

The Calcium-binding Protein Calreticulin Is a Major Constituent of Lytic Granules in Cytolytic T Lymphocytes

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Summary

Cytolytic T lymphocytes (CTL), natural killer cells, and lymphokine-activated killer (LAK) cells are cytolytic cells known to release the cytolytic protein perforin and a family of proteases, named granzymes, from cytoplasmic stores upon interaction with target cells. We now report the purification of an additional major 60-kD granule-associated protein (grp 60) from human LAK cells and from mouse cytolytic T cells. The NH₂-terminal amino acid sequence of the polypeptide was found to be identical to calreticulin. Calreticulin is a calcium storage protein and carries a COOH-terminal KDEL sequence, known to act as a retention signal for proteins destined to the lumen of the endoplasmic reticulum. In CTLs, however, calreticulin colocalizes with the lytic perforin to the lysosome-like secretory granules, as confirmed by double label immunofluorescence confocal microscopy. Moreover, when the release of granule-associated proteins was triggered by stimulation of the T cell receptor complex, calreticulin was released along with granzymes A and D. Since perforin is activated and becomes lytic in the presence of calcium, we propose that the role of calreticulin is to prevent organelle autolysis due to the protein's calcium chelator capacity.

Cytolytic T cells represent a major arm of the cell-mediated immune response. They recognize and lyse cells carrying nonself epitopes, such as virus-infected cells, tumor cells, or grafted tissues (for review see references 1 and 2). A current view of the mechanism of destruction of the target cell includes the vectorial secretion of the content of cytoplasmic granules by the effector cell into the intercellular space at the site of contact (3–5). This process of degranulation releases, in addition to typical lysosomal enzymes, a pore-forming protein (PFP) called perforin (cytolysin), a family of serine esterases, known as granzymes, and proteoglycan molecules.

Isolated cytoplasmic granules are cytolytic to various tumor cell lines and erythrocytes (3, 6), indicating the importance of the granule-associated molecules in the cytolytic event. Only for perforin, however, has the lytic potential clearly been demonstrated. In the presence of calcium, perforin polymerizes on target cell membranes forming transmembrane, ring-like pores, structurally similar to those caused by the complement membrane attack complex or bacterial toxins (7, 8). Any target cell, except activated cytolytic T cells, succumbs to this attack (9, 10).

The role of other granule-harbored molecules in cytotoxicity is less clear. Purified granzymes are not lytic, despite the fact that various protease inhibitors suppress CTL-mediated cy-

tolysis (11–13). Granzymes A and B, however, seem to participate in the degradation of the target cell's nuclear DNA, which is observed during the CTL attack in parallel to the impairment of the cytoplasmic membrane (14–16). An identical role has also been attributed to TIA-1, a recently described granule-associated poly(A)-binding protein (17).

We have considered the possibility that additional granule proteins may regulate the process of cell lysis. Here we report the purification of a 60-kD, granule-associated protein that copurifies with perforin.

Materials and Methods

Cells. The murine B6.1 CTL cell line (7) was grown in RPMI supplemented with 5% FCS; 10% supernatant of Con A-activated rat spleen cells as a source of IL-2; 100 U/ml penicillin; 100 µg/ml streptomycin, 25 mM Hepes, and 2 mM L-glutamine. The murine CTL clones Cw3 1.1 and Pb CS F12 specific for the *Plasmodium berghei* circumsporozoite (CS) epitope (249–260) were kindly provided to us by Dr. P. Romero (18) and maintained in culture by weekly restimulation with peptide-pulsed P815 and spleen cells. Human LAK cells were prepared from PBMC of healthy donors (19). Mononuclear cells were isolated by density gradient centrifugation using Ficoll-Hypaque. Monocytes and B cells were depleted by plastic adherence and passage through nylon wool columns (20).

HL60¹ cells are human promyelocytic leukemia cells obtained through the American Type Culture Collection (Rockville, MD).

Isolation of Granules. The method for isolation of CTL granules from mouse cell lines has been previously described in detail (21). The identical procedure was also used for the isolation of LAK-derived granules. In brief, cells were washed twice in PBS, resuspended in 12 ml PIPEG (100 mM KCl, 3.5 mM NaCl, 3.5 mM MgCl₂, 1 mM ATP, 1.25 mM EGTA, and 10 mM Pipes, pH 6.8) and broken by nitrogen cavitation. After removal of nuclei and aggregates, the organelles were separated on a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden). The gradient consisted of 15.9 ml of 85% (vol/vol) Percoll, and 12.1 ml of 39% Percoll in PIPEG. The tube was centrifuged at 19,000 rpm in a rotor (model JA20; Beckman Instruments, Inc., Palo Alto, CA) for 30 min at 4°C. 1-ml fractions were collected and tested for hemolytic activity. For the isolation of soluble granule proteins, granules were made 1.5 M in NaCl and 10 mM in benzamidine, thereby disrupting the granule membranes. They were ultracentrifuged to eliminate Percoll and membranes (170,000 g for 2 h at 4°C) and the supernatant recovered.

Separation of Granule Proteins. The solubilized granule preparations (10⁹ human LAK cell or mouse CTL equivalent) were dialyzed against 10 mM Tris-HCl (pH 8.0) and 50 mM NaCl. The mixture was applied to a Mono Q anion exchange column (fast protein liquid chromatography [FPLC] Pharmacia). After the column had been washed with 10 ml 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, the proteins were eluted with a 20-ml linear salt gradient of 50 mM–500 mM NaCl in 10 mM Tris-HCl (pH 8.0). 1-ml fractions were collected. Aliquots (50 µl) of each fraction were analyzed by SDS-PAGE.

Amino Acid Sequencing. The Mono Q fractions containing the protein of interest were pooled, concentrated 20-fold on membranes (Centricon PM 30, Amicon, Beverly, MA) and resolved by SDS-PAGE in the presence of 100 mM thioglycolate and 0.05 mM glutathione (Sigma Chemical Co., Buchs, Switzerland). Proteins were transferred to polyvinylidene difluoride [PVDF] (Immobilon; Millipore, Zürich, Switzerland) microporous membrane and stained with Ponceau red (Serva Biochemicals, Heidelberg, Germany). The 60-kD protein was excised and subjected to automated Edman degradation in a gas phase sequencer (model 470A; Applied Biosystems, Inc., Foster City, CA) using standard protocols.

SDS-PAGE, Immunoblots, and Antibodies. Samples were electrophoresed on 10% slab gels according to Laemmli (22). Gels were developed with the silverstain procedure. For immunoblots, proteins from the gels were transferred electrophoretically to nitrocellulose, and immunoblot analysis was performed according to the procedure of Towbin et al. (23). For the detection of calreticulin, a polyclonal rabbit antiserum was raised against the NH₂-terminal 20 amino acids of the human sequence of calreticulin coupled to KLH. It was used at a dilution of 1:200. Perforin was detected with the mAb CB. 5.4 raised against the recombinant mouse protein, whereas a rabbit polyclonal antiserum (21) was used to reveal granzyme D. The blots were developed with alkaline-phosphatase-coupled secondary antibodies and the 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (Sigma Chemical Co.) detection system. Calreticulin from HL60 cells was purified according to Krause et al. (24).

Degranulation of CTL. 100-mm petri dishes (model 1029; Falcon, Zürich, Switzerland) were coated with rat anti-mouse CD3

mAb 17 A2 (25) by incubating 10 ml of antibody solution (20 µg/ml) per plate in 50 mM Tris-HCl, pH 9.5, for 2 h at 37°C. After incubation, each plate was washed twice with PBS and 10⁷ cells were added per dish. Subsequently, CTL Pb CS F12 were incubated for 4 h at 37°C in dishes coated with solid-phase anti-CD3 mAb, or for control purposes, devoid of activating antibodies.

Protein Analysis of the Cell Supernatant. After 4 h stimulation in serum-free medium as described above, 10⁷ cells were centrifuged at 300 g and cell supernatant was harvested, passed through 0.22-µm filters, dialyzed for 2 h against 20 mM Tris (pH 8) 50 mM NaCl, and finally loaded onto the Mono Q column. Fractions of 1 ml were eluted with a gradient of 50–500 mM NaCl and analyzed by immunoblotting. The amount of secreted granzyme A activity (BLT-esterase) from 10⁵ CTL was measured in 0.1 ml DMEM with 25 mM Hepes in a 96-well microtiter plate using the method described by Pasternack et al. (26). The activity of lactate dehydrogenase, as marker enzyme of the cytosolic compartment, was tested in supernatants of CTL harvested after 4 h incubation of cells in wells as described (27).

Immunofluorescence Microscopy. Cells from mouse CTL clone Cw3 1.1 were plated onto glass slides (pretreated with polylysine at 50 µg/ml for 30 min) 4 d after stimulation. Cells were fixed with 3% paraformaldehyde in PBS for 30 min at room temperature. Unreacted aldehyde groups were quenched by incubation in 50 mM NH₂Cl for 10 min. Cells were then permeabilized by incubation in PBS, 0.2% BSA, and 0.05% saponin (Sigma Chemical Co.) for 2 × 10 min. Perforin was detected by staining with a monoclonal rat anti-mouse perforin antibody CE2.2 conjugated to Texas red (Fluka Chemical Co., Buchs, Switzerland) for 30 min. Calreticulin was detected by incubation with a rabbit anti-human antiserum directed against the 20 NH₂-terminal residues of calreticulin. After extensive washing, bound antibody was visualized using a FITC-conjugated secondary antibody (Amersham Corp., Zürich, Switzerland) at a dilution of 1:1,000. For double labeling, cells were sequentially incubated with the different antibodies. Confocal microscopy was carried out using a light microscope (model 35M; Zeiss, Oberkochen, Germany) equipped with a confocal laser system (model MRC-600; Bio-Rad Laboratories, Zürich, Switzerland). Specimens were observed under epifluorescence illumination with a 488- or 514-nm scanning argon laser beam.

Results

Purification of a Granule-associated 60-kD Protein (grp 60) with NH₂-terminal Identity to Human Calreticulin (Ro/SS-A antigen). Previous studies in our laboratory have shown that the adsorption of solubilized granule-associated proteins to a Mono S cation exchange column allowed to the separation and isolation of the highly basic granzymes (21). In contrast, perforin binds to the anion exchanger Mono Q. While using this column, we repeatedly observed that an additional protein was coeluting with perforin and its lytic activity.

Typically, granules isolated from 2 × 10⁹ human LAK cells were solubilized in 1.5 M NaCl and, after dialysis, subjected to FPLC Mono Q chromatography. The bound proteins were desorbed with a salt gradient, revealing several protein species eluting at low ionic strength. We focused our attention on two predominant proteins consistently coming off at 400 mM NaCl with apparent molecular masses of 60 and 70 kD, respectively (Fig. 1). The 70-kD protein was identified as perforin by immunoblotting (see below). Sev-

¹ Abbreviations used in this paper: ER, endoplasmic reticulum; FPLC, fast protein liquid chromatography; grp, granule-associated protein; HL60, human promyelocytic leukemia 60 cells.

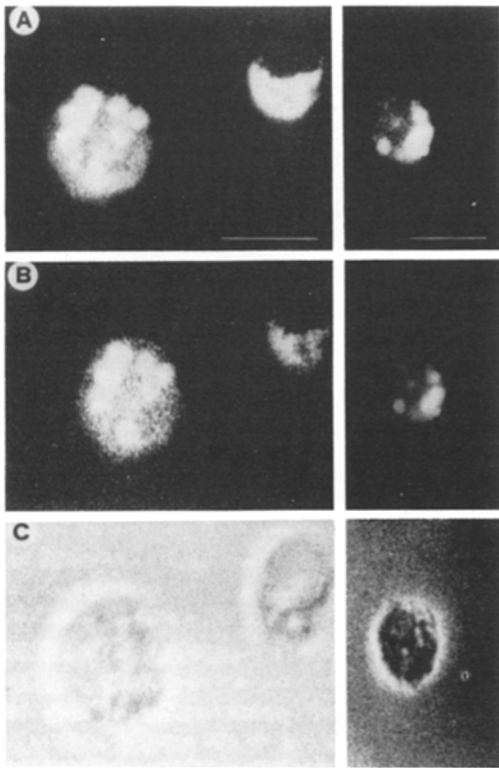


Figure 4. Colocalization of calreticulin with perforin in the cytolytic granules. The murine CTL clone Cw3 1.1 was analyzed using confocal microscopy. CTLs were double-labeled with anticalreticulin and mAb against perforin. Indirect labeling of calreticulin with FITC (A) is similar to the staining of the same cell with antiperforin mAb directly coupled to Texas red (B). (C) Phase contrast imaging of the analyzed cells. Bars, 10 μm .

routing of KDEL proteins and calreticulin in particular has not been investigated. Because of the inherent pitfall of localization studies by ultracentrifugation techniques, it was important to corroborate calreticulin's intracellular distribution by different, independent methods. Using immunofluorescence confocal microscopy, cytolytic granules are easily distinguishable because of their distinct morphology (Fig. 4). When a rat mAb directed against mouse perforin was used, these organelles were clearly stained. Calreticulin was present in the same organelle as detected by confocal double label immunofluorescence.

Release of Calreticulin in the Supernatant upon TCR Complex Occupation. The specific secretion of intragranular proteins in CTL is induced by the occupation of the CTL antigen receptor by the target cell surface antigens or by immobilized anti-TCR complex mAbs (35). Subsequent to these stimuli, granzyme A and B activity can be measured in the supernatant, and granzyme C, D, E, and F antigen can be detected. Degranulation is specific for proteins residing in the cytoplasmic lytic granules and no markers of other organelles are released, thus providing indirect biochemical evidence for the intracellular localization of these molecules. Evidence for the TCR-regulated secretion of calreticulin is provided by the results of the experiment shown in Fig. 5. By immunoblot analysis, calreticulin was detected in incuba-

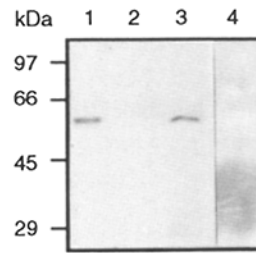


Figure 5. Calreticulin is released upon TCR complex stimulation. The mouse CTL clone Cw3 1.1 was incubated with solid phase 17.A2 anti-CD3 mAbs previously adsorbed onto petri dishes. After 4 h stimulation, the cell supernatant was dialyzed and concentrated by Mono Q chromatography. The fraction eluting at 400 mM salt from stimulated cells (lane 1) or from cells without stimuli (lane 2) were analyzed by immunoblotting. Lane 3 corresponds to purified calreticulin. The efficacy of degranulation after the stimulation was assayed by testing granzyme D release using a polyclonal rabbit anti-granzyme D antiserum (lane 4).

tion medium of CTL which had been stimulated during 4 h with immobilized anti-CD3 mAb (Fig. 5, lane 1), whereas no calreticulin was detected in incubation medium of CTL that were incubated without stimuli (Fig. 5, lane 2). It is interesting that the minor 62-kD form of calreticulin found in isolated granules (see Fig. 1) was never revealed in the releasate. Based on the granzyme A activity recovered in the cell supernatant, 40% of granule proteins were secreted under these conditions, whereas the activity of the cytoplasmic enzyme lactate dehydrogenase, which served as a control to exclude self-lysis of the cells during stimulation, did not increase above background levels (data not shown).

Taken together, the results described in Figs. 1–5 provide evidence for grp 60 being calreticulin, and for its localization to granules of CTL and LAK cells.

Discussion

The major constituents of cytolytic granules so far characterized are proteoglycan molecules of the chondroitin sulfate A type, granzymes, and perforin. Here we describe the isolation of an additional polypeptide with an apparent molecular mass of 60 kD. It is present in granules of murine CTLs and human LAK cells at an apparent concentration equal to that of perforin.

We were able to devise a purification strategy that allowed us to obtain sufficient protein to analyze the first 10 NH_2 -terminal amino acids. The sequence data obtained for the human protein showed its complete identity to the Ro/SS-A antigen, a protein which encodes the human homologue of calreticulin. An antiserum raised against the NH_2 -terminal peptide of human calreticulin reacted with purified human and mouse grp 60. Both lymphocyte-derived grp 60 and calreticulin isolated from HL60 cells revealed the same molecular weight, further corroborating the identity of the two molecules.

Calreticulin is a ubiquitous calcium binding protein. The protein has a highly zonal structure with an approximal neutral (net charge) NH_2 -terminus, forming a globular domain followed by a proline-rich repetitive region and an acidic, Ca^{2+} -binding COOH -terminal domain (28). Calreticulin is a prototype of a protein carrying a COOH -terminal KDEL sequence which is known to act as a retention signal for pro-

teins destined for the lumen of the ER. In lymphocytes, the majority of calreticulin is associated with the lytic granules as shown by immunofluorescence studies. This localization was substantiated by the observation that the occupation of the TCR complex leads to the secretion of not only well-identified granule-specific proteins such as granzyme A, but also of calreticulin.

We can only speculate on the reasons leading to the surprising sorting behavior of calreticulin in lymphocytes. Perhaps the form of calreticulin sorted into granules of CTL may have lost the KDEL sequence by the action of a peptidase. In isolated granules, two immunoreactive proteins, a major product of 60 kD and a minor species of 62 kD, are detected. However, only the 60-kD species is released upon TCR stimulation, raising the possibility that the larger molecules represent ER-associated calreticulin, and that these two forms contain different sorting signals. The presence of a minor 62-kD band has already been observed by Peter et al. (33) in ER-derived preparations from liver cells. Calreticulin may associate with a protein destined for the regulated secretory pathway during biosynthesis. Assuming that calreticulin forms a complex with perforin or granzymes during the biosynthetic pathway, the built-in sorting signal of perforin or granzymes — it has been described that granzymes are sorted via the mannose-6-phosphate receptor (36) — may overrun or mask the KDEL signal, and sorting of calreticulin to the granules would ensue. Alternatively, the KDEL receptor may be present in this lytic, storage organelle. The KDEL signal does not prevent the diffusion of the proteins in the ER or the export from it, but allows escaped proteins to be retrieved from later compartments of the secretory pathway (34). That such a retrieval occurs was demonstrated by the finding that proteins with retention signals undergo carbohydrate modifications that only occur in the Golgi compartments (33, 37). Indeed, most of the receptor is found in a salvage compartment between ER and Golgi (34). Saturation of the KDEL receptor is another possible explanation. The receptor is a minor transmembrane protein (38). During the activation of lymphocytes and granule biogenesis, high quantities of proteins destined for the granules and secretion in general are synthesized (39). Among these, calreticulin's or other KDEL proteins' synthesis may be induced, thereby saturating the capacity of the retrieval system. The excess of molecules may thus be secreted or routed to organelles with high storage capacity. This hypothesis can be tested, since other KDEL proteins should be found in the granules in addition to calreticulin.

The marked domain structure enables calreticulin to exert different functions. Apart from its role as calcium binding molecule, it is known to stabilize the flavin-containing monooxygenase in rabbit lungs by forming a 1:1 complex, thereby decreasing its susceptibility to inactivation by detergents (40). In addition, the synthesis of calreticulin is stress induced (41), and therefore it has been proposed that calreticulin is used as a chaperone-like molecule to facilitate processing or folding of proteins, similarly to heat shock proteins.

Both the calcium-binding and chaperone-like properties of calreticulin could explain and justify its presence in storage

granules of lymphocytes, where it colocalizes with the lytic perforin. Perforin is stored in a nonstable conformation, and the presence of only 100 μM free calcium ions (1, 42) is known to induce a hydrophilic-amphiphilic transition which leads to its insertion into membranes. Moreover, preincubating granules with identical calcium concentrations, before a lysis test, leads to a rapid assembly and inactivation of perforin. It is therefore important for lymphocytes to keep free calcium concentration as low as possible along perforin's biosynthetic pathway to avoid both autolysis of the ER and Golgi and to prevent perforin's inactivation. In the ER of fibroblasts, for instance, the free calcium concentration amounts to only 0.5 μM (43). Most of the remaining ions are supposedly bound to calreticulin and not freely available. Little, however, is known about the Ca^{2+} concentration found in other organelles along the secretory pathway, in particular in lytic granules of lymphocytes, but again calreticulin may fulfil an important role as Ca^{2+} buffer to maintain the lytic activity of perforin.

Akin to the lung monooxygenase, perforin in its monomeric conformation is unstable and highly sensitive to inactivation by detergents (44). Calreticulin may complex to perforin during biosynthesis and storage, thereby keeping the pore-forming protein in its metastable, globular conformation in a chaperone-like manner. The coelution of calreticulin with perforin from the Mono Q column suggests that this may be the case, since the pI of perforin (6.85) and calreticulin (4.1) are quite different.

Previous results demonstrated that calreticulin is identical to the Ro/SS-A antigen. The presence of anti-Ro/SS-A/calreticulin autoantibodies in patient sera is a distinct feature of autoimmune diseases such as systemic lupus erythematosus (40% of cases) and Sjögren's syndrome (70% of cases) (45). These findings were recently challenged by Rokeach et al. (30), who demonstrated that calreticulin is not identical with one of the Ro/SS-A autoantigens, but rather defines a different autoantigen. Autoantibodies to calreticulin, however, have also been clearly demonstrated in patients suffering from onchocerciasis, or river blindness (30). *Onchocerca volvulus*, the causative agent of this disease, is a filarial nematode which causes blindness, sclerosing lymphadenitis, and dermatologic diseases in humans (46). The mechanisms leading to the production of the autoantibodies remain unclear, as does their role in the pathogenesis of the disease. There has been mounting evidence that foreign microbial or parasite-derived antigens may trigger an inappropriate immune response against self-antigens through molecular mimicry (47). It is interesting that calreticulin's sequence shares a 64% overall identity with the RAL-1 antigen, the major antigen of *Onchocerca volvulus* (48). Antibodies generated against the calreticulin-like RAL-1 protein (29) cross-react with calreticulin (30). We propose that the antibody response against the common epitopes will be stimulated and thus maintained upon the release of calreticulin from stimulated lymphocytes. Immune complexes will be formed, leading to an increased inflammatory response. The immunologic consequence of the permanent release of calreticulin from lymphocytes during infection is currently under investigation.

The authors wish to thank Drs. A. Chonn, T. Glaser, and R. Etges for critical reading of the manuscript.

This work was supported by grants from the Swiss National Science and Marie Heim Vögtlin Foundations.

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Received for publication 9 September 1992 and in revised form 13 October 1992.

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