

Misfolding of Major Histocompatibility Complex Class I Molecules in Activated T Cells Allows *cis*-Interactions with Receptors and Signaling Molecules and Is Associated with Tyrosine Phosphorylation*

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Knowledge of the origin and biochemical status of β_2 -microglobulin-free or misfolded major histocompatibility complex (MHC)-I molecules is essential for understanding their pleiotropic properties. Here we show that in normal human T cells, misfolding of MHC-I molecules is turned on upon activation and cell division and is proportional to the level of proliferation. Immunoprecipitation showed that a number of proteins are associated with MHC-I heavy chains at the surface of activated T cells, including the CD8 $\alpha\beta$ receptor and the chaperone tandem calreticulin/ERp57, associations that rely upon the existence of a pool of HC-10-reactive molecules. Biochemical analysis showed that misfolded MHC-I molecules present at the cell surface are fully glycosylated mature molecules. Importantly, misfolded MHC-I molecules are tyrosine phosphorylated and are associated with kinase activity. *In vitro* kinase assays followed by reprecipitation indicated that tyrosine phosphorylation of the class I heavy chain is probably mediated by a Src tyrosine kinase because Lck was found associated with HC-10 immunocomplexes. Finally, we show that inhibition of tyrosine phosphorylation by using the Src-family tyrosine kinase inhibitor PP2 resulted in enhanced release of MHC-I heavy chains from the cell surface of activated T cells and a slight down-regulation of cell surface W6/32-reactive molecules. This study provides new insights into the biology of MHC-I molecules and suggests that tyrosine phosphorylation may be involved in the regulation of MHC-I misfolding and expression.

Major histocompatibility complex (MHC)¹ class I molecules are composites of a 44–49-kDa heavy chain, a 12-kDa light

chain (β_2m), and a 9–11 amino acid peptide (1, 2). These composites are assembled and folded in the endoplasmic reticulum with the involvement of a growing number of chaperones (3). Once the complex is properly assembled and folded it follows the exocytic pathway to the cell surface. MHC-I molecules that fail to fold properly are retro-translocated into the cytosol and degraded (4). In addition to their well known role in presenting peptides to CD8⁺ T cells, MHC-I molecules are also involved in the regulation of other biological processes in a number of cell types, including T cells. Early reports using soluble antibodies against MHC-I molecules showed inhibition of T cell proliferation induced by mitogens (5–8). In contrast, immobilized or cross-linked antibodies induced optimal proliferation of stimulated human T cells (9–11). Subsequent studies demonstrated that ligation of surface MHC-I molecules influenced early as well as late activation events including tyrosine phosphorylation, cell adhesion, and apoptosis (12–15).

The molecular mechanisms whereby MHC-I molecules are involved in the regulation of intracellular signals are uncertain. Studies by Lipsky and co-workers (16) suggested that the cytoplasmic domain of MHC-I molecules is not needed for signal transduction. At the same time, two interesting studies reported that activated human T cells and lymphoblastoid cell lines expressed MHC-I heavy chains not associated with β_2m or misfolded (17, 18). Based on earlier studies demonstrating the physical association between several cell surface receptors endowed with signaling machinery and MHC-I molecules (19–22), some authors suggested that associations between misfolded MHC-I molecules and cell surface receptors were the basis for the regulation of T cell activation-related events by MHC-I molecules (17, 23). One early study demonstrated that clustering of folded MHC-class I molecules at the cell surface was correlated with the appearance of free heavy chains (24), and a recent report implicates the clustering of class I molecules in the efficiency of recognition by specific cytotoxic T lymphocytes (25), thus linking the presence of misfolded class I molecules at the cell surface with the control of T cell activation. Expression of misfolded MHC-I molecules at the cell surface of activated T cells has been shown to depend on endocytosis of folded MHC-I conformers (26). Moreover, the cytoplasmic domain of MHC class I molecules contains a tyrosine motif that is involved in endocytosis and is essential for Nef-induced down-modulation of class I molecules (27, 28). Interestingly, expression of misfolded MHC-I molecules at the cell surface of activated T cells in association with the chaperone calreticulin has been reported suggesting that plasma

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¹ The abbreviations used are: MHC, major histocompatibility complex; β_2m , β_2 -microglobulin; HIV, human immunodeficiency virus; PBL, peripheral blood T lymphocytes; CFSE, 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester; mAb, monoclonal antibody; HRP, horseradish peroxidase; Endo-H, endoglycosidase H; Gly-F, *N*-glycanase F.

membrane MHC-I molecules may be subjected to quality control mechanisms (29).

Despite the above studies, data addressing biochemical aspects of the MHC-I *cis*-associations and their relationship with the misfolding process in normal human T cells are scarce. This is a crucial issue because of studies suggesting: (i) that MHC-I misfolding and involvement in signaling could be associated with trafficking between the plasma membrane and endosomes (26, 30–32) and (ii) that a conserved tyrosine in the cytoplasmic domain of MHC-I molecules seems to play a crucial role in MHC-I endocytosis under certain pathological situations such as during HIV infection (33, 34). In this study we present data linking the unfolding of cell surface MHC class I molecules with undergoing phosphorylation and their association with other polypeptides.

MATERIALS AND METHODS

Cells and Reagents—Human peripheral blood mononuclear cells were obtained from buffy coats after centrifugation over Lymphoprep (Nycomed, Oslo, Norway). Partially purified peripheral blood T lymphocytes were obtained by conventional adherence techniques and referred to as PBL. Activated T cells were obtained after stimulation of PBL in RPMI media (1% fetal calf serum, 1% penicillin/streptomycin, 1% glutamine) with 5 μ g/ml of phytohemagglutinin. After 1 to 5 days in an incubator at 37 °C, 5% CO₂, and 99% humidity, activated T cells were processed for subsequent studies. Phytohemagglutinin, antibiotic/antimycotic solution, and Brij 96 were obtained from Sigma-Aldrich. RPMI 1640, Hanks' balanced salt solution, and fetal calf serum were from Invitrogen. Methyl[³H]thymidine ([³H]TdR) was purchased from Amersham Biosciences and 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) was purchased from Molecular Probes (Amsterdam, The Netherlands). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine, a Src-family selective tyrosine kinase inhibitor also known as PP2, was from Calbiochem.

Antibodies—The following antibodies were used in this study: W6/32 (Dakopatts, Copenhagen, Denmark) is a mouse mAb that recognizes a monomorphic epitope on all HLA heavy chains, dependent on the presence of β_2 m (35). HC-10 (a gift from Dr. Hidde Ploegh, Harvard Medical School, Boston, MA) is a mouse mAb that reacts preferentially with misfolded HLA-B and -C heavy chains not associated with β_2 m (36). 19Thy5D7, 21Thy2D3 and 2ST85H7 (a gift from Dr. Chris Rudd, Imperial College, London, UK) are mouse mAbs against the extracellular domains of CD4, CD8 α , and CD8 β , respectively (37). SPA-891 (Stressgen Biotechnologies Corp., London, UK) is a mouse mAb that reacts with protein disulfide isomerase. SPA-600 (Stressgen Biotechnologies Corp.) is a rabbit polyclonal Ab that recognizes calreticulin. Poly57 is a rabbit polyclonal Ab that reacts with Erp57 (a gift from Neil Bulleid, University of Manchester, UK). 4G10 (a gift from Paul Crocker, University of Dundee, UK) is a mouse mAb that recognizes phosphorylated tyrosine residues. Goat anti-mouse HRP-conjugated IgG were from Molecular Probes (Leiden, The Netherlands). Fluorochrome conjugated secondary antibodies (from mouse, rabbit, and swine) were from Dakopatts. Rabbit sera against the intracellular tyrosine kinase Lck were a gift from Dr. Chris Rudd (37). Polyclonal anti-ZAP-70 was from Santa Cruz Biotechnology (Santa Cruz, CA).

Flow Cytometry—Staining steps of resting and activated T cells for flow cytometry studies were performed at 4 °C for 30 min in staining solution (phosphate-buffered saline, 0.2% bovine serum albumin, 0.1% NaN₃) in round-bottom microtiter plates (Greiner, Nürtingen, Germany) with $\sim 0.5 \times 10^6$ cells/well. First, cells were labeled with HC-10, calreticulin, and Erp57 antibodies followed by F(ab')₂ fragments of rabbit anti-mouse (HC-10) or swine anti-rabbit fluorescein isothiocyanate (calreticulin and Erp57)-conjugated antibodies. Irrelevant mouse and rabbit antibodies were used as negative controls to define background staining. Cells were then counterstained with anti-TCR $\alpha\beta$ -RPE antibodies (Caltag Laboratories, BioAtlântico, Portugal), washed three times, and immediately acquired without fixation in a FACScalibur (BD Biosciences). For each sample 10,000–20,000 viable lymphocytes were acquired using forward scatter/side scatter characteristics and analyzed using CellQuest software.

Determination of T Cell Activation, Transformation, and Division—T cell activation and proliferation were studied by two methods: (i) thymidine uptake and (ii) CFSE fluorescence loss. For thymidine uptake, 0.5 μ Ci of [³H]TdR (specific activity 5.0 Ci/mmol, Amersham-Pharmacia Biotech, England) was added 4 h prior the end of the culture and cells

harvested on glass fiber filters (Filter MAT, Skatron Instruments, Suffolk, UK), using a semiautomatic cell harvester (Skatron, Norway). The incorporated [³H]TdR was measured in a Beckman liquid scintillation counter, and results were expressed as counts per minute (cpm). For CFSE fluorescence loss PBL were first labeled with CFSE prior to stimulation, and rounds of cell division were determined by sequential halving of CFSE fluorescence intensity as described (38).

Cell Labeling and Lysis—For cell surface biotinylation resting or activated PBLs were incubated with 0.25 mg/ml of EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in phosphate-buffered saline for 10 min at room temperature followed by four washes in phosphate-buffered saline. After washing, 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 experiments where phosphorylation was studied a 1:100 dilution of a phosphatase inhibitor mixture (Sigma) was added during the lysis procedure. The lysates were centrifuged at 10,000 $\times g$ to remove cell debris and precleared for 1 h with protein A-Sepharose (Amersham Biosciences).

Immunoprecipitation and Enzymatic Treatment—Precleared lysates were immunoprecipitated with the proper antibodies followed by Sepharose beads for 2 h at 4 °C by end-over rotation. Washed immunoprecipitates were boiled for 5 min in 2 \times SDS buffer and resolved by SDS-PAGE. Immunoprecipitates to be treated enzymatically were boiled for 3 min in lysis buffer containing 0.1% SDS, cooled on ice for 2 min, and incubated for 2 h at 37 °C with 1 μ l of *N*-glycosidase F or endoglycosidase H (Roche Applied Science). The reaction was stopped by the addition of non-reducing sample buffer. For reprecipitation experiments, the beads containing the primary immunoprecipitates were boiled for 5 min in 2% SDS and diluted 8-fold with lysis buffer. The beads were spun down; the supernatants were recovered and cleared for 1 h with protein A-Sepharose beads. Proteins of interest were immunoprecipitated with the proper antibodies followed by Sepharose beads overnight. Immunoprecipitates were washed three times, boiled for 5 min in sample buffer, and resolved by SDS-PAGE. For immunoprecipitation of MHC-I molecules from the culture supernatants, 1 mM phenylmethylsulfonyl fluoride was added and supernatants centrifuged at 500 $\times g$ for 15 min. Supernatants were precleared with Sepharose beads for 2 h at 4 °C followed by another centrifugation. Immunoprecipitation with W6/32 and HC-10 antibodies followed by Sepharose beads was performed overnight at 4 °C by end-over rotation. Immunoprecipitates were washed three times, boiled for 5 min in 2 \times SDS, and resolved by SDS-PAGE.

In Vitro Kinase Assays—For immunocomplex kinase assays, washed immunoprecipitates were washed two additional times in Brij 96 assay buffer (25 mM HEPES, pH 7.5, 0.1% Brij 96). Brij assay buffer (30 μ l) containing 10 mM MnCl₂ and 5 μ Ci of [γ -³²P]ATP was added to the dried beads, and *in vitro* kinase reactions were allowed to occur for 10 min at room temperature. Reactions were stopped by the addition of 30 μ l of 2 \times SDS buffer. The samples were boiled for 5 min, and the products of the reaction were resolved by SDS-PAGE. Gels were either blotted onto nitrocellulose filters or dried directly and were exposed to Kodak Bio-max MR-1 films (Sigma-Aldrich, Madrid, Spain).

Western Blots and Immunodetection—Polyacrylamide gels from biotin-labeled samples were electroblotted to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), blocked with 5% nonfat dry milk in TBS-T Tris-buffered saline (0.1%) Tween 20 and incubated for 1 h with a 1:7500 dilution in TBS-T of Extravidin-conjugated horseradish peroxidase (Sigma). Proteins were visualized using SuperSignal West Pico (Perbio). For immunodetection of MHC class I heavy chains the HC-10 antibody was used. For immunodetection of phosphotyrosine the membranes were blocked with 2% bovine serum albumin in TBS-T and incubated with 4G10 antibody (as culture supernatant) or the PY-Plus antibody mixture (clones PY-7E1 and PY20, Zymed Laboratories, Inc.). The membranes were then incubated with HRP-conjugated goat anti-mouse antibodies (Molecular Probes), and visualization was performed using chemoluminescence, as described above.

RESULTS

Conformational Changes of Cell Surface MHC-I Molecules on Human T Cells upon Activation—Previous results have shown that activated human T cells and transformed T cell lines display an increase in the proportion of misfolded MHC class I molecules present at the cell surface. However, this biological event has not been fully characterized on normal human T cells. Human PBL were activated for 5 days and at time points after activation T cells were harvested, cell surface-labeled

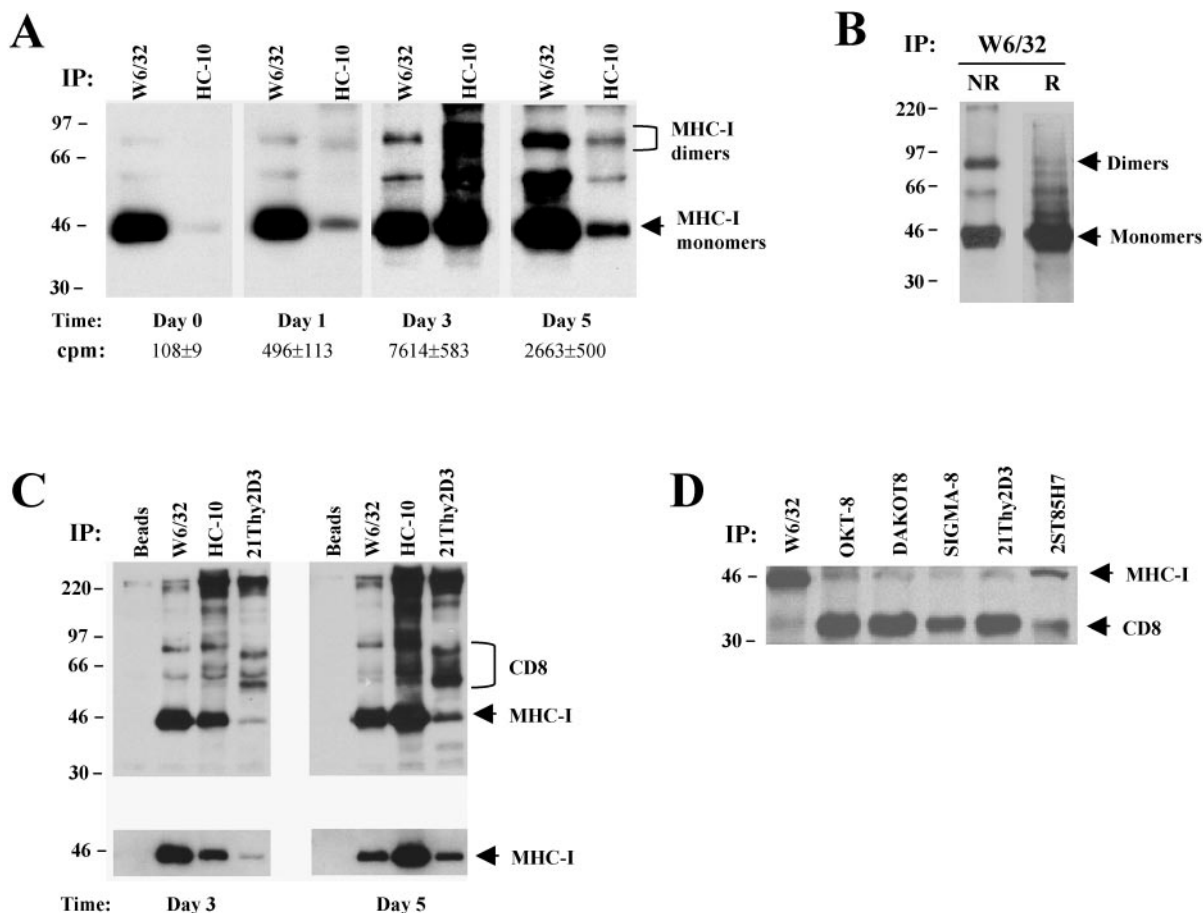


FIG. 1. Kinetics of MHC class I expression at the cell surface of activated human PBL. *A*, resting and one-, three-, and five-day activated human T cells were cell surface biotinylated, lysed in 1% Brij 96, and immunoprecipitated with W6/32 and HC-10 antibodies. Samples were resolved in 10% SDS-PAGE gels under non-reducing conditions and blotted onto nitrocellulose membranes. Filters were incubated with HRP-ExtrAvidin, and biotinylated proteins were visualized by ECL. Molecular mass markers are indicated in kDa. For determination of T cell proliferation, tritiated thymidine (0.5 μ Ci/well) was added 4 h prior to the end of the culture; cells were harvested on fiber filters, and incorporated thymidine was determined by scintillation counting. Results show thymidine uptake (cpm, mean \pm S.D. of triplicates). *B*, three-day activated T cells were biotinylated, lysed, and immunoprecipitated with W6/32 antibodies as indicated above. Samples were resolved in 10% SDS-PAGE gels under non-reducing (NR) and reducing (R) conditions and blotted onto nitrocellulose membranes. Filters were incubated with HRP-ExtrAvidin, and proteins were visualized as above. Molecular mass markers are indicated in kDa. *C*, three- and five-day activated T cells were biotinylated, lysed, and immunoprecipitated with W6/32, HC-10, and 21Thy2D3 antibodies as indicated. About 50% of each sample was directly resolved by 10% SDS-PAGE under non-reducing conditions and transferred onto nitrocellulose membranes (*upper panels*). The remainder of the primary immunoprecipitates was denatured in 2% SDS and reprecipitated with HC-10 antibodies. Reprecipitates were boiled in 1% SDS and resolved in 10% SDS-PAGE gels under reducing conditions (*bottom panels*). Filters were incubated with HRP-ExtrAvidin, and biotinylated proteins were visualized by ECL. Molecular mass markers are indicated in kDa. *D*, resting human PBL were biotinylated, lysed, and immunoprecipitated with the indicated antibodies. OKT-8, DAKOT8 and SIGMA-8 are commercially available antibodies against the CD8 α chain. Samples were resolved in 10% SDS-PAGE gels under reducing conditions and transferred onto nitrocellulose membranes. Filters were incubated with HRP-ExtrAvidin, and biotinylated proteins were visualized by enhanced chemoluminescence. Molecular mass markers are indicated in kDa.

with biotin and lysed. Cell lysates were then immunoprecipitated with antibodies against folded (W6/32) and misfolded (HC-10) MHC-I molecules. Fig. 1A shows the levels of the two MHC-I conformational states studied on the cell surface of resting (day 0) and activated (days 1, 3, and 5) T cells. Although the levels of folded MHC-I molecules remained steady from day 0–3, misfolded MHC-I molecules showed a time-dependent increase; they were barely detected in resting T cells, started to appear 1 day after stimulation, and peaked by day three (Fig. 1A). Maximal expression of misfolded MHC-I molecules varied between individuals but in general reached a peak between days 3 and 5, coinciding with the peak of cell proliferation as determined by thymidine uptake (Fig. 1A). Noteworthy, both W6/32 and HC-10 antibodies immunoprecipitated a band of ~90-kDa, corresponding to the estimated molecular weight of MHC-I dimers. To ascertain this possibility the immunoprecipitates were resolved under non-reducing and reducing conditions. As depicted in Fig. 1B, the 90-kDa protein band observed under non-reducing conditions essentially disappeared

when the samples were reduced, leading to an increase in 46-kDa class I heavy chain monomers. Interestingly, the peak of expression of misfolded MHC-I molecules at day 3 correlated with the co-precipitation of a high number of protein bands, other than the MHC-I dimers, with molecular masses between 56 and 110 kDa. These co-precipitated proteins were barely observed by day 5, when the decrease in HC-10 reactive heavy chains was paralleled by an increase in folded MHC-I molecules (Fig. 1A).

Physical Associations between MHC-I Molecules and Cell Surface Proteins on Human T Cells Depend on Misfolding— The immunoprecipitation results illustrated in Fig. 1A pointed to the existence of physical associations between MHC-I molecules and other receptors at the cell surface of activated T cells. Also, these associations were apparently dependent on the level of expression of misfolded MHC-I molecules. To ascertain this assumption, a number of T cell surface receptors (*i.e.* CD2, CD3, CD4, CD5, and CD8 $\alpha\beta$) as well as MHC-I molecules were immunoprecipitated from cell lysates of cell surface biotinylated

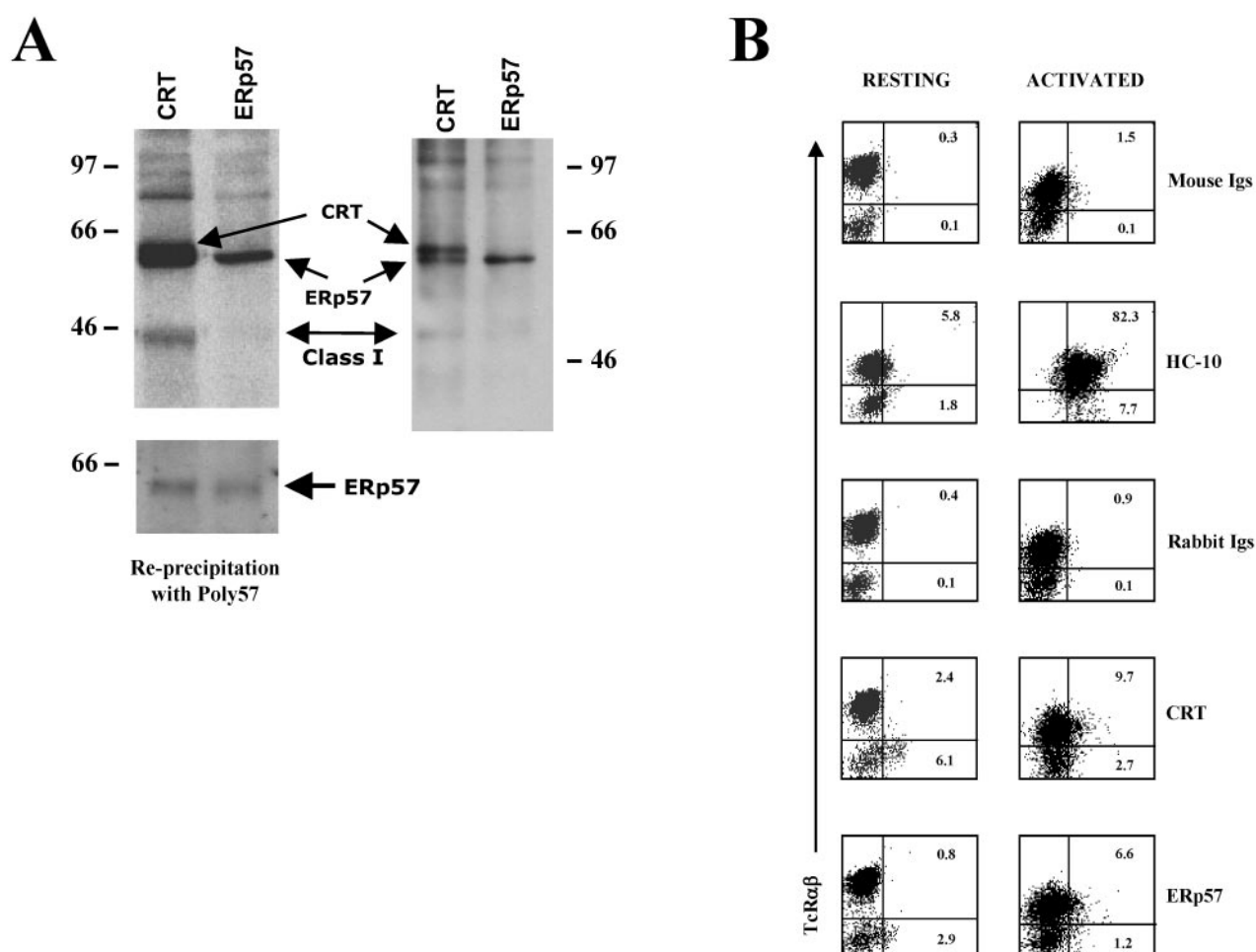


FIG. 2. Expression of calreticulin and ERp57 at the cell surface of human PBL. A, three-day activated human T cells were surface biotinylated, lysed in Brij 96, and immunoprecipitated with anti-calreticulin and anti-ERp57 antibodies. About 20% of each sample was directly resolved by 10% SDS-PAGE under non-reducing conditions and blotted onto nitrocellulose membranes. Results from two separate experiments are shown (*upper panels*). The remainder of the primary immunoprecipitates of one of the experiments (*left*) was denatured in 2% SDS and reprecipitated with anti-ERp57 antibodies. Re-precipitates were boiled in 1% SDS and resolved by 10% SDS-PAGE under non-reducing conditions (*bottom panel*). Filters were incubated with HRP-ExtrAvidin, and biotinylated proteins were visualized by enhanced chemoluminescence. Calreticulin, ERp57, and MHC-I heavy chains are indicated. B, resting and three-day activated human T cells were stained with antibodies against misfolded MHC-I (HC-10), calreticulin, and ERp57 as first-step antibodies. Second-step antibodies were fluorescein isothiocyanate-conjugated rabbit-anti-mouse (for HC-10) and swine anti-rabbit (for calreticulin and ERp57) antibodies. Mouse and rabbit Igs were used as control to define background staining. Cells were then stained with phycoerythrin-conjugated anti-TcR $\alpha\beta$ antibodies. After extensive washes, cells were acquired in a FACScalibur and analyzed using the CellQuest software. From *top to bottom*, dot plots show expression of TcR $\alpha\beta$ versus mouse Igs, HC-10, rabbit Igs, calreticulin, and ERp57 in resting (*left dot plots*) and activated (*right dot plots*) cells gated in viable lymphocytes. The percentage of positive cells in each relevant quadrant is indicated.

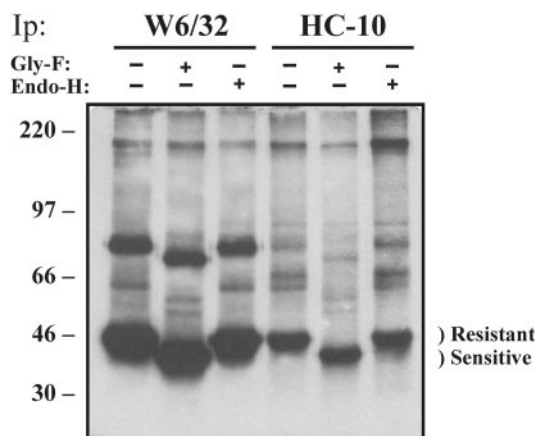
lated T cells at different phases of the activation process and examined for the presence of 46-kDa heavy chains. Of the up to 10 unrelated samples studied, only the CD8 $\alpha\beta$ co-receptor was consistently found to co-precipitate a biotin-labeled protein band with a molecular weight similar to the MHC-I heavy chain from the cell surface of activated T cells, and reprecipitation studies with HC-10 antibodies confirmed that the band corresponded to MHC-I heavy chains (Fig. 1C). The results depicted in Fig. 1C also demonstrated that the higher the level of misfolded MHC-I molecules present at the cell surface the higher the amount of MHC-I heavy chain co-precipitated with the CD8 $\alpha\beta$ co-receptor (Fig. 1C, compare *days 3* and *5*). Importantly, co-precipitation of MHC-I heavy chains with the CD8 $\alpha\beta$ co-receptor was more effective when antibodies against the CD8 β chain were used (Fig. 1D).

Finally, in view of our previous studies showing that the chaperone calreticulin was present at the cell surface of activated T cells in association with misfolded MHC-I molecules (29), we examined for the presence of other related chaperones. Significant levels of calreticulin and ERp57 but not protein

disulfide isomerase were found on the cell surface of activated T cells (Fig. 2A). As expected from our previous studies, antibodies against calreticulin co-precipitated a pool of misfolded MHC-I heavy chains (Fig. 2A, Ref. 29), something not observed in ERp57 immunoprecipitates. Surprisingly, antibodies against calreticulin also co-precipitated a protein band of the same molecular weight as ERp57 (Fig. 2A, *upper panels*), and reprecipitation showed that this protein was indeed ERp57 (Fig. 2A, *lower panel*). Expression of the tandem calreticulin-ERp57 at the cell surface of normal human T cells (resting and activated) was confirmed by flow cytometry (Fig. 2B). Interestingly, two-color flow cytometry showed that in resting but not in activated lymphocytes, calreticulin and ERp57 were mostly at the cell surface of TCR-negative cells (Fig. 2B).

Misfolded MHC-I Molecules Are Fully Glycosylated Proteins—To rule out the possibility that misfolded MHC-I molecules present at the cell surface of activated T cells originated from immature molecules that have escaped the quality control mechanisms of the endoplasmic reticulum, we examined their glycosylation status. Folded and misfolded molecules were im-

A



B

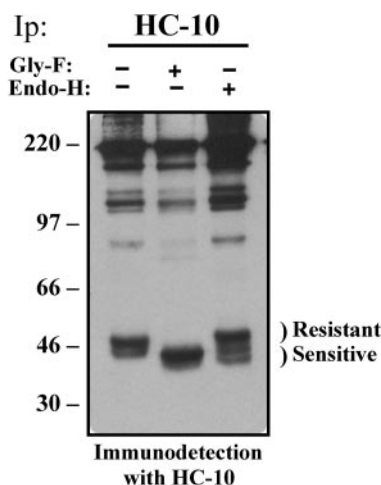


FIG. 3. Glycosylation status of folded and misfolded cell surface MHC class I molecules. A, three-day activated human T cells were cell surface biotinylated, lysed in 1% Brij 96, and immunoprecipitated with W6/32 and HC-10 antibodies. Washed samples were either left untreated or treated with Gly-F or Endo-H for 2 h at 37 °C. Samples were resolved in 8% SDS-PAGE under non-reducing conditions and blotted onto nitrocellulose membranes. Filters were incubated with HRP-ExtrAvidin, and biotinylated proteins were visualized by enhanced chemoluminescence. B, three-day activated human PBL were lysed in Brij 96 and immunoprecipitated with HC-10 antibodies. Washed immunoprecipitates were treated with Gly-F or Endo-H, resolved by SDS-PAGE, and blotted onto nitrocellulose membranes as above. Filters were incubated with HC-10 antibodies, followed by HRP-conjugated goat anti-mouse antibodies. MHC-I heavy chains were visualized by enhanced chemoluminescence. Resistant and sensitive MHC-I heavy chains and molecular mass markers in kDa are indicated.

munoprecipitated from cell lysates of cell surface biotinylated activated T cells and subjected to endoglycosidase H (Endo-H) or *N*-glycanase F (Gly-F) treatment. The products of the enzymatic reaction were resolved by SDS-PAGE under non-reducing conditions, blotted to nitrocellulose membranes, and visualized with ExtrAvidin-HRP followed by SuperSignal West Pico. As shown in Fig. 3A both folded and misfolded MHC-I heavy chain monomers present at the cell surface of activated T cells were resistant to Endo-H although sensitive to Gly-F. The 90-kDa dimers of MHC-I molecules showed a similar pattern of resistance to Endo-H and Gly-F. As a control, we immunoprecipitated total misfolded MHC-I molecules from cell lysates of non-biotinylated activated T cells with HC-10. In

contrast to the results obtained with misfolded MHC-I molecules from the cell surface, Endo-H treatment of total misfolded MHC-I molecules resulted in the digestion of a fraction of these molecules, most likely immature heavy chains residing in the endoplasmic reticulum/Golgi compartments (Fig. 3B). Thus, cell surface misfolded MHC-I molecules contain mature glycans indicating that they originate from folded MHC-I molecules.

Misfolded MHC-I Molecules Are Phosphorylated in Tyrosine Residues—Next, we wanted to ascertain the tyrosine phosphorylation status of the two conformational states of MHC-I molecules present at the cell surface of activated human T cells. Several studies have suggested that a conserved tyrosine motif within the cytoplasmic tail of MHC-I molecules is important for signaling events coupled with intracellular trafficking (16, 33). Besides, studies in mice have suggested that dephosphorylation of serine residues in the cytoplasmic domain of cell surface MHC-I molecules is associated with the misfolded conformational state of H-2D^d molecules (39). We immunoprecipitated W6/32- and HC-10-reactive molecules from post-nuclear supernatants of activated T cells that had been lysed in the presence of tyrosine phosphatase inhibitors. Aliquots of the immunoprecipitates were resolved by SDS-PAGE under non-reducing conditions, and proteins were blotted onto nitrocellulose filters and probed with anti-phosphotyrosine (4G10) or anti-MHC-I heavy chains (HC-10) followed by HRP-conjugated secondary antibodies and detection by ECL. In the majority of the experiments performed, probing with 4G10 antibodies revealed that HC-10 reactive heavy chain monomers (misfolded MHC-I) were phosphorylated in tyrosine whereas W6/32 reactive heavy chain monomers (folded MHC-I) were not phosphorylated (Fig. 4A, left panel). This was also true for the MHC-I heavy chain dimers. Even though in some experiments W6/32-reactive molecules appeared also to be phosphorylated, the level of phosphorylation was very weak especially when compared with the total levels of MHC-I heavy chains (data not shown). Probing with HC-10 antibodies showed that the differences in phosphorylation between folded and misfolded MHC-I heavy chains were not caused by deficient immunoprecipitation of W6/32-reactive molecules (Fig. 4A, right panel). To rule out the possibility that the phosphorylated misfolded heavy chains corresponded to immature MHC-I molecules captured from the endoplasmic reticulum/Golgi, HC-10 immunoprecipitates were subjected to Endo-H treatment. As shown in Fig. 4B, the pool of tyrosine phosphorylated misfolded MHC-I molecules were Endo-H resistant and therefore were mature glycosylated molecules.

Misfolded MHC-I Molecules Are Associated with *in Vitro* Kinase Activity: a Role for the Tyrosine Kinase Lck?—Considering that phosphorylation in tyrosine residues may reflect a timely regulated and highly specific biochemical event mediated by a tyrosine kinase, we wondered whether MHC-I molecules could be found associated with a kinase activity. Therefore, immunoprecipitates of W6/32, HC-10, and CD8 $\alpha\beta$ were subjected to an *in vitro* kinase assay. As expected, CD8 $\alpha\beta$ immunoprecipitates were associated with kinase activity, mostly because of the Src kinase Lck (Fig. 5, A and B). Interestingly, HC-10-reactive molecules were also associated with significant levels of kinase activity, levels that were higher than the associated with W6/32-reactive molecules (Fig. 5A). Among the *in vitro* phosphorylated proteins in MHC-I immunocomplexes a strong 46-kDa phosphorylated band was consistently observed in all the kinase assays performed. Reprecipitations using HC-10 antibodies confirmed that the 46-kDa phosphorylated band was the MHC-class I heavy chain (Fig. 5B). It is noteworthy that by using antibodies against known tyrosine kinases we demonstrated that Lck but not ZAP-70 or

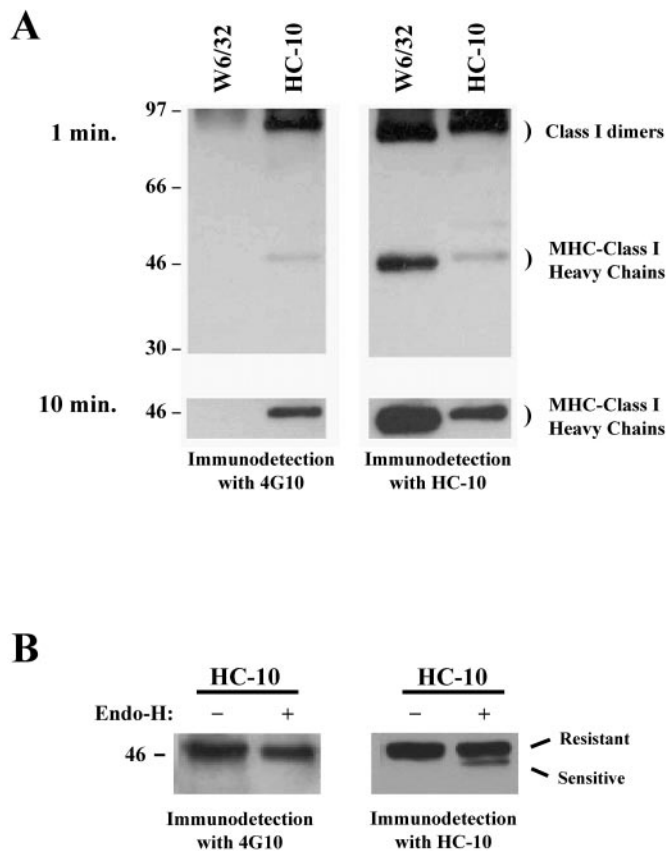


FIG. 4. Misfolded cell surface MHC-I molecules are phosphorylated in tyrosine residues. *A*, two-day activated human PBL cells were harvested, washed, and lysed in Brij 96 lysis buffer in the presence of tyrosine phosphatase inhibitors. Precleared cell lysates were immunoprecipitated with W6/32 and HC-10 antibodies, and duplicate samples were resolved by 10% SDS-PAGE under non-reducing conditions and blotted onto nitrocellulose membranes. Filters were incubated either with 4G10 (*left*) or HC-10 (*right*) antibodies followed by HRP-conjugated goat anti-mouse antibodies. Proteins were visualized by enhanced chemoluminescence. *Upper and lower panels* show different time exposures (1 min *versus* 10 min) of the same filters. The MHC-I heavy chain monomers and dimers are shown. *B*, two-day activated human PBL were harvested, washed, lysed, and immunoprecipitated with HC-10 antibodies. Samples were treated with Endo-H as described in the legend of Fig. 3, resolved in duplicate in 8% SDS-PAGE, and blotted onto nitrocellulose membranes. Filters were incubated either with 4G10 (*left*) or HC-10 (*right*) antibodies followed by HRP-conjugated goat anti-mouse antibodies. MHC-I heavy chain monomers were visualized by enhanced chemoluminescence. Resistant and sensitive molecules are indicated, and molecular mass markers are shown in kDa.

Csk is present in the MHC-I immunocomplexes, namely of misfolded MHC-I (Fig. 5*B* and data not shown).

The Tyrosine Kinase Inhibitor PP2 Reduces the Levels of Phosphorylation of Misfolded MHC-I Molecules and Enhances Their Release from the Cell Surface—Finally, we wanted to ascertain the significance of the tyrosine phosphorylation of misfolded MHC-I molecules. In the context of previous studies reporting that a pool of misfolded MHC-I molecules is cleaved and released from the plasma membrane of activated T cells (31), we examined the effect of the Src-kinase inhibitor PP2 on MHC-I phosphorylation status and shedding. PP2 is a potent and highly specific Src-family inhibitor that inhibits T cell proliferation. Because inhibition of T cell proliferation results in inhibition of MHC-I misfolding,² we performed these studies on activated T cells and for short periods. As illustrated in Fig. 6*A*, short-term treatment of activated T cells with PP2 resulted

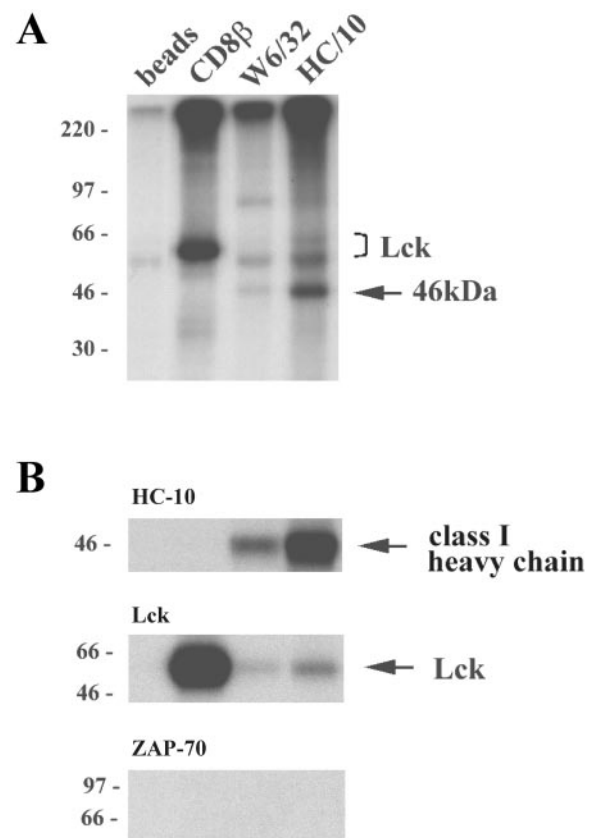


FIG. 5. MHC class I molecules are associated with kinase activity. *A*, three-day activated human PBL were lysed in 1% Brij 96 lysis buffer. Immunoprecipitates of CD8β, W6/32, and HC-10-reactive molecules were subjected to *in vitro* kinase assays as described in under "Materials and Methods." About 25% of the immunoprecipitates were resolved by 10% SDS-PAGE under reducing conditions, and phosphorylated products were visualized after exposure of the dried gels to Biomax MR-1 films. *B*, the remainder of the immunoprecipitates was denatured in 2% SDS, and aliquots were reprecipitated with antibodies against misfolded MHC-I (HC-10), against Lck, or against ZAP-70. Reprecipitates were boiled in 1% SDS and resolved in 10% SDS-PAGE gels under reducing conditions. Phosphorylated MHC-I heavy chains and Lck were visualized after exposure of the dried gels to Biomax MR-1 films. Lck and MHC-I heavy chain are indicated, and molecular mass markers are shown in kDa.

in a reduction in the amount of tyrosine phosphorylated misfolded MHC-I molecules. The reduction in tyrosine phosphorylation was not caused by a decrease in HC-10 reactive molecules at the cell surface (Fig. 6*B* and data not shown). Instead, it correlated with a down-modulation in W6/32 reactivity (Fig. 6*B*). Interestingly, examination of soluble MHC-I heavy chains present in the culture media showed a higher amount of MHC-I heavy chains in cultures of activated T cells treated with PP2 (Fig. 6*C*). In addition to an increase in 46-kDa heavy chains, several cleaved forms of MHC-I were also detected in the media of cultures of activated T cells treated with PP2 but not in untreated cultures (Fig. 6*C*).

DISCUSSION

Protein misfolding is being acknowledged as a predisposing factor in the development of many diseases, and certain MHC-I molecules such as HLA-B27 are particularly prone to misfold (40, 41). Recent studies suggest that cell surface MHC-I proteins may have fundamental biological roles that rely in the interaction, in *cis* or *trans*, with molecular structures recognizing amino acid motifs that are hidden in the heterodimeric folded MHC-I molecules but that become unmasked when the MHC-I molecules misfold after losing the peptide and/or the

² S. G. Santos, S. J. Powis, and F. A. Arosa, unpublished data.

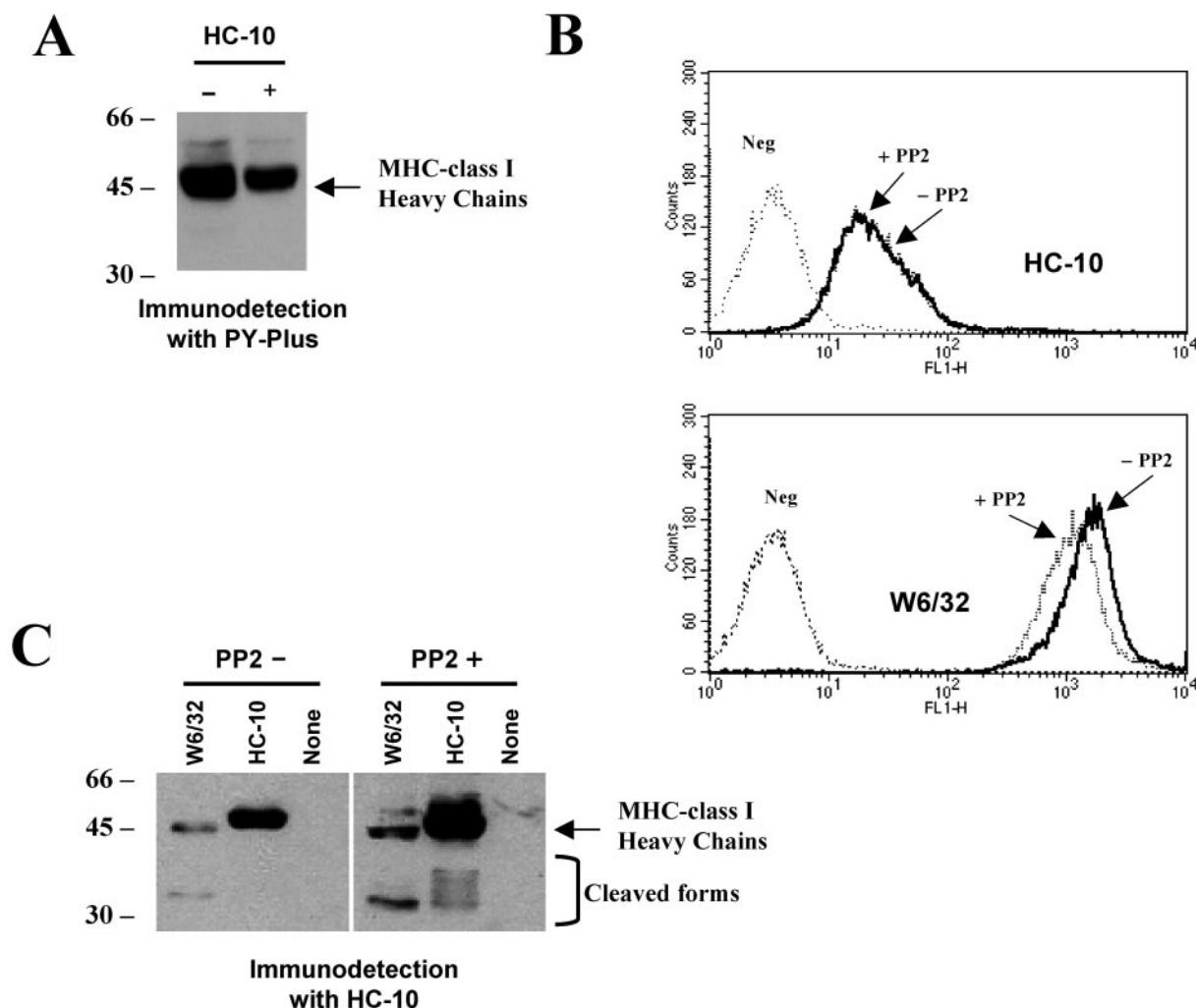


FIG. 6. The Src family inhibitor PP2 inhibits MHC-I phosphorylation and enhances MHC-I shedding. Three-day activated human PBL were incubated for 3 h in the absence (–) or presence (+) of 10 μ M PP2. **A**, cells were then harvested, washed, and lysed in Brij 96 in the presence of tyrosine phosphatase inhibitors, and cell lysates were immunoprecipitated with HC-10 antibodies. Samples were resolved by 10% SDS-PAGE under non-reducing conditions, and proteins were blotted onto nitrocellulose filters. Membranes were then incubated with anti-phosphotyrosine antibodies (PY-Plus) followed by HRP-goat anti-mouse Igs. Phosphorylation of MHC-I heavy chain was visualized by enhanced chemoluminescence. **B**, cells were harvested and stained with W6/32, HC-10, or mouse Igs followed by fluorescein isothiocyanate-conjugated rabbit-anti-mouse Igs. After extensive washes, cells were acquired in a FACScalibur and analyzed using the CellQuest software. Histograms show expression of HC-10 (upper histogram) and W6/32 (lower histogram) reactive molecules at the cell surface of activated T cells untreated (–PP2) and treated (+PP2) with the Src-kinase inhibitor. **C**, culture media from the cultures of activated human PBL incubated for 3 h in the absence (PP2–) or presence (PP2+) of 10 μ M PP2 were collected and immunoprecipitated with W6/32 and HC-10 antibodies. Samples were resolved by 10% SDS-PAGE under non-reducing conditions, and proteins were blotted onto nitrocellulose filters. Membranes were then incubated with HC-10 antibodies followed by HRP-goat anti-mouse Igs. MHC-I heavy chains as well as several cleaved forms were visualized by enhanced chemoluminescence. Molecular mass markers are shown in kDa.

light chain β_2m (42, 43). Therefore, knowledge of the molecular mechanisms that govern the folding and misfolding of MHC-I molecules at the plasma membrane is important for understanding their participation in a number of immunological and non-immunological processes such as natural killer recognition, T cell activation, receptor-mediated signaling and endocytosis, and viral interference.

In the present study evidence is presented that in activated normal human T cells, cell surface MHC-I molecules co-exist into two conformational states, folded and misfolded, identified by the use of the monoclonal antibodies W6/32 and HC-10, respectively. The increase of free heavy chains at the cell surface of activated T cells together with previous data correlating the presence of those free heavy chains with MHC-class I clustering (24) suggest that the equilibrium between folded and misfolded class I molecules at the cell surface of activated T cells may play a role in regulating the intensity of activation signals. Moreover, we have demonstrated that the temporal

co-existence of two conformational states allows misfolded MHC-I molecules to associate with other molecules and receptors, such as CD8 $\alpha\beta$. Early studies reporting the physical association between CD8 $\alpha\beta$ and MHC-I molecules used cross-linking reagents and antibodies against the CD8 α chain (20, 21). In our studies we also used anti-CD8 α antibodies, and the levels of co-precipitated MHC-I molecules were very similar to these earlier studies even in the absence of cross-linking reagents. Importantly, we have shown that antibodies against the CD8 β chain co-precipitated higher amounts of MHC-I molecules than antibodies against the CD8 α chain, which may suggest that anti-CD8 α antibodies may disrupt the physical *cis*-association. In our opinion, the interaction in *cis* between misfolded MHC-I molecules and the CD8 $\alpha\beta$ receptor is the result of a time-regulated event that takes place in activated CD8+ T cells to fine tune the signaling delivered by the CD8-Lck complex, and that ultimately leads to cell division and expansion. It is anticipated that interference with this *cis*-

association will likely impact negatively the expansion of CD8⁺ T cells. The preferential association of MHC-I molecules with CD8 $\alpha\beta$ but not other co-receptors could be caused by the existence of molecular constraints defined by the MHC-I sequence. Although our studies do not allow us to distinguish if the *cis*-association takes place with the CD8 α chain, the CD8 β chain, or both, a scenario where misfolded MHC-I heavy chains preferentially associate with the CD8 β is favored. Previous studies demonstrated that peptide free H-2L^d mouse heavy chains could interact with CD8 α and - β chains through their binding groove, and computational predictions revealed an amino acid motif in human CD8 β that scored high for their ability to bind the HLA-A2 allele (42).

We have previously reported the presence of calreticulin at the cell surface of activated normal human PBL (29). In the present study we extend those results by demonstrating that ERp57 is also present at the cell surface of resting and activated T cells and is found in association with calreticulin. Two color flow cytometry using antibodies against the T cell receptor confirmed the results obtained by cell surface biotinylation and immunoprecipitation and revealed two important facts. First, in resting human PBL samples calreticulin and ERp57 are mainly expressed by TcR $\alpha\beta$ negative (–) cells. Preliminary results indicate that these cells are CD8⁺ natural killer cells.² Second, the increase in the amount of calreticulin and ERp57 observed in activated human PBL takes place in TcR $\alpha\beta$ ⁺ cells. The presence of chaperones involved in the folding and assembly of MHC-I molecules inside the endoplasmic reticulum at the cell surface is likely to have biological relevance. Thus, reprecipitation studies showed that calreticulin and ERp57 form a complex at the cell surface with a pool of misfolded MHC-I molecules. An association between calreticulin and ERp57 in the absence of tapasin has been observed during the folding of a class I-like molecule inside the endoplasmic reticulum (44); therefore we hypothesize that the calreticulin-ERp57 complex is present in the clusters of misfolded MHC-I molecules at the cell surface of actively dividing T cells for the purpose of stabilizing this conformational state. This hypothesis is in agreement with a recent report by Wearsch *et al.* (45) showing that calreticulin can bind class I molecules independent of their assembly status, being unable to distinguish between peptide-loaded class I molecules and free heavy chains. Stabilization of misfolded MHC-I molecules at the cell surface may allow them to associate *cis* with other receptors thus facilitating fine tune modulation of the receptor-mediated signals. Our inability to detect MHC-I heavy chain associated with calreticulin (29) or the calreticulin/ERp57 tandem (present study) at the cell surface of resting PBL supports this assumption.

The use of Endo-H digestion as an indicator of MHC-I maturation confirmed that the biotinylated molecules captured at the cell surface of activated T cells were mature, fully glycosylated proteins. Our results are in agreement with earlier studies indicating that MHC-I molecules begin their lives first as HC-10-reactive, then as W6/32-reactive, and later regain the HC-10 reactivity (46). Furthermore, we have shown that MHC-I heavy chain homodimers are present at the cell surface of normal human dividing T cells. MHC-I homodimers were rarely seen in resting T cells, which suggests a relationship between T cell activation, misfolding, and dimerization as described by other authors using different models (47, 48). More recently, it has been shown that homodimer formation involves cysteines at position 67 and possibly also 164 (49). Interestingly, Cys⁶⁷ is located within the peptide-binding groove and therefore is inaccessible for disulfide bonding unless the heavy chain is misfolded. Some authors have suggested that MHC-I

homodimers might be linked with the development of autoimmune diseases, such as ankylosing spondylitis, whereas others suggest that such associations may be part of clusters that fulfill significant functions in cell-to-cell contacts and signal transduction (reviewed in Refs. 50 and 51). The appearance of significant levels of MHC-I dimers at the cell surface of activated but not resting normal human T cells argues in favor of the view that these molecules are functionally important for dividing T cells.

In this study, we have shown for the first time that misfolded MHC-I molecules are phosphorylated in tyrosine residues and are associated with significant levels of kinase activity. These results are provocative if we take into consideration that several authors have suggested that recycling pathways are involved in MHC-I misfolding and dimerization (48, 52) and that a conserved tyrosine residue in the cytoplasmic domain of MHC-I molecules (Tyr³²⁰) plays a key role in these processes (33, 34). Furthermore, early studies demonstrated that this conserved tyrosine could be phosphorylated *in vitro* by pp60^{v-src} (53). Even if we cannot discern whether tyrosine phosphorylation is the cause or the consequence of MHC-I misfolding, our results clearly indicate that the phosphorylation process is related to the conformational change of MHC-I molecules at the cell surface of activated T cells. This conformational change may allow a kinase(s) to phosphorylate the tyrosine residue. This hypothesis would validate a scenario where tyrosine-phosphorylated misfolded MHC-I molecules could play a role in the signaling pathways that are turned on upon T cell activation. In this context, the higher *in vitro* kinase activity associated with HC-10 reactive molecules suggested an association with an intracellular kinase(s). Indeed, the Src tyrosine kinase Lck was present in HC-10 immunocomplexes. Although we could not detect ZAP-70 or Csk,² we could not rule out the possibility that other tyrosine kinases contribute to the kinase activity of misfolded MHC-I molecules. Associations between kinases and their substrates are usually transient events that may not be detected by immunoprecipitation. Yet, the finding that Lck is associated with misfolded MHC-I molecules together with the results obtained with the Src-family specific tyrosine kinase inhibitor PP2 places this tyrosine kinase as a likely candidate for the tyrosine phosphorylation of MHC-I heavy chains both *in vitro* and *in vivo*.

Although the physiological significance of the tyrosine phosphorylation of MHC-I molecules at the cell surface of activated T cells *in vivo* requires further study, we have demonstrated that tyrosine phosphorylation of class I may be linked in part with the half-life of these molecules at the cell surface. Thus, by inhibiting tyrosine phosphorylation with PP2 we enhanced the release of MHC-I molecules, including minor cleaved forms, which correlated with a down-modulation of W6/32-reactive molecules. These data suggest that tyrosine phosphorylation of misfolded MHC-I heavy chains may be a signal that reduces the shedding of folded MHC-I molecules (that upon cleavage are recognized by HC-10 antibodies) and allows establishment of *cis*-associations that rely upon endocytosis and intracellular trafficking. These results are important in the context of reports demonstrating that soluble MHC-I molecules play an important role in the regulation of CD8⁺ numbers and function by triggering apoptosis of activated CD8⁺ T and natural killer cells (54, 55). Another possible implication of class I phosphorylation relates to Nef down-modulation of class I molecules in HIV infected cells. Nef is known to interfere with class I cytoplasmic tyrosine motif (28) both in the Golgi and at the cell surface. Nef also contains a motif that binds Src family kinases, which is necessary for class I down-modulation (56). In this study we have shown that misfolded class I molecules are tyrosine-phosphorylated on activated

T cells by a Src family kinase. It is therefore tempting to speculate that Nef could use a cellular Src kinase to phosphorylate class I molecules as a specific down-regulation mechanism.

Further characterization of the molecular mechanisms regulating the folded to misfolded equilibrium at the cell surface and its relationship with endocytosis and intracellular trafficking by exploring how phosphorylation of class I may interfere with the endocytosis process may unravel novel aspects of the biology of MHC-I molecules that may help in our understanding of the participation of MHC-I molecules in apparently unrelated biological processes as well as contribute to a better understanding how Nef down-modulation of MHC-class I molecules takes place.

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Misfolding of Major Histocompatibility Complex Class I Molecules in Activated T Cells Allows *cis*-Interactions with Receptors and Signaling Molecules and Is Associated with Tyrosine Phosphorylation

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