Physical association between the CD8 and HLA class I molecules on the surface of activated human T lymphocytes

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ABSTRACT  Immune recognition by cytotoxic effector T cells requires participation of the CD8 and major histocompatibility complex class I antigens. We found that the CD8 molecule is noncovalently associated with the HLA class I heavy chain on the surface of human T cells activated by Con A. Accordingly, anti-CD8 monoclonal antibodies precipitated a heterodimer containing polypeptides of 32 and 43 kDa from the lysates of activated T cells. The 43-kDa chain of this heterodimer can be adsorbed from cell lysates with anti-HLA-A, -B, and -C antibodies. Endoglycosidase F treatment and chymotryptic peptide mapping identified a structural similarity between this 43-kDa molecule and the HLA class I heavy chain precipitated by the anti-HLA-A, -B, and -C antibody W6/32. Analysis of anti-CD8 precipitates under nonreducing and reducing conditions indicated a lack of interchain disulfide bonding between the CD8 and HLA heavy chain molecules. The CD8–HLA heavy chain complex was also detected in mixed lymphocyte cultures and a cloned cytotoxic T-lymphocyte line but not in purified natural killer cells. The present study indicates that CD8 is complexed with HLA heavy chain on the same cells, and the complex may have functional relevance in the T-cell recognition process.

Peripheral T lymphocytes recognize a repertoire of cell-associated antigens in the context of class I or class II molecules encoded by the major histocompatibility complex (MHC). It is generally accepted that cytotoxic T lymphocytes (CTL) recognize cell-associated viral or tumor antigens in association with MHC class I molecules (1–4) and that the α/β-chain T-cell receptor molecules on CTL recognize antigen and polymorphic MHC determinants (5–8). This recognition appears to be a complex process, and at least one additional cell surface component, the CD8 molecule, is implicated (4, 9–11).

CD8 has been characterized as a 32- to 34-kDa integral membrane protein in its reduced form (12, 13). Under nonreducing conditions, CD8 has been identified in a series of multimeric forms differing in inter- or intrachain disulfide bonding (13). Studies have suggested (1–4, 14–16) that CD8 can bind to MHC class I molecules and thereby increase T-cell–target or T-cell–accessory-cell avidity that allows recognition of antigen and T-cell activation. To date, however, the actual physical interaction between CD8 and MHC class I molecules on T-cell surface is not well understood. This report demonstrates a noncovalent association between CD8 and HLA heavy chain molecules on the surface of activated human T cells.

MATERIALS AND METHODS

Cells. Human thymocytes and peripheral blood T cells isolated by forming rosettes with sheep erythrocytes (17) were used directly for radioiodination. For Con A activation, T cells were incubated at 10⁶ cells per ml with 10⁴ non-T cells per ml in complete medium [RPMI 1640 medium with 5% (vol/vol) fetal bovine serum, glutamine (0.06 mg/ml), streptomycin (100 µg/ml), and penicillin (100 units/ml)] with Con A (10 µg/ml) for 2 days. Before radioiodination, the Con A-activated T cells were washed once with 50 mM α-methyl mannoside (Sigma) and twice with isotonic phosphate-buffered saline (pH 7.2). For allogeneic activation of T cells, unseparated peripheral blood lymphocytes from two healthy donors were incubated in complete medium at 2 × 10⁹ cells per ml for 5 days. A natural killer (NK)-enriched cell population obtained by discontinuous Percoll gradient centrifugation (18) was 80% positive for CD16, recognized by monoclonal antibody B73.1 (19). A cloned CTL line (CD3⁺, CD4⁻, CD8⁺), derived from peripheral blood lymphocytes sensitized with irradiated allogeneic non-T cells, was maintained in RPMI 1640 medium with 20% (vol/vol) pooled human AB serum (Flow Laboratories) and human interleukin 2 (20 units/ml) (Boehringer Mannheim). The human T-cell leukemia line HPB-ALL was from J. Minowada (Roswell Park Memorial Institute, Buffalo, NY) and was maintained in complete medium.

Antibodies. The monoclonal antibodies OKT8A (Ortho Diagnostics), anti-Leu-2a (Becton Dickinson), and C8 (20) (a gift of C. Y. Wang, United Biomedical, Lake Success, NY) recognize CD8. The anti-HLA-A, -B, and -C monoclonal antibodies W6/32 (21) and A1.4 (22) were also used.

Internal Labeling. Cells (5 × 10⁶ cells) were washed twice, resuspended in 4 ml of RPMI 1640 medium deficient in methionine and cystine with 5% (vol/vol) dialyzed pooled human AB serum, and incubated for 30 min at 37°C. Then 100 µCi of [35S]methionine and 100 µCi of [35S]cysteine (New England Nuclear; 1 Ci = 37 GBq) were added to the cell cultures and incubated for 4 hr.

Radioiodination, Immunoprecipitation, and NaDodSO₄/PAGE. Cells were radioiodinated by the lactoperoxidase method and lysed with 2 ml of isotonic phosphate-buffered saline, pH 8.3/0.5% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride/10 mM iodoacetamide (22). The lysates were adsorbed with goat anti-mouse IgG-agarose beads (Sigma) alone and immunoprecipitated with goat anti-mouse IgG-agarose beads coated with specific monoclonal antibodies. The material was eluted from the beads with Laemml sample buffer (23) for reducing [with 2% (vol/vol) 2-mercaptoethanol] or nonreducing NaDodSO₄/PAGE. In sequential immunoprecipitation experiments, 100-µl aliquots of labeled cell lysates, preincubated three times with excess antibody W6/32-coated goat anti-mouse IgG-agarose beads, were subsequently adsorbed with antibody A1.4-coated beads (twice), immunoprecipitated with antibody C8-coated beads, and analyzed by NaDodSO₄/PAGE. Vertical slab gels of

Abbreviations: β₂m, β₂-microglobulin; CTL, cytotoxic T lymphocytes; MHC, major histocompatibility complex; NK, natural killer.

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11.5% and 9% acrylamide were used for reducing and nonreducing NaDodSO₄/PAGE, respectively (23). A Bio-Rad video densitometer (model 620) was used to quantitate individual protein bands of NaDodSO₄/PAGE autoradiograms.

Treatment with Endoglycosidase F. The material, eluted from immunoprecipitates with 50 mM Tris-buffered saline, pH 7.8/1% Nonidet P-40/0.3% NaDodSO₄/10 mM phenanthroline monohydrate (Sigma), was digested with endoglycosidase F (provided by A. Pinter, Public Health Research Institute) for 4 hr at 37°C and analyzed by NaDodSO₄/PAGE.

Peptide Mapping. Chymotryptic peptide mapping was performed as described by Elder et al. (24).

RESULTS

Immunoprecipitation of a Heterodimer by Anti-CD8 Antibodies from Con A-Activated Peripheral Blood T Cells. Antibody C8 immunoprecipitated a heterodimer containing polypeptides of 32 and 43 kDa (32/43-kDa heterodimer) from Con A-activated T-cell lysates (Fig. 1, lane 5), whereas only the 32-kDa CD8 band was immunoprecipitated from untreated T-cell lysates as shown by reducing NaDodSO₄/PAGE (Fig. 1, lane 2). The 32/43-kDa heterodimer was also precipitated by OKT8A and anti-Leu-2a antibodies from Con A-activated but not from untreated T-cell lysates (data not shown). The 43-kDa band precipitated by anti-CD8 appeared to be identical in size to the HLA class I heavy chain that can be precipitated by antibody W6/32 from the same cell lysates (Fig. 1, lane 4). In addition, treatment of precipitates from Con A-activated T cells with endoglycosidase F, which cleaves complex N-linked carbohydrates and carbohydrates high in mannose (25), resulted in a shift in the apparent molecular mass of the 43-kDa band to 40 kDa (Fig. 1, lanes 4-7).

We next examined the Con A-activated T cells from seven other normal donors for the 32/43-kDa heterodimer in anti-CD8 immunoprecipitates. In six donors, anti-CD8 precipitates from Con A-activated T cells yielded the 32/43-kDa heterodimer, whereas untreated cells produced a single 32-kDa band (data not shown). However, untreated T-cell lysates of one donor (donor M) unexpectedly yielded the 32/43-kDa heterodimer, and treatment of T cells from donor M with Con A did not change its quantity. Because a small quantity of Con A remained bound to T cells after treatment with a-methyl mannoside and because the iodinated Con A migrated closely to the CD8 band on NaDodSO₄/PAGE (Fig. 1B), characterization of the 32/43-kDa heterodimer was partially performed with the untreated cells of donor M.

Identification of the 43-kDa Molecule Coprecipitated by Anti-CD8 as an HLA Heavy Chain. Whereas antibody W6/32 precipitated the 43-kDa HLA heavy chain and β₂-microglobulin (β₂m) (26) from the cell lysates, antibodies C8, OKT8A, and anti-Leu-2a precipitated a 32-kDa CD8 and a 43-kDa molecule without 12-kDa β₂m (Fig. 1B). Nevertheless, there are striking similarities in molecular mass and the extent of glycosylation between the 43-kDa proteins precipitated by antibodies anti-CD8 or W6/32. To determine whether the 43-kDa molecule precipitated by anti-CD8 was indeed identical with the HLA heavy chain, sequential adsorption experiments and chymotryptic peptide mappings were performed with T-cell lysates from donor M. An aliquot of the cell lysate adsorbed with antibody W6/32 was subjected to sequential precipitations with antibodies A1.4 and C8. Incubation of W6/32-adsorbed cell lysate with antibody A1.4, which recognizes β₂m-associated and β₂m-free HLA heavy chains (22), resulted in the precipitation of β₂m-free, 43-kDa HLA heavy chain (Fig. 2, lane 3). Subsequent incubation of this HLA-depleted cell lysate with antibody C8 resulted in the precipitation of a single 32-kDa band (lane 5), whereas when the lysate was first adsorbed with beads alone and then incubated with antibody C8, the 32/43-kDa heterodimer was immunoprecipitated (lane 6). By densitometric scanning, on average 10% (5-16%) of the total cell-surface HLA class I heavy chain appears to be associated with CD8.

Fig. 1. NaDodSO₄/PAGE of radioiodinated peripheral blood T-cell lysates precipitated with anti-CD8 (C8) and anti-HLA class I (W6/32) antibodies. (A) Untreated T-cell lysate incubated with beads alone (−) or with antibody C8. (B) Con A-activated T-cell lysate incubated with beads alone (−), antibody W6/32, or antibody C8 before (−) or after (+) digestion with endoglycosidase F (endo-F). Open arrowheads indicate the position of CD8. The band migrating at 30 kDa (B) is radioactive Con A that contaminates cell lysates.
By using a reciprocal preadsorption procedure, we next determined whether depletion of CD8 from the cell lysate resulted in a decrease in quantity of \( \beta_m \)-m-free HLA heavy chain precipitated by antibody A1.4. To avoid the reaction of \( \beta_m \)-m-associated HLA heavy chain with antibody A1.4, the cell lysate was adsorbed with antibody W6/32 prior to sequential precipitation with antibodies C8 and A1.4. However, we were unable to completely remove \( \beta_m \)-m-free HLA heavy chain with antibody C8, suggesting that not all available \( \beta_m \)-m-free HLA heavy chains were complexed with CD8 (data not shown). Interestingly, precipitation with antibody A1.4 did not yield detectable CD8 (Fig. 2, lane 3), probably due to a dissociation of CD8 and HLA molecules upon binding of antibody A1.4. This hypothesis is supported by the observation that antibody A1.4 can actually precipitate CD8 from the lysates of chemically cross-linked T cells (Y. B., unpublished data).

Chymotryptic peptide mapping further suggested that the HLA heavy chain precipitated by antibody W6/32 was identical to the 43-kDa molecule precipitated by antibody C8. Tyrosine-containing peptides of both 43-kDa molecules appeared to be identical, with some differences only in intensity of label, probably due to different accessibility of \( \beta_m \)-m-associated and CD8-complexed HLA heavy chains to iodination (Fig. 3).

Noncovalent Bonding Between CD8 and HLA Heavy Chain.
Our adsorption experiments suggested that CD8 and HLA heavy chain were noncovalently associated, since depletion of HLA heavy chain by antibodies W6/32 and A1.4 from cell lysates did not coincidently remove CD8 (Fig. 2). In contrast, in human thymocytes, CD8 was reported to be linked through disulfide bonds to another MHC class I-like molecule, CD1 (27, 28). To characterize the association of CD8 with HLA heavy chain, anti-CD8 precipitates from the T cells (donor M) and thymocytes were compared on NaDodSO4/PAGE under nonreducing conditions. As reported (13), both anti-CD8 precipitates yielded a series of multimeric forms of CD8 with approximate molecular masses of 60, 80, 150, and 200 kDa (Fig. 4A). Only the anti-CD8 precipitate from the T-cell lysate, however, yielded the 43-kDa band in the absence of monomeric 32-kDa CD8, suggesting that CD8 is noncovalently associated with the HLA heavy chain (Fig. 4, lane 1). Each of these multimeric species was then eluted from the gel and submitted to NaDodSO4/PAGE under reducing conditions. The results indicated that the 60-kDa species, found in thymocyte and T-cell lysates, were disulfide-linked dimers of 32-kDa CD8 (data not shown). The 80, 150, and 200 kDa species of human thymocytes contained the 32-kDa CD8 linked by disulfide bonds to the 46-kDa molecule, presumably CD1 (Fig. 4, lanes 6–8), whereas the 80, 150, and 200 kDa species precipitated from the T-cell lysate contained only the 32-kDa CD8 without the 43-kDa protein (Fig. 4, lanes 3–5). We concluded from these experiments that the association between CD8 and HLA heavy chain in T cells is noncovalent.

Presence of the CD8–HLA Heavy Chain Complex in Activated T Cells. Since the CD8–HLA heavy chain complex was found in Con A-activated T cells but generally not in resting T cells (Fig. 1), we subsequently examined whether this complex exists exclusively in activated T cells. For this purpose, a cloned CTL line, purified NK cells, mixed lymphocyte cultures, and HPB-ALL leukemia cells were radioiodinated or labeled metabolically with \( ^{35} \)S)cysteine and \( ^{35} \)Smethionine, and then lysates of these cells were immunoprecipitated with anti-CD8 and analyzed on NaDodSO4/PAGE under reducing conditions. As seen in Fig. 5 (lanes 1–6), CTL lysates contained the CD8–HLA heavy chain complex. Existence of intrinsically labeled 32/43-kDa heterodimer (lane 2) indicated that the formation of CD8–HLA heavy chain complex was not an artifact resulting from iodination. In addition, since the CTL line represented a cloned population, the complex between CD8 and HLA heavy chain was likely to be formed on the same cells. The CD8–HLA heavy chain complex was also detected in mixed lymphocyte cultures (lane 8) but not in purified NK cells and in the HPB-ALL leukemia cell line, although these cells expressed CD8 (lanes 7 and 9). Therefore, the CD8–HLA heavy chain complex appears to be formed only when mature T cells are activated by Con A or antigen stimulation.

DISCUSSION
Our results indicate that the CD8 molecule is associated with HLA heavy chain on the surface of activated human T cells. Resting T cells generally contain no detectable CD8–HLA heavy chain complex, the appearance of which can be induced by Con A or allogeneic stimulation. The expression of the CD8–HLA heavy chain complex in untreated T cells of donor M may be due to an in vivo activation of these T cells, as stimulation of these T cells with Con A did not alter the overall pattern of the expression of the CD8–HLA heavy chain complex. This is clearly unrelated to the HLA alleles (HLA-A26, -29 and HLA-B33, -38) of this donor, because untreated T cells of other donors with the same type of HLA alleles were negative for the CD8–HLA heavy chain complex (data not shown).

Formation of the CD8–HLA heavy chain complex is likely to occur on the same cells, rather than from two adjacent cells. This conclusion is based on the following experiments. Con A-activated T cells were first incubated with antibody C8 and then lysed, and the immune complexes were isolated...
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with goat anti-mouse IgG-agarose beads. NaDodSO₄/PAGE analysis showed the presence of the CD8–HLA heavy chain complex, suggesting that the complex was formed before its reaction with antibody C8. Formation of the complex after cell lysis is unlikely, since no CD8–HLA heavy chain complex was evident in most resting T-cell preparations albeit CD8 and HLA heavy chains were present in the lysates. In another experiment, Con A-activated T cells were allowed to adhere at low density to Petri dishes precoated with poly (l-lysine) (Sigma) to avoid cell-to-cell contact during cell lysis. The CD8–HLA heavy chain complexes in the cell lysates were again found (data not shown), suggesting formation of the complex occurs on the same cells.

Appearance of the CD8–HLA heavy chain complex only in activated T cells may result from changes during the activation process in the relative disposition of these molecules that leads to an increased spatial association on the cell surface. Alternatively, formation of the CD8–HLA heavy chain complex may occur intracellularly. Thus, HLA heavy chains spontaneously internalized in activated T cells (29) can be dissociated from β₂m in acidic endosomes (B. Pernis, personal communication), and then, by an unknown mechanism,

**Fig. 3.** Peptide mapping of the 43-kDa protein precipitated by anti-CD8 antibodies and the HLA heavy chain precipitated by antibody W6/32. Peptide maps of chymotryptic digests were developed on TLC plates by electrophoresis in the first dimension and chromatography in the second dimension. (A) Peptide map of the HLA heavy chain precipitated by antibody W6/32 (from Fig. 2, lane 1). (B) Peptide map of the 43 kDa chain precipitated by antibody C8 (from Fig. 2, lane 6). (C) Mixture of samples shown in A and B. (D) Peptide map of CD8 precipitated by antibody C8 (from Fig. 2, lane 6). T cells from donor M were used for this experiment.

**Fig. 4.** Association between CD8 and MHC class I heavy chains on the surface of human T cells. (A) Lysates of radioiodinated T cells from donor M and thymocytes from another donor were incubated with antibody C8, and precipitates were submitted to NaDodSO₄/PAGE under nonreducing conditions. Lanes 1 and 2 show T-cell and thymocyte lysates incubated with antibody C8, respectively. (Overexposure was used to visualize the HLA heavy chain indicated by the arrow.) (B) Bands a, b, and c from both lanes were excised and analyzed by NaDodSO₄/PAGE under reducing conditions. Lanes 3–5 indicate that the bands c, b, and a, derived from T-cell lysates (lane 1), contained the 32-kDa CD8 only. Lanes 6–8 show that bands a–c, derived from thymocytes (lane 2), were composed of CD8 and CD1 linked by disulfide bonds. Lane 9 shows a C8 precipitate from the thymocyte lysate analyzed under reducing conditions. Open arrowhead, CD1 coprecipitated by antibody C8. The nature of the 55-kDa doublet seen in lanes 3 and 4 is presently unknown.
HLA heavy chains can complex with CD8 and be recycled to the cell surface as a CD8–HLA heavy chain complex. Enhanced expression of CD8 in activated T cells has been documented (30), and it is inferred that increased synthesis of CD8 is required for the formation of this CD8–HLA heavy chain complex.

Interaction of immature thymocytes with MHC gene products present in thymic parenchyma appears to be essential for the generation of appropriate MHC-restricted T-cell clones with various functions (31, 32). The covalent CD8–CD1 complex in thymocytes and noncovalent CD8–HLA heavy chain complex in activated mature T cells may be of functional importance. An interaction between the CD8 of effector cells and the HLA heavy chain of target cells may occur only when CD8 is present in its HLA-complexed form. Accordingly, the interaction between the effector and target cells may largely depend on the recognition between the two complexes—i.e., CD8–HLA on the effector cell and β₂m–HLA on the target cell. Since the noncovalent association of β₂m with HLA class I heavy chain is loose enough to allow replacement by exogenous β₂m (33, 34), the interaction between effector cell CD8 and target cell HLA molecules may be facilitated by transfer of β₂m between these two complexes.

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