FIGURE 1: CRT283 antibody is specific for KDEL amino acid sequence in calreticulin. GST−calreticulin fusion proteins were expressed in *E. coli* and purified as described under Experimental Procedures. Fifty micrograms of *E. coli* extract containing GST−calreticulin fusion proteins was separated on SDS−PAGE, transferred to nirocellulose membrane, and probed with the CRT283 anti-calreticulin (KDEL) antibodies. Lane 1, recombinant GST−calreticulin fusion protein truncated and lacking the KDEL ER retrieval amino acid sequence; lane 2, full-length mature GST−calreticulin fusion protein ending with the KDEL sequence. The arrow indicates location of the GST−calreticulin fusion protein.

Antibodies and Immunoblotting. Goat anti-rabbit skeletal muscle calreticulin antibodies were extensively characterized by Milner et al. (*38*). Rabbit anti-calreticulin (designated CRT283) was raised against a synthetic peptide QAKDEL, which encodes the C-terminal amino acids 386−401 of rabbit calreticulin (*39*). The peptide was synthesized on a peptide synthesizer (Model 430A; Applied Biosystems, Inc., Foster City, CA) and then coupled to keyhole limpet hemocyanin by the Alberta Peptide Institute, University of Alberta. Rabbits were immunized with 0.1 mg of keyhole limpet hemocyanin conjugated peptide emulsified in Freund's complete adjuvant. The immunization was repeated using Freund's incomplete adjuvant after 2 weeks and 4 weeks. The CRT283 antibody was affinity purified using a BSA-coupled QAKDEL synthetic peptide affinity column. Antibodies were eluted with ImmunoPure Gentle Ag/Ab elution buffer (Pierce). Specificity of the anti-CRT283 antibody was tested with recombinant calreticulin missing KDEL carboxy-terminal ER retrieval sequence and expressed in *E. coli* as GST fusion protein (Figure 1). Full-length calreticulin expressed as GST fusion protein was used as a control (*15*). Calreticulin minus KDEL GST fusion protein was generated by PCR-driven amplification as described by Baksh and Michalak (*15*) except that a reverse primer contained a stop codon in front of the KDEL amino acid sequence of mature calreticulin. These proteins were expressed in *E. coli*, purified, separated on SDS−PAGE, and tested for their reactivity with CRT283 antibodies (Figure 1). The antibody did not recognize the recombinant calreticulin missing the KDEL carboxyl-terminal amino acid sequence (Figure 1, lane 1) and did recognize KDEL-containing recombinant calreticulin indicating its specificity for the carboxyl-terminal amino acid sequence of calreticulin (Figure 1, lane 2). Furthermore,

the CRT283 was specific for calreticulin. It did not recognize either calsequestrin or other KDEL-containing ER resident proteins including PDI, Grp78(BiP), ERp72, and Grp96(endoplasmin) (not shown). We concluded that CRT283 was specific for the KDEL-containing, ER form of calreticulin. This antibody is available from UBI. For immunoblotting all anti-calreticulin antibodies were used at 1:300 dilution. Anti-SERCA2 antibody IID8 (a generous gift from K. Campbell, University of Iowa) was used at the concentration of 1:500. Anti-PDI was used as described by Baksh et al. (*35*). Polyclonal anti-BiP antibodies were made against the synthetic peptide KEEDTSEKDEL corresponding to the C-terminus of BiP. Anti-BiP antibody was used at 1:100 dilution. Another rabbit polyclonal antibody was raised against KETEKESTEKDEL synthetic peptide, and it recognized BiP, PDI, and Grp96 (endoplasmin). This antibody was used at 1:100 dilution. Both antibodies were generous gifts of S. Fuller (EMBL, Heidelberg, Germany). Rabbit anti-ERp72 antibody was a generous gift of M. Green (St. Louis University), and it was used at 1:300 dilution. Antibody binding was detected with the ECL light detection system. T-cells were stimulated with concanavalin A (ConA) and lysed for SDS−PAGE as described by Burns et al. (*13*). Affinity-purified anti-perforin antibodies were generated to a peptide representing amino acid residues 464−475 of perforin (*40*).

Interactions of Perforin with GST−Calreticulin. The purified GST−calreticulin fusion protein was bound to the glutathione−agarose beads (200 μL of 10% solution) (*35*). The beads were extensively washed with phosphate-buffered saline followed by washing with a buffer containing 20 mM Tris, pH 7.0, 150 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, and 0.1 mM $CaCl_2$ or 0.1 mM EGTA. Perforin (Co^{2+}−IMAC) was added, and the mixture was incubated for 60 min at 4 °C followed by centrifugation for 3 min at 2000*g* in an Eppendorf centrifuge. Pellets containing the beads and GST−calreticulin-bound proteins were washed with the buffer and spun down for 3 min at 2000*g*, and proteins bound to beads were subjected to SDS−PAGE. The proteins were transferred to nitrocellulose membrane and probed with anti-perforin antibodies.

Protein Blots. Full-length mouse perforin was radioactively labeled with [^{35}S]methionine using the Promega T7 coupled TNT Reticulolysate System. GST−calreticulin and the GST−calreticulin domains were separated on 10% SDS−PAGE and transferred to nitrocellulose. The blots were blocked for 60 min with 5% bovine serum albumin, in a buffer containing 20 mM Tris, pH 7.0, and 150 mM KCl in the presence or absence of 5 mM EGTA. Then, blots were incubated for 60 min with [^{35}S]methionine-labeled perforin in blocking buffer, washed three times for 60 min with blocking buffer, air dried, and exposed to X-ray film.

Immunoprecipitation. Perforin−calreticulin complex was immunoprecipitated using anti-calreticulin antibodies (*11*) (generous gift of K.-H. Krause) either from purified CTL granules or from [^{35}S]methionine-labeled cell extracts from mouse B6.1 T-cell line. For immunoprecipitation purified CTL granules were solubilized in RIPA buffer (1% NP40, 0.5% deoxycholate, 150 mM NaCl, 1 mM EDTA, 25 mM Tris, pH 7.5) containing 1 mg/mL BSA followed by centrifugation at 100000*g* for 45 min. Solubilized granule proteins were incubated with 5 μL of anti-calreticulin antibody in the absence or presence of 1.5 mM Ca^{2+} (free Ca^{2+} ~600 μM) for 2 h at room temperature. The mixture was then incubated with 50 μL of 10% protein A/G beads equilibrated in a RIPA buffer containing BSA. After 30 min incubation the beads were spun down for 15 s at 10000*g* followed by washing with RIPA buffer containing 500 mM NaCl and RIPA buffer containing 0.1% SDS, and finally with 0.1% NP40, 25 mM Tris, pH 7.5. All washes were carried out either in the absence or in the presence of 1.5 mM Ca^{2+}. Washed beads were suspended in a Laemmli sample buffer containing 1% SDS, heated for 2 min at 60 °C, and loaded onto SDS−PAGE. Proteins were transferred to nitrocellulose and tested for the presence of perforin using anti-perforin monoclonal antibodies (*41*). Immunoprecipitation of perforin−calreticulin complexes from [^{35}S]methionine-labeled B6.1 T-cell line extracts was carried out in the absence of Ca^{2+}. Perforin was identified in these immunoprecipitates by Western blot analysis using a monoclonal anti-mouse perforin antibody.

SDS−Polyacrylamide Gel Electrophoresis. SDS−PAGE was on 10% polyacrylamide gels as described by Laemmli (*42*). After gel electrophoresis, either gels were stained with Coomassie Blue or proteins were transferred electrophoretically onto nitrocellulose membrane according to the method of Towbin et al. (*43*). Standards were Bio-Rad low-range molecular weight proteins.

Immunofluorescence Microscopy. For intracellular localization of calreticulin in CTL the cells were attached to polylysine (Sigma) treated cover slips followed by immunostaining. Polyclonal goat anti-calreticulin antibodies were used at 1:50 dilution in PBS. FITC-conjugated secondary antibodies were used at 1:30 dilution in PBS. For fluorescence microscopy, cells were fixed in 3.8% formaldehyde in PBS for 10 min, extracted with 0.1% Triton X-100 in a buffer containing 100 mM Pipes, 1 mM EGTA, and 4% (w/v) poly(ethylene glycol) 8000 (pH, 6.9) for 30 min, washed in PBS for 10 min, and then processed for labeling with primary antibodies followed by appropriate FITC-conjugated secondary antibodies. All incubations were carried out for 3 min at room temperature, followed by a 10 min wash with PBS. After final wash, the slides were mounted in Vinol 205S, which contained 0.25% 1,4-diazobicyclo-(2,2,2)-octane and 0.002% *p*-phenylenediamine to prevent photobleaching. Confocal fluorescence microscopy was done using a Bio-Rad MRC-600 microscope.

Identification of Calreticulin in Perforin Knock-Out Mice. Calreticulin was identified in T-cells isolated from perforin gene knock-out mice by both immunocytochemistry and cell extract fractionation. Perforin knock-out and wild-type CTLs (Figure 4) were adhered to glass slides by cytospin and fixed in 2% paraformaldehyde for 15 min at room temperature. Cells were permeabilized in PBS with 0.1% saponin and blocked in 3% nonfat skim milk powder in PBS/saponin for 30−60 min at room temperature. Mouse anti-granzyme B and goat anti-calreticulin antibodies were both added at a dilution of 1:50 and incubated for 2 h at room temperature. Cells were washed up to 5 times in PBS/saponin and incubated for 2 h with secondary antibody consisting of donkey anti-mouse FITC and donkey anti-goat Texas Red (Jackson Immuonoresearch). Cells were washed up to 5 times in PBS/saponin, dried, and mounted for viewing by a confocal laser scanning microscope (Department of Cell

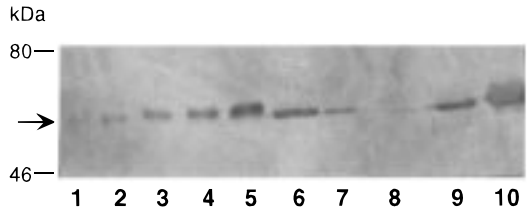

FIGURE 2: Calreticulin of T-cell granules contains the KDEL ER retrieval amino acid sequence. Purification of T-cell granules, perforin, and ConA stimulation of T-cells was carried out as described under Experimental Procedures. Fractionated proteins or cell lysate were separated by SDS−PAGE, transferred to a nitrocellulose membrane, and probed with the CRT238 anti-calreticulin KDEL antibodies. Lanes 1−5, increasing amounts of purified calreticulin, 10, 20, 50, 100, and 500 ng of protein/lane, respectively; lane 6, unfractionated granule extract; lane 7, Co^{2+}-IMAC isolated perforin; lane 8, lysate from the resting T-cells; lane 9, lysate from the ConA stimulated T-cells; lane 10, purified calreticulin. The arrow indicates the position of calreticulin.

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Cytoplasmic extracts of CTL from mixed lymphocyte cultures from wild-type or perforin null mice were fractionated on Percolle gradient. Percoll gradient fractions were tested for the presence of granzyme A by estimation of their esterolytic activity and for the presence of calreticulin by Western blot analysis using anti-calreticulin antibodies.

Yeast Two-Hybrid Gene Expression System. To examine the interaction between perforin and calreticulin the yeast two-hybrid system was employed (*44*). Fusion proteins of the GAL4 binding domain with either the N-, N1-, N2-, P- or P1-domain were generated by PCR as previously described (*35*). Schematic representation of calreticulin domains used in this study is shown in Figure 7.

Full-length perforin fused to the GAL4 activating domain was generated by PCR amplification of mouse perforin that was isolated from a mouse T-cell cDNA library. Primers used are as follows:

primer 1, 5′-ATATGAATTCATGCCCTGCTACACTGCCAC-3′

primer 2, 5′-ATATGGATCCTTACCACACAGCCCCACTG-3′

Primer 1 contained an *Eco*RI site and nucleotides encoding the first six amino acids of mature mouse perforin. Primer 2 contained a *Bam*HI site and nucleotides encoding for the last five amino acids of mature mouse perforin. Amplification was carried out for 25 cycles with each cycle consisting of 1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C and then followed by one cycle extension for 10 min at 72 °C. The PCR products were cloned into the *Eco*RI and *Bam*HI sites of the phosphatase-treated pGAD424 vector to generate the construct designated pGAD-perf. Every construct was confirmed by nucleotide sequencing.

Interactions between perforin and calreticulin were detected by transforming the yeast strain SFY526 with pGB-N1, pGB-N2, pGB-P1, or pGAD-perf or by cotransforming with either pGB-N1, pGB-N2, or pGB-P1 and pGAD-perf using 100 ng of DNA for single transformations and 200 ng of each construct for double transformations. As negative controls the pGBT9 and pGAD424 vectors were cotransformed as well as each of pGB-N1, pGB-N2, and pGB-P1 were cotransformed with pGAD424 and pGAD-perf was cotransformed with pGBT9. The interaction was monitored by a liquid assay for β-galactosidase activity according to the manufacturer's protocol. Color development was monitored for 24 h.

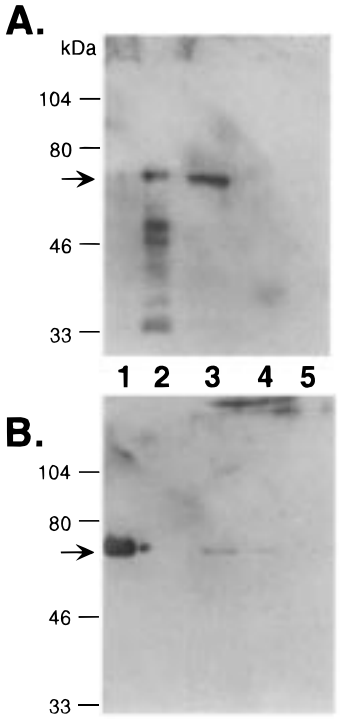

FIGURE 3: Coisolation of calreticulin and perforin after fractionation of cytotoxic granule proteins. Granules were isolated by Percoll density. The proteins were extracted with high salt and fractionated by Cu^{2+} IMAC as described under Experimental Procedures. Proteins present in various fractions were separated on SDS−PAGE, transferred to nitrocellulose membrane, and probed with anti-perforin antibodies (A) or with the goat anti-calreticulin antibodies (B). Lane 1, purified calreticulin (2 μg of protein); lane 2, unfractionated granule proteins; lane 3, perforin-containing fraction; lane 4, unbound proteins (including proteoglycan); lane 5, granzymes. Arrows indicate the location of perforin (A) or calreticulin (B). In A, perforin is detectable in the initial granule extract (lane 2) and the perforin fraction (lane 3). The low molecular weight protein bands in lane 2 are likely degradation products of perforin. In B, calreticulin is detected with perforin (lane 3). Calreticulin was not detected in the unfractionated granule extract (lane 2) due to the low relative abundance of calreticulin in the extract and the low affinity of the goat anti-calreticulin antibody.

Miscellaneous. All recombinant techniques were conducted according to standard protocols (*45*). Protein was determined by the method of Lowry et al. (*46*) or Bradford (*47*).

RESULTS

Calreticulin Is a Component of the Cytolytic Granules. Calreticulin and perforin copurify and colocalize to CTL granules (*11*). Calreticulin belongs to the family of ER resident proteins that are retrieved to the lumen of the ER by a KDEL-dependent receptor mechanism (*48*). This observation raised the question of whether the granule form of calreticulin contains the KDEL carboxyl-terminal ER retrieval amino acid sequence. To examine this issue we generated and employed specific antibodies (designated CRT283) that recognize the QAKDEL carboxyl-terminal amino acid sequence of calreticulin. Specificity of the antibody was tested using truncated recombinant calreticulin which was missing the KDEL carboxyl-terminal amino acids. Figure 1 shows that the CRT283 antibody recognized full-

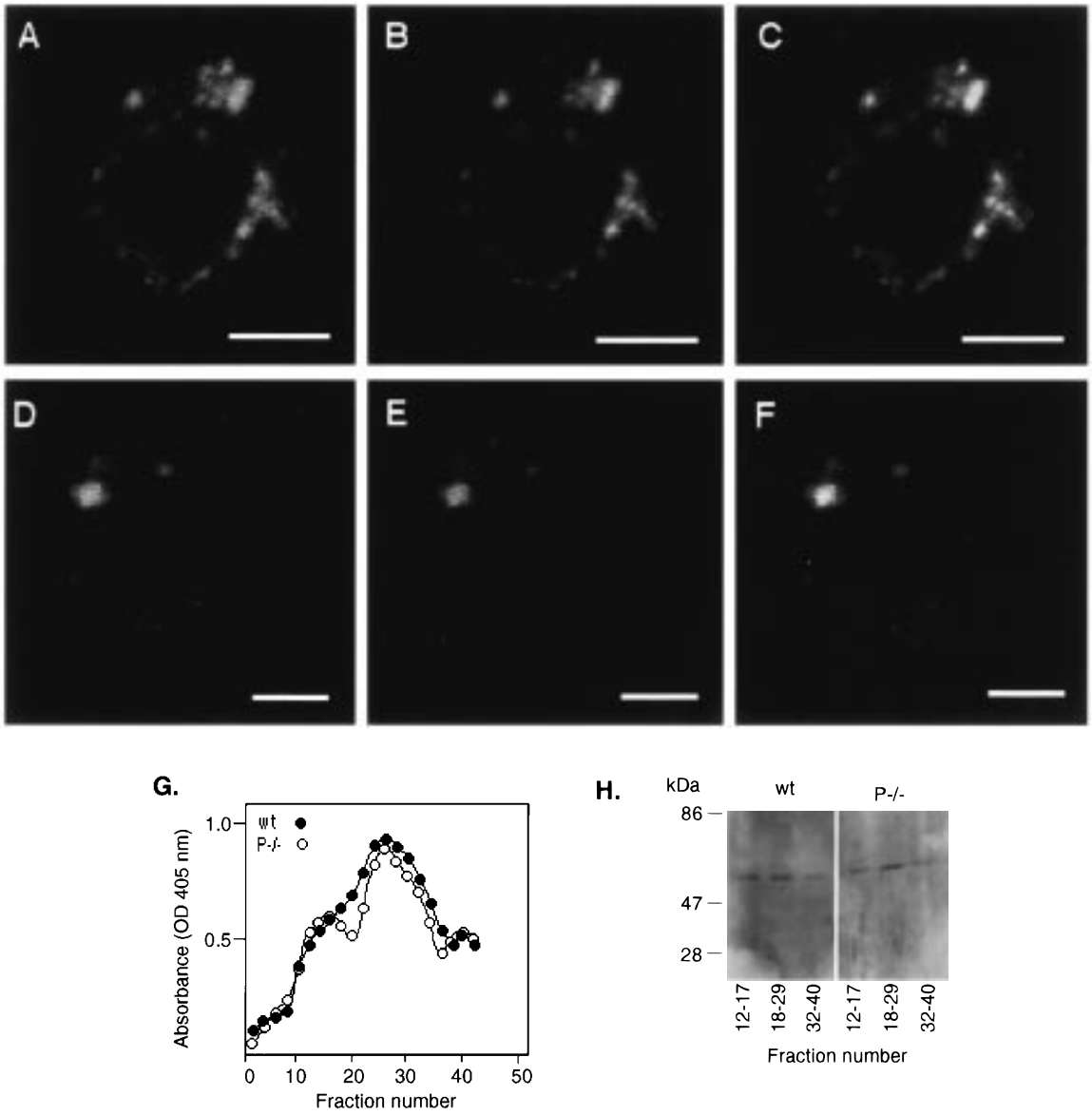

FIGURE 4: Localization of calreticulin to CTL granules. Calreticulin is found in the CTL granules in normal (D) and perforin gene knock-out (A) mice, and it colocalizes with granzyme B (C and F). Indirect immunofluorescence staining was carried out with anti-calreticulin (A, D) and anti-granzyme (B, E) antibodies as described under Experimental Procedures. (G, H) Calreticulin cofractionates with granzyme A in wild-type and perforin knock-out mice. Cytoplasmic extracts of T-cells from wild-type (wt) and perforin null (p−/−) mice were fractionated on a Percoll gradient followed by analysis of distribution of granzyme A (G) and calreticulin (H).

length mature calreticulin (Figure 1, lane 2) but failed to bind truncated protein (Figure 1, lane 1). Importantly, the CRT283 antibodies recognized calreticulin in the extracts of cytolytic granules and within preparations of perforin (Figure 2, lanes 6 and 7). Figure 2 also shows that, in agreement with our earlier observations (*13*), ConA-activated T-cells expressed more calreticulin than unstimulated T-cells (Figure 2, compare lanes 8 and 9). These experiments revealed that calreticulin found in the stimulated T-cells, isolated granules, or the purified perforin fraction did contain the KDEL. Calreticulin was the only one of six proteins of the KDEL family that was detected in purified granules. Using specific antibodies we did not detect PDI, BiP, Grp96, ERp72 (ER lumenal proteins that belong to the KDEL family of proteins), and SERCA2 in purified granules or in the preparations of perforin (data not shown).

In addition to perforin, granzymes are another major component of the CTL granules (*2*, *3*, *7*, *8*, *49*). In order to test if calreticulin associates selectively with these proteases we separated granule proteins by metal-affinity chromatography. The fractions were probed with the anti-perforin antibodies (Figure 3A) and with the goat anti-calreticulin antibodies (Figure 3B). Figure 3A shows that perforin was detected in the solubilized granules as well as the fractions eluted from the IMAC column. Perforin was not detected in the column flow through or in the protease fraction of the granule extract (Figure 3A). Calreticulin was detected with perforin and was barely detected in the pool of unbound proteins (Figure 3B, lanes 3 and 4). However, calreticulin was not found in the protease fraction (Figure 3B, lane 5). We conclude that calreticulin is an exceptional ER resident protein that is targeted to the cytotoxic granules and that in the granules the protein associates with perforin but not with granzymes.

Sorting of Calreticulin to CTL Granules in the Perforin Null Mouse. In order to test if targeting of calreticulin to

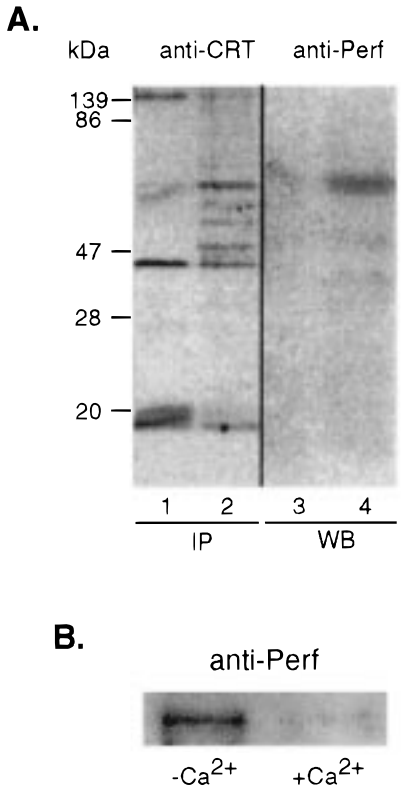

FIGURE 5: Ca^{2+}-dependent association of perforin with calreticulin in vivo. The perforin−calreticulin complex was immunoprecipitated with anti-calreticulin antibodies from [^{35}S]methionine-labeled cell extracts from mouse B6.1 T-cell line (A) and from purified CTL granules (B). In A, lanes 1 and 3, control serum; lanes 2 and 4 anti-calreticulin antibodies; IP, immunoprecipitate; WB, western blot analysis with anti-perforin antibodies. In B, immunoprecipitation with anti-calreticulin antibodies was carried out in the presence or absence of Ca^{2+} as described under Experimental Procedures.

the cytotoxic granules depended on the presence of perforin, we carried out indirect immunofluorescence staining and biochemical analysis of the CTL granules in normal and perforin gene knock-out mice. By using immunocytochemical methods we showed that calreticulin was localized to CTL granules (Figure 4A−F). In both wild-type and perforin null cells, calreticulin was targeted to the granules as revealed by its colocalization with granzyme B, a marker of the CTL granules (Figure 4A−F). Furthermore, Percoll gradient fractionation of cytoplasmic extracts of T-cells showed that calreticulin cofractionates with granzyme A in wild-type and perforin null cells (Figure 4, panels G and H). We concluded that calreticulin was targeted to the granules in perforin gene knock-out mice (Figure 4), indicating that this localization was not dependent on the presence of perforin.

Calreticulin Perforin Interaction is Ca^{2+}-Regulated. Calreticulin is a Ca^{2+}-binding protein, and perforin activation requires Ca^{2+} (50, 51). We employed immunoprecipitation and GST-affinity chromatography techniques to directly investigate interactions between calreticulin and perforin and to establish whether these were Ca^{2+}-dependent. First, [^{35}S]-methionine-labeled extracts from the mouse B6.1 T-cell line were incubated with anti-calreticulin or control antibodies (Figure 5A, lanes 1 and 2). The immunoprecipitates were further analyzed by SDS−PAGE and probed by Western blot analysis using anti-perforin antibodies (Figure 5A, lanes 3 and 4). Figure 5A (lane 4) shows that perforin was present

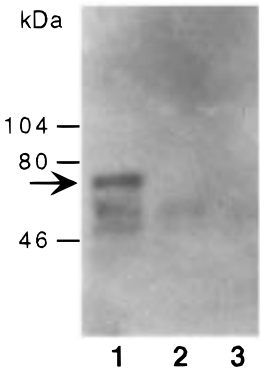

FIGURE 6: Binding of perforin to the calreticulin affinity column is Ca^{2+}-dependent. GST and GST−calreticulin were expressed in *E. coli* and purified as described by Baksh and Michalak (*15*). The GST and GST fusion proteins were bound to the Glutathione−agarose beads and then incubated with purified perforin as described under Experimental Procedures. The beads were spun down to obtain the pellet containing bound material. The pellet was solubilized in a Laemmli sample buffer, separated by SDS−PAGE, transferred to nitrocellulose membrane, and probed with anti-perforin antibodies. Lane 1, GST−calreticulin-bound material in the absence of Ca^{2+} and in the presence of 0.1 mM EGTA; lane 2, GST control; lane 3, GST−calreticulin-bound material in the presence of 0.1 mM Ca^{2+}. The arrow depicts the position of perforin.

in the calreticulin immunoprecipitate but not in the control sample, confirming that the two proteins interacted. To test the Ca^{2+} dependence of the interactions, detergent-solubilized CTL granule proteins were incubated with anti-calreticulin antibodies in the presence or absence of exogenous Ca^{2+} (Figure 5B). Perforin was found only in the calreticulin immunoprecipitate obtained in the absence of Ca^{2+} (Figure 5B). We conclude that in vivo perforin and calreticulin interact only in the absence of Ca^{2+}.

To further investigate perforin−calreticulin interactions and their Ca^{2+} dependence we used GST-affinity chromatography techniques. Purified GST−calreticulin fusion protein coupled to glutathione−agarose was incubated with perforin in the presence of either 0.1 mM EGTA or 0.1 mM Ca^{2+}. GST−agarose beads were spun down and washed extensively. Recombinant GST was used as a control. Figure 6 (lane 1) shows that perforin was retained by the GST−calreticulin affinity column when incubated in the absence of Ca^{2+}; however, in the presence of 0.1 mM Ca^{2+} perforin did not bind (Figure 6, lane 3). Perforin did not interact with the recombinant GST in the presence or in the absence of Ca^{2+} (Figure 6, lane 2). These results indicate that perforin and calreticulin interact only in the absence of Ca^{2+}.

Perforin Interacts with the N- and P-Domains of Calreticulin. To establish the specificity of the calreticulin-perforin interactions and to determine which region of calreticulin binds perforin we employed a protein ligand blotting technique. Bovine serum albumin, calsequestrin, PDI, GST−calreticulin, and fusion proteins containing GST with calreticulin domains (15) were separated on 10% SDS−PAGE and transferred to nitrocellulose. The blot was probed with [^{35}S]methionine-labeled perforin in the absence or presence of Ca^{2+}. Perforin binding was visualized using autoradiography. Perforin did not interact with any proteins in the presence of Ca^{2+} (data not shown), supporting our findings (see above) that perforin interacts with calreticulin only in

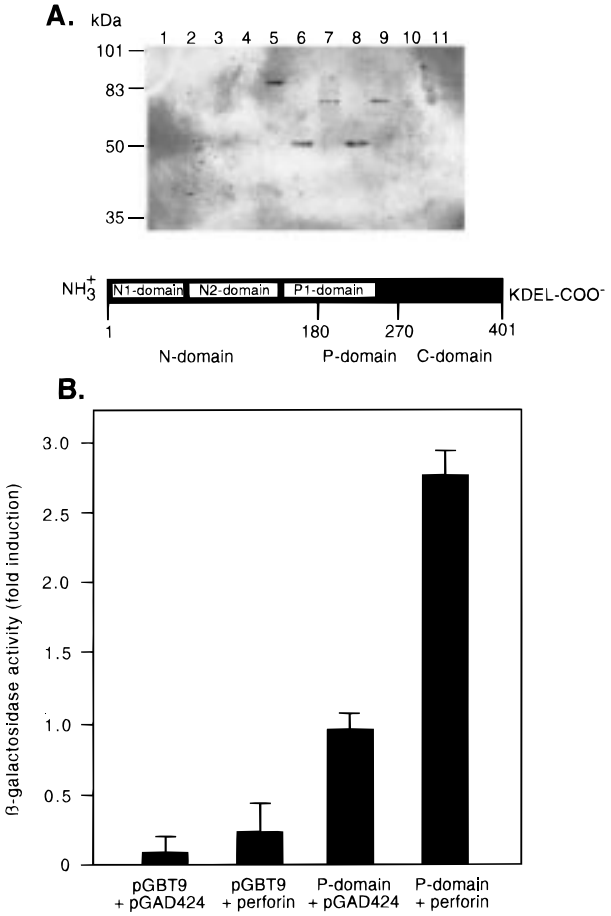

FIGURE 7: The P-domain of calreticulin interacts with perforin in ligand blotting and in the yeast two-hybrid system. GST, GST−N-domain, and GST−P-domain, and GST−C-domain of calreticulin were expressed in *E. coli* and purified as described by Baksh and Michalak (*15*). Cardiac calsequestrin and PDI were purified as described under Experimental Procedures. Purified proteins were separated on SDS−PAGE, transferred to nitrocellulose membranes, and incubated in the absence of Ca^{2+} with [^{35}S]methionine-labeled perforin as described under Experimental Procedures. In A, lane 1, bovine serum albumin; lane 2, canine cardiac calsequestrin; lane 3, recombinant GST; lane 4, GST−calreticulin; lane 5, GST−N-domain; lane 6, GST−N+P-domain; lane 7, GST−P-domain; lane 8, GST−P+C-domain; lane 9, GST−C-domain; lane 10, GST−C-domain. Two micrograms of protein was loaded per lane. In B, for the yeast two-hybrid experiments, appropriate expression vectors were transformed into yeast strain SFY526 as described under Experimental Procedures. β-Galactosidase activity was measured by liquid assay according to the manufacturer's protocol using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate. Schematic representation of calreticulin domains is shown.

the apparent absence of Ca^{2+}. Therefore, the experiments presented were carried out in the absence of Ca^{2+} and in the presence of 0.5 mM EGTA. Figure 7A shows that perforin did not bind to bovine serum albumin (lane 1), calsequestrin (a Ca^{2+}-binding protein of similar physicochemical properties to calreticulin) (lane 2), PDI (an ER lumenal protein) (lane 3), or recombinant GST (lane 4). Five regions of calreticulin were expressed as GST fusion proteins: the GST−N-domain (encompassing the NH_2-terminal 182 amino acids of the protein), the GST−N+P-domain (the NH_2-terminal 273 amino acids), the GST−P-domain (residues 139−273), the GST−P+C-domain (residues 139−401), and the GST−C-domain (residues 270−401). Figure 7A shows that [^{35}S]-methionine-labeled perforin bound to the GST−calreticulin (lane 5), GST−N-domain (lane 6), N+P-domain (lane 7), P-domain (lane 8), and P+C-domain (lane 9) but not to the GST−C-domain (lane 10) of calreticulin. We concluded that in this in vitro system perforin can interact with both the N- and P-domain.

Interaction between Perforin and the Domains of Calreticulin Inside Cells. We employed the yeast two-hybrid system (44) to test if perforin and calreticulin interact inside cells. Calreticulin was exposed as a fusion protein with the DNA binding domain of the GAL4 transcriptional activator. Full-length perforin was expressed as a fusion protein with the activating domain of GAL4. In addition to the full-length N-domain (amino acids 1−174) two regions of the N-domain of calreticulin were also expressed: the N1-domain encompassing residues 1−86 and the N2-domain residues 83−174. The full-length P-domain and a shorter region of the P1-domain (residues 169−240) were also used in this study (Figure 7). The P1-domain contains the NH_2-terminal portion of the P-domain and included P-x-x-I-x-D-P-D-A-x-K-P-E-D-W-D-E amino acid repeats conserved in all calreticulins and calnexins (14, 34, 52).

The N-, P-, N1- and P1-domain fusion proteins did not activate reporter gene expression on their own when coexpressed with the GAL4 transcriptional activation domain. The perforin fusion protein also did not activate the reporter gene when coexpressed with the GAL4-DNA binding domain. When jointly expressed as GAL4 fusion proteins, only the P- and P1-domain of calreticulin expressed with perforin induced expression of β-galactosidase, indicating that the two proteins interacted under these conditions (Figure 7B). Furthermore, these results indicate that concentration of Ca^{2+} in the yeast cell nucleus must be sufficiently low ($\sim 10^{-7}$ M) to promote these protein−protein interactions (Figure 7B). Growing yeast cells in the presence of either 50 mM Ca^{2+} or 200 mM EGTA had no effect on cell growth or observed calreticulin−perforin interactions (data not shown), suggesting that, under these conditions, yeast cells maintain low intracellular Ca^{2+} concentrations. Surprisingly, the N- or N1-domains when coexpressed with perforin did not induce expression of β-galactosidase, suggesting that this region did not interact with perforin under the yeast two-hybrid conditions, an observation in contrast to the protein blot experiments (see Figure 7A, lane 6). We conclude that calreticulin interacts with perforin and that these interactions are strongly dependent upon the P-domain of calreticulin.

DISCUSSION

In this report we show that calreticulin is a component of CTL and NK cell granules in both normal and perforin gene knock-out mice. Calreticulin interacts with perforin as demonstrated by copurification, immunoprecipitation, ligand overlay, and the yeast two-hybrid techniques. The two proteins bind in the absence of Ca^{2+}. Our results suggest that calreticulin may act as perforin chaperone and play a role in both perforin sorting and cytotoxic lymphocyte-mediated killing of target cells.

Perforin interacts with the N-domain and P-domain of calreticulin in ligand-blotting experiments but only with the P-domain in the yeast two-hybrid system. The P-domain of calreticulin (residues 181−280) is one of the most interesting regions in calreticulin. It is rich in proline and

contains two sets of three sequence repeats (amino acid sequences P-x-x-I-x-D-P-D-A-x-K-P-E-D-W-D-E and G-x-W-x-P-P-x-I-x-N-P-x-Y-x, respectively (*12*). The P-domain of calreticulin binds Ca^{2+} with a high affinity ($K_m = 1\ \mu M$; *15*), interacts with PDI (*35*), and has lectin-like, chaperone activity (*32*). Furthermore, this region shares striking sequence similarity with calnexin, an ER membrane chaperone (*12*, *52*). The two proteins are unusual as chaperones because they are lectins, interacting specifically with partially trimmed, monoglucosylated, N-linked oligosaccharides (*29*, *53*). The P-domain of calreticulin binds to oligosaccharides in the presence of Ca^{2+} (*32*). The cationic conditions that favor lectin binding will dissociate calreticulin and perforin complexes, suggesting that calreticulin−perforin interactions are not due to oligosaccharide binding. Furthermore, mature perforin contains only complex glycans (*54*) of a type not recognized by calreticulin (*53*). Therefore, the calreticulin−perforin nature of the binding is similar to the calreticulin−PDI interaction described by Baksh et al. (*35*).

An important finding of this study is that the granule form of calreticulin terminates with the KDEL ER retrieval amino acid sequence. How calreticulin overcomes the KDEL-dependent ER retrieval system is unclear at present. Following synthesis in the ER perforin may associate with calreticulin and travel via the Golgi apparatus to be finally packaged into lysosome-like cytoplasmic granules (*8*, *55*). It is possible that association between calreticulin and perforin masks the KDEL sequence to allow the complexed calreticulin to escape its normal retrieval system. However, perforin-dependent masking of the KDEL appears unlikely since calreticulin is targeted to CTL granules in perforin knock-out mice. Furthermore, perforin interacts with the P-domain of calreticulin, whereas the KDEL ER retrieval amino acid sequence is found at the C-terminus of the C-domain of the protein.

Analysis of calreticulin reveals that it has two potential lysosome targeting signals at amino acid residues 42−48 and 347−353 (*56*). These sequences are found in the N-domain and C-domain of calreticulin, respectively (*14*). It is possible that when stimulated T-cells express large quantities of the protein (*13*) the transiently elevated concentration of calreticulin favors recognition by lysosome targeting signals. Recognition of these targeting sequences could result in targeting of the protein complexes to the granules. This mass action-based scenario is further supported by the observation that calreticulin appears to be the only resident ER membrane protein of the KDEL family that is targeted to the granules. We have not detected BiP, PDI, Erp72, and Grp96 (which lack lysosomal targeting signals) in any of the granule preparations tested.

Granzymes are sorted to the granules by a mannose-6-phosphate receptor pathway (*57*). The exact mechanism for trafficking of perforin is unknown. Perforin is a dangerous protein since it is able to bind to phospholipid membrane and insert into the lipid bilayer to form pores. Within the lytic granules such events are prevented by the low levels of Ca^{2+}, the acidic pH, and the presence of proteoglycan and calreticulin. However, other compartments of the cell through which perforin passes are not so well protected. Recently, Griffiths' group (*54*) demonstrated that perforin is synthesized on rough ER as an inactive precursor and converted (past the ER compartment) to the mature, active form by proteolysis. This is clearly an important mechanism for protecting CTL and NK from perforin-dependent self-destruction as the protein is synthesized and travels through the ER and the Golgi. Proteolysis of perforin leads to the removal of the last 20 amino acids and to exposure of the C2 phospholipid binding domain of the protein (*54*). Calreticulin and perforin are unlikely to form a complex in the lumen of the ER where Ca^{2+} concentrations are about >750 μM (*58*, *59*). Our results suggest that calreticulin interacts with mature perforin past the ER compartment, where these interactions would result in formation of an inactive complex. Thus as perforin is proteolytically activated it is packaged into granules as an inactive complex with calreticulin. This would then protect the cell from self-destruction brought about by the fortuitous polymerization of perforin and its membranolytic activity.

The Ca^{2+}-dependent interaction between calreticulin and perforin is of considerable physiological significance. It has potential ramifications for both the fate of the target cell and the ability of the effector to deal with expression of a lethal protein. Perhaps the most obvious importance lies in the regulation of the perforin lytic activity toward the target cell. It is well-documented that granule-mediated killing is Ca^{2+}-dependent and that prior exposure to Ca^{2+} activates perforin (*50*, *51*). This has previously been interpreted as being caused by a Ca^{2+}-dependent interaction of perforin with phosphatidylcholine head groups in the lipid bilayer of the target cell plasma membrane with a subsequent conformation change and polymerization to form a channel. Our results suggest that the self association and phosphatidylcholine-binding domains of perforin may be masked by calreticulin and may only become exposed when the complex is disrupted by Ca^{2+}.

In conclusion we show that calreticulin is a component of the cytotoxic T lymphocytes and NK cell granules that selectively binds perforin from among many other granule proteins. The sustained interaction between the two molecules could be a key controlling mechanism for perforin action after degranulation, where the activation of perforin would be dependent on Ca^{2+}-dependent interaction of the two molecules. Regulation of perforin−calreticulin interactions clearly represents a potentially pivotal control point for the life or death decision.

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