Calreticulin Is Expressed on the Cell Surface of Activated Human Peripheral Blood T Lymphocytes in Association with Major Histocompatibility Complex Class I Molecules*

(Received for publication, November 18, 1998, and in revised form, March 9, 1999)

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Calreticulin is an endoplasmic reticulum resident molecule known to be involved in the folding and assembly of major histocompatibility complex (MHC) class I molecules. In the present study, expression of calreticulin was analyzed in human peripheral blood T lymphocytes. Pulse-chase experiments in [35S]methionine-labeled T cell blasts showed that calreticulin was associated with several proteins in the endoplasmic reticulum and suggested that it was expressed at the cell surface. Indeed, the 60-kDa calreticulin was labeled by cell surface biotinylation and precipitated from the surface of activated T cells together with a protein with an apparent molecular mass of 46 kDa. Cell surface expression of calreticulin by activated T lymphocytes was further confirmed by immunofluorescence and flow cytometry, studies that showed that both CD8⁺ and CD4⁺ T cells expressed calreticulin in the plasma membrane. Low amounts of cell surface calreticulin were detected in resting T lymphocytes. By sequential immunoprecipitation using the conformation independent monoclonal antibody HC-10, we provided evidence that the cell surface 46-kDa protein co-precipitated with calreticulin is unfolded MHC I. These results show for the first time that after T cell activation, significant amounts of calreticulin are expressed on the T cell surface, where they are found in physical association with a pool of β₂m-free MHC class I molecules.

Calreticulin is a highly conserved and widely tissue distributed calcium-binding protein with a C-terminal KDEL endoplasmic reticulum (ER) retrieval sequence (1–3). However, calreticulin has also been found outside the ER, such as within the secretory granules of cytotoxic lymphocytes, the cell surface of melanoma cells and virus-infected fibroblasts, and the cytosol and nucleus of several cell types (Ref. 4–8, reviewed in Ref. 9). Given its lectin-like properties, calreticulin is considered to be an ER chaperone involved in the assembly and folding of nascent glycoproteins (10–13).

Mature MHC class I molecules are composites of a 44–49-kDa polymorphic heavy chain and a 12-kDa light chain (β₂m) complexed with a cytosolic-processed peptide that are expressed on the plasma membrane of almost every nucleated cell (14). A number of ER resident molecular chaperones, such as calnexin, BiP, and transporter-associated protein, are involved in the assembly of the different composites (15–18). Recently, calreticulin has also been shown to function as a chaperone in the assembly and folding of MHC class I molecules in the ER (19–22). Contrary to calnexin, calreticulin binds to MHC class I-β₂m dimers and to transporter-associated protein via another chaperone, tapasin (19, 21). After peptide loading and deglycosylation of N-linked glycans, calreticulin dissociates from the MHC class I-β₂m dimers, thus allowing the final transport of mature MHC class I molecules to the cell surface (20).

Recent studies on the biosynthesis of TcR-CD3 complexes suggest that some chaperones such as calnexin can escape the ER retention mechanisms and be expressed on the cell surface of immature thymocytes in association with TcRβ chains (23–27). On the contrary, studies on the biosynthesis of MHC class I molecules have never reported associations of ER chaperones with MHC class I molecules outside the ER and Golgi compartments (19–22, 28–30). Given that calreticulin displays multiple functions and has long been associated with several chronic diseases (31, 32), we undertook a study of calreticulin expression on human peripheral blood T lymphocytes to gain new insights into possible biological functions of this molecular chaperone. In the present study, we report the finding that calreticulin is expressed at the cell surface of activated human peripheral blood T lymphocytes, where it is physically associated with a pool of unfolded MHC class I molecules.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Fresh human peripheral blood mononuclear cells were obtained from buffy coats of regular healthy blood donors after centrifugation over Lymphoprep (Nycomed, Norway). Partially purified resting peripheral blood T lymphocytes (usually >80%) were obtained by conventional adherence techniques and referred to as PBLs as described previously (33). T cell blasts were obtained after stimulation of PBLs in RPMI complete media (1% fetal calf serum, 1% penicillin/streptomycin, 1% glutamine, 25 mM HEPES) with 5 μg/ml phytohemagglutinin (Sigma). After 5 days in an incubator at 37 °C, 5% CO₂, and 99% humidity, T cell blasts (usually >95% CD3⁺ cells) were processed for subsequent studies.

Antibodies—The following antibodies were used in the present study. W6/32 (Dakopatts, Copenhagen, Denmark) is a mouse monoclonal Ab that recognizes a monomorphic epitope on all HLA heavy chains, dependent on the presence of β₂m (34). PA3–900 (Affinity Bioreagents, Golden, CO) is a polyclonal rabbit serum that recognizes the molecular
chaperone calreticulin (35). HC-10 is a mouse mononclonal Ab that reacts preferentially with unfolded HLA B and C heavy chains not associated with \( \beta_2 \)-m (36). Anti-C1q-R Ab is a rabbit polyclonal serum that recognizes the C1q receptor (37). A rabbit serum against the intracellular tyrosine kinase FYN was a gift of Dr. Chris E. Rudd (Dana Farber Cancer Institute, Boston, MA). A rabbit serum against the intracellular tyrosine phosphate SHP-1 was a gift of Dr. R. J. Matthews (Howard Hughes Medical Institute, St. Louis, MO). Mouse anti-human CD6-P-E and CD4-Cy5PE and swine anti-rabbit fluorescein isothiocyanate (F(ab\(^\prime\))\_2 fraction) were from Dakopatts.

**Cell Staining and Immunofluorescence**—Staining steps of resting and activated T cells for flow cytometry studies were performed at 4°C for 30 min in staining solution (PBS, 0.2% bovine serum albumin, 0.1% NaN\(_3\)) in round-bottom microtiter plates (Greiner, Nürtingen, Germany) with \( \approx 0.5 \times 10^6 \) cells/well as described previously (38). After staining, the cells were washed three times in staining solution and analyzed in a FACScan (Becton and Dickinson, Mountain View, CA).

For immunofluorescence studies, cytopsins of resting and activated T cells (100,000 cells/cytopsin) were air-dried and fixed with 3.7% formaldehyde. Cells were blocked for 1 h at room temperature with 5% nonfat dry milk in PBS. Cytopsins were incubated for 1 h with PA3–900 (1:100 dilution) followed by fluorescein isothiocyanate-conjugated secondary Ab (1:20 dilution) for 1 additional h. Preparations were mounted with Vectastain H1000 mounting media (Vector Laboratories, USA).

**Cell Labeling, Cell Lysis, and Immunoprecipitation**—For cell surface biotinylation, resting or activated T cells (10 \(^6\) cells/ml) were incubated with 0.5 mg/ml sulfo-succinimidyl 6-biotinamido-hexanoate (EZ-Link\(^\text{TM}\) Sulfo-NHS-LC-biotin) (Pierce) in PBS for 10 min at room temperature followed by four washes in PBS. For metabolic labeling, activated T cells were cultured in methionine-deficient media (Sigma) for 1 h and pulse-labeled with 100 \( \mu \)Ci of \([^{35}\text{S}]\)methionine (Amersham Pharmacia Biotech) for 15 min. Cells were chased at different times, as indicated in the results, after removing the labeling media and adding complete RPMI media. Labeled cells were lysed in lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1% Brij for 30 min on ice. In some experiments, Triton X-100 was used in the lysis buffer (see Fig. 2B legend). The lysates were centrifuged at 12,000 \( \times g \) to remove cell debris and precleared for 30 min by end-over-end rotation with protein-A-Sepharose CL-4B beads (Amersham Pharmacia Biotech). Precleared detergent lysates corresponding to 10 \( \times 10^6 \) cells, unless otherwise indicated, were immunoprecipitated with the proper antibodies and protein-A-Sepharose CL-4B beads for 2 h at 4°C by end-over-end rotation. In some experiments, biotinylation of whole cell lysates was performed as follows. Resting or activated T cells were lysed in lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1% Brij) for 30 min on ice and processed as above. Precleared detergent lysates were incubated with 0.5 mg/ml of Link\™ Sulfo-NHS-LC-biotin (Pierce) in PBS for 10 min at room temperature followed by centrifugation at 12,000 \( \times g \). Supernatants were used for subsequent immunoprecipitations as described above. Washed immunoprecipitates were boiled for 5 min in 1% SDS sample buffer and resolved by SDS/PAGE. Polyacrylamide gels from \([^{35}\text{S}]\)-labeled samples were fixed and processed for autoradiography using Biomax MR-1 films (Eastman Kodak Co.). For re-purification experiments, the primary immunoprecipitates were boiled for 5 min in 2% SDS and diluted 10-fold with lysis buffer. The beads were spun down, and the supernatants were recovered and precleared for 30 min with protein A-Sepharose CL-4B beads. Proteins were precipitated with the proper Abs plus protein-A-Sepharose CL-4B beads overnight. Immunoprecipitates were washed three times, boiled for 5 min in 1% SDS buffer, and resolved by SDS/PAGE.

**Western Blots**—Polyacrylamide gels from biotin-labeled samples were electroblotted to nitrocellulose membranes (Hybond C super, Amersham Pharmacia Biotech) and blocked with 5% nonfat dry milk in Tris-buffered saline, 0.1% Tween. Proteins were visualized after incubation with ExtrAvidin-conjugated horseradish peroxidase (Sigma) and exposure to Biomax MR-1 film using enhanced chemiluminescence (ECL or ECL+, Amersham Pharmacia Biotech). For immunodetection of MHC class I heavy chains in blocked nitrocellulose filters, the HC-10 Ab was used, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (Transduction Laboratories, Lexington, UK).

**RESULTS**

Activated human PBLs were metabolically labeled with \([^{35}\text{S}]\)methionine for 15 min and chased in methionine-supplemented media at different times. Brij 96 detergent lysates were immunoprecipitated with anti-calreticulin Abs, and the immunoprecipitates were analyzed by SDS/PAGE. Numerous ER proteins co-precipitated with the 60-kDa calreticulin at time 0 after the 15-min pulse (Fig. 1). Among those, several have been previously identified as transporter-associated protein (70–72 kDa), tapasin (48 kDa), and MHC class I (46 kDa) (21). Additional bands corresponding to unidentified proteins of ~97, 50, and 42 kDa were also visible. Interestingly, most of the pulse-labeled proteins, including calreticulin, rapidly disappeared 1 h after the initial pulse, with the marked exception of the 46-kDa band. Thus, 4 h after the pulse, although barely detectable labeled calreticulin was observed, a strong 46-kDa labeled protein was still co-precipitated by anti-calreticulin Abs (Fig. 1). Four h after the initial pulse, all proteins associated with calreticulin that are targeted to the cell surface would have reached the plasma membrane unless they were endocytosed and/or degraded. Among these, the survival of surface MHC class I molecules in activated human T cells is known to be increased (39). Therefore, we suspected that newly synthesized “unlabeled” calreticulin was present in the cell surface of activated T cells in association with the mature 46-kDa MHC class I heavy chain.

To address this question, resting and activated human PBLs were cell surface-labeled with biotin, lysed in Brij 96, and immunoprecipitated with the PA3–900 and W6/32 Abs. PA3–900 immunoprecipitated the 60-kDa calreticulin from lysates of biotinylated resting and activated PBLs (Fig. 2A). The amount of precipitated calreticulin was, however, approximately 3-fold higher in activated PBLs. To note, a protein of 46 kDa similar to the one immunoprecipitated by W6/32 Abs, which recognize mature \( \beta_2 \)-m-associated MHC I, was again co-precipitated with calreticulin in activated but not resting PBLs (Fig. 2A). These results provided evidence that the ER resident chaperone calreticulin was expressed in the cell surface of human PBLs, that its expression increased after T cell activation, and that it was associated with a 46-kDa protein corresponding to the mobility of the MHC I heavy chain. The use of more stringent conditions during the cell lysis procedure (i.e. 2% Triton X-100), although decreasing markedly the amount of the co-precipitated 46-kDa protein, did not completely disrupt the association (Fig. 2B). The observed difference between detergents did not reflect less

**FIG. 1. Co-precipitation of calreticulin and a 46-kDa protein from lysates of \([^{35}\text{S}]\)methionine-labeled activated human PBLs.** Five-day-activated human PBLs (40 \( \times 10^6\) cells) were metabolically labeled for 15 min with 100 \( \mu \)Ci of \([^{35}\text{S}]\)methionine and chased for the periods indicated in hours. At each time point, 10^7 cells were solubilized in 1% Brij 96 lysis buffer, and lysates were immunoprecipitated with PA3–900. Precipitates were boiled in 1% SDS sample buffer, and aliquots were separated on 12% SDS, PAGE under denaturing conditions. Gels were dried and exposed to Biowax MR-1 film for 7 days. Calreticulin (CRT) and the co-precipitated 46-kDa protein band are indicated by arrows. Molar mass markers in kDa are indicated on the left.
protein extraction by Triton X-100, as both detergents extracted similar amounts of calreticulin (PA3–900 Ab) and MHC I heavy chain (W6/32 Ab). These results suggest that this association might be of physiological importance and rules out the possibility that the co-precipitation of the 46-kDa protein with the PA3–900 Ab is the consequence of an artifact resulting from cross-reactivity. It should be emphasized that there was some variation in the amount of the 46-kDa protein co-precipitated with calreticulin between the individuals studied. Calreticulin or calreticulin-like proteins have been described in almost every compartment of the cell. Recent reports have shown that human T cells express receptors for the C1q protein and that one of those receptors (cC1q-R) shows homology with calreticulin (40, 41). To rule out the possibility that our PA3–900 antibody was recognizing the cC1q receptor, we performed depletion experiments. As shown in Fig. 3, depletion of T cell surface calreticulin by four rounds of immunoprecipitation with the PA3–900 Ab did not abolish immunoprecipitation of the cC1q receptor. These data provide evidence that the calreticulin expressed in the surface of activated human T lymphocytes is apparently different from the C1q receptor. Next, we wanted to rule out the possibility that the calreticulin we were detecting on the cell surface of human PBLs could be result of intracellular biotinylation because of background labeling during the handling of the cells. Using activated human PBLs, immunoprecipitation of Brij 96 cell lysates from cell surface-biotinylated cells allowed the detection of cell surface-expressed molecules, such as calreticulin, but not of intracellular molecules such as the protein-tyrosine kinase FYN and the protein-tyrosine phosphatase SHP-1 (Fig. 4, left panel). On the contrary, immunoprecipitation of biotinylated Brij 96 cell lysates allowed the detection of both sets of molecules, together with a number of co-precipitated proteins (Fig. 4, right panel). Interestingly, antibodies against the intracellular tyrosine kinase FYN were able to co-precipitate a strong band of similar mobility to the MHC class I heavy chain (Fig. 4, right panel).

To further characterize calreticulin expression on human PBLs, immunofluorescence microscopy and flow cytometry were performed. For that purpose, cytopsin of resting and activated human PBLs were stained with PA3–900 followed by a Fab′/2 fraction of swine anti-rabbit fluorescein isothiocyanate conjugated Ab to avoid nonspecific binding to Fc receptors in activated T cells. The results demonstrated a faint cell surface expression of calreticulin in resting PBLs that markedly increased in activated T cells (Fig. 5A), which is in agreement with the immunoprecipitation results (Fig. 2). Permeabilized PBLs, either resting or activated, showed a conspicuous cytoplasmic staining (data not shown). To determine whether calreticulin was differentially expressed by the two major peripheral blood T cell subsets, two color flow cytometry studies were performed. The results showed that both CD8+ and CD4+ activated T cells expressed calreticulin at similar levels (Fig. 5B).

Finally, we wanted to confirm the identity of the 46-kDa protein co-precipitated with calreticulin. Because the W6/32 Ab was unable to co-precipitate a protein of similar mobility with calreticulin, we decided to include in our studies the conformation-independent Ab HC-10, which detects β2m-free MHC class I molecules. As shown in Fig. 6A, calreticulin Abs were able again to co-precipitate low but significant amounts of a protein with 46 kDa. Re-precipitation of the primary immunoprecipitates with HC-10 demonstrated that the 46-kDa protein co-precipitating with calreticulin was indeed the MHC class I heavy chain (Fig. 6B). Immunodetection using the HC-10 Ab further confirmed that the 46-kDa band co-precipitating with
calreticulin corresponds to the MHC class I heavy chain (data not shown). On the other hand, HC-10 but not W6/32 Abs were able to co-precipitate a protein of the same mobility as calreticulin from the surface of activated human PBLs (Fig. 6A). Re-precipitation of the primary immunoprecipitates with the PA3–900 Ab confirmed that calreticulin could be re-precipitated from HC-10 but not W6/32 primary immunoprecipitates (Fig. 6C), therefore demonstrating that calreticulin is associated with a pool of unfolded β₂m-free MHC class I molecules on the cell surface of activated human PBLs.

**DISCUSSION**

Recent studies on calreticulin are disclosing the key role played by this molecular chaperone in the folding and assembly of MHC class I molecules in the ER (19–22). However, a number of studies indicate that calreticulin is involved in a number of biochemical and physiological functions unrelated with its ER chaperone role. These studies have shown that calreticulin can be found in different locations within the cell, from the nucleus to the plasma membrane of several cell lines (reviewed in Ref. 31). How calreticulin can escape the ER retention mechanisms and move into the cytoplasm, nucleus, or plasma membrane is an enigma that challenges current biology. In addition, calreticulin is thought to be involved in a number of diseases, all sharing an immunological basis (32, 42). All these findings have led to the suggestion that calreticulin may represent a family of highly homologous proteins.

The present study addressed the characterization of calreticulin expression in human peripheral blood T lymphocytes. Our interest in studying calreticulin relies 1) on our previous work demonstrating that T lymphocytes and classical MHC class I molecules play a regulatory role in tissue damage under...
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excessive iron accumulation caused by a primary defect in an MHC class I like molecule (reviewed in Ref. 43) and 2) that calreticulin has also been described as an iron-binding protein (44). The few studies of calreticulin in human T lymphocytes have shown that the levels of calreticulin mRNA and protein increase after mitogenic stimulation and that calreticulin is a constituent of lytic granules in lymphokine-activated killer cells (4, 45). The present results extend these studies by showing that calreticulin is expressed on the plasma membrane of peripheral blood human T lymphocytes and that cell surface expression increases after T cell activation.

Previous studies have shown that calreticulin or calreticulin-like molecules are expressed on the plasma membrane of transformed cell lines of different origin. These studies raised the question of how calreticulin can be expressed at the cell surface because it lacks a transmembrane domain. A likely explanation is that cell surface calreticulin is anchored to other(s) surface molecules (46, 47). By cell surface biotinylation and immunoprecipitation, we have provided evidence that calreticulin co-precipitates with several T cell surface molecules, and we have identified MHC class I molecules as one of these T cell surface molecules. Escape of ER resident molecular chaperones to the plasma membrane has previously been reported in several cell types including thymic lymphoma cells (24, 27, 31, 42). It has been argued that this may result from changes in the relative availability of KDEL receptors present in post-ER compartments and known to be involved in the retrograde transport of proteins containing this C-terminal retrieval sequence (48–50). Because we have detected calreticulin on the surface of normal human T lymphocytes, it is not unlikely to anticipate that other KDEL-containing ER chaperones such as BiP and GRP94 could also be expressed at the T cell surface as well.

The association of calreticulin with MHC class I molecules on the surface of activated human T cells is intriguing considering the properties displayed by these polymorphic molecules. T cell surface MHC class I molecules are subjected to spontaneous cycles of endocytosis/re-expression via coated pits (39, 51–53). Moreover, endocytosed β₂m-associated MHC class I molecules are re-expressed at the cell surface as β₂m-free unfolded MHC class I molecules (54, 55). On the other hand, recent studies have shown that calreticulin preferentially associates with unfolded MHC class I molecules in the ER and that the oligosaccharide moiety in the α1 domain and a residue within the α3 domain are critical for interaction with calreticulin (29, 30).

Considering that the calreticulin-MHC I association was detected in activated but not resting PBLs, it is interesting to notice that the increase in the translocation of ER calreticulin to the plasma membrane observed in activated human PBLs parallels the increase in the amount of unfolded MHC class I molecules that arise after T cell activation (Fig. 6A and data not shown). Therefore, if under T cell activation large amounts of calreticulin escape the ER retention mechanisms and are translocated to the cell surface, interaction with unfolded MHC class I molecules would be expected. Indeed, we have shown that T cell surface calreticulin is associated with unfolded but not folded MHC class I molecules, as determined using the HC-10 Ab. This is in agreement with previous studies showing that W6/32 Abs do not co-precipitate calreticulin in the ER (19) and with the fact that large amounts of unfolded MHC class I molecules can be precipitated from the surface of activated human PBLs (Fig. 6 and data not shown). The low amount of calreticulin co-precipitated with the HC-10 Ab observed in this study may reflect the preferential reactivity of the HC-10 Ab with unfolded MHC class I molecules of the B and C locus (36). Consequently, any possible calreticulin associated with unfolded MHC class I molecules of the A locus would not be detected. This is important, taking into consideration that in the ER, calreticulin appears to show different patterns of association with MHC class I molecules, associating strongly with alleles of the A locus, such as A29 and A3, but weakly with alleles of the B locus (19).

The similarities between the behavior of the calreticulin-MHC class I association in the ER and the T cell surface of activated human T cells raise intriguing questions regarding the possible physiological significance of this association. Although in the ER the association appears to be related with the folding and assembly of immature MHC class I molecules, in the plasma membrane it could be related to an important previously uncharacterized biological function related to the proliferative status of the cells. In this context, the present findings could be of particular relevance to the recent demonstration that calreticulin-bound peptides obtained from tumors can elicit a peptide-specific CD8+ T cell response (56). Calreticulin has also been described as an Fe²⁺-binding protein that is found in endocytic vesicles containing iron-transferrin/transferrin receptor complexes, where it becomes labeled with iron released from transferrin (57). On the other hand, surface MHC class I molecules are known to regulate endocytosis of several receptors, such as the transferrin receptor, a finding consistent with earlier studies demonstrating that MHC class I molecules and transferrin receptors co-localize in endocytic vesicles in T lymphocytes (58, 59). In this context, it is tempting to suggest that T cell surface calreticulin, by means of its association with MHC class I molecules, may be an intermediary in the process of iron uptake and transfer to intracellular compartments in dividing T lymphocytes, a hypothesis that can be easily tested. The recent demonstration that the hemochromatosis gene product, an MHC class I-like protein, associates with the transferrin receptor must be taken into consideration (60–62). Alternatively, T cell surface calreticulin may also perform a chaperone function by mediating the refolding of unfolded MHC class I molecules that arise as a result of T cell division (63), a function that may uncover a new role for calreticulin in protein quality control.

Acknowledgments—We thank the personnel of the Blood Bank of the Hospital Geral Santo Antônio in Porto for their cooperation and support in collecting blood samples. We thank Dr. Hidde Ploegh (Harvard Medical School, Boston, MA) and Dr. Bob Sim (Oxford University, Oxford) for providing the HC-10 and Cd4-H antibodies, respectively. We also thank Dr. Robert A. Clark (University of Texas) for helpful comments during the revision of this manuscript.

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