HLA-derived Peptides which Inhibit T Cell Function Bind to Members of the Heat-Shock Protein 70 Family

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Summary

Synthetic peptides corresponding to sequences of HLA class I molecules have inhibitory effects on T cell function. The peptides investigated in this study have sequences corresponding to the relatively conserved region of the α1 helix of HLA class I molecules that overlaps the “public epitope” Bw4/Bw6. These HLA-derived peptides exhibit inhibitory effects on T lymphocytes and have beneficial effects on the survival of allogeneic organ transplants in mice and rats. Peptides corresponding to the Bw4a epitope appear most potent as they inhibit the differentiation of T cell precursors into mature cytotoxic T lymphocytes (CTL) and target cell lysis by established CTL lines and clones. To elucidate the mechanism through which these peptides mediate their inhibitory effect on T lymphocytes, peptide binding proteins were isolated from T cell lysates. We show that the inhibitory Bw4a peptide binds two members of the heat-shock protein (HSP) 70 family, constitutively expressed HSC70 and heat-inducible HSP70. Peptide binding to HSC/HSP70 is sequence specific and follows the rules defined by the HSC70 binding motif. Most intriguing, however, is the strict correlation of peptide binding to HSC/HSP70 and the functional effects such that only inhibitory peptides bind to HSC70 and HSP70 whereas noninhibitory peptides do not bind. This correlation suggests that small molecular weight HLA-derived peptides may modulate T cell responses by directly interacting with HSPs. In contrast to numerous reports of HSP70 expression at the surface of antigen-presenting cells and some tumor cells, we find no evidence that HSC/HSP70 are expressed at the surface of the affected T cells. Therefore, we believe that the peptides’ immunomodulatory effects are not mediated through a signaling event initiated by interaction of peptide with surface HSP, but favor a model similar to the action of other immunomodulatory compounds, FK506 and cyclosporin A, with a role for HSC/HSP70 similar to that for immunophilins, FKBP5 and CyP40.
conserved regions in the α1 domain of HLA class I molecules demonstrated that peptides from the α1 helix (amino acid residues 60-84) also affected T cell function (10). The activity of these peptides was mapped to the COOH-terminal ten amino acids and found to correspond to the region of the HLA class I molecule that was recognized in early antiserum typing studies as the mutually exclusive "public epitope," Bw4/Bw6 (11, 12). The limited heterogeneity of this region, with only six different sequences reported to date (Bw4a-d, Bw6a,b), and the conservation of these motifs throughout hominoid evolution (13) suggest that these sequences might have a critical function in the immune response. Indeed, recent data indicate that amino acids in this region are involved in the specificity and regulation of cytolysis by NK cells (14-16).

The effect on T cell function of synthetic peptides corresponding to the Bw4a/Bw6a sequences has been further analyzed (17, 18). Synthetic peptides from both groups, Bw4a (represented by the sequence of the HLA-B2702 allotype) and Bw6a (represented by the sequence of the HLA-B0701 allotype), prevented the differentiation of precursors into effector stage CTL. In addition, Bw4a peptides blocked lysis by established CTL lines. These peptide effects were found to be nonallelic specific, that is, inhibition was observed for CTL specific for a variety of target cells expressing different HLA allelic products. Because of these strong effects on T cell function in vitro, peptides from both groups were investigated in animal transplantation models. Permanent acceptance (tolerance) of heart allografts was achieved in rats when the Bw6a peptide was administered in combination with subtherapeutic doses of cyclosporin A (CsA) (19). Furthermore, both the Bw4a and Bw6a peptide prolonged the survival of skin or heart allografts when administered as monotherapy (20, 21).

To elucidate the mechanism by which these HLA-derived peptides mediate their effect on T cells, experiments were designed to identify peptide binding proteins. We found that the inhibitory peptide B2702 binds two proteins with apparent molecular masses of 70 and 74 kD that are members of the heat-shock protein (HSP) 70 family. The binding is sequence specific and restricted to peptides with T cell inhibitory activity. The physiologic significance of this binding and the potential role of HSP70 family members in mediating the T cell immunomodulatory effects are discussed.

Materials and Methods

HLA-derived Peptides. All peptides were synthesized as described (17). The peptides synthesized include (Tables 1 and 2): peptides B0701.60-84 and B2702.60-84, encompassing the entire α1 helix (amino acids 60-84) of the respective HLA class I molecules (Table 1); and peptides B0701.75-84, B2702.75-84, and B2705.75-84, representing the COOH-terminal halves (amino acids 75-84) of the former peptides (Table 2). Amino acid residues which comprise the public epitope Bw4a (B2702) or Bw6a (B0701), respectively, are underlined in Tables 1 and 2. HLA class I heavy chain sequences are from Zemmour and Parham (22). B2702.60-84 and B2702.75-84 performed similarly in vitro T cell assays with some variability in the activity of the B2702.75-84 peptide dependent upon the sources of serum used in the assays. This variability might be due to the activity of serum proteases to which the shorter peptides are more susceptible than are their longer derivatives. To improve the activity of the B2702.75-84 peptide, dimers were synthesized. B0701.84-75/75-84 and B2702.84-75/75-84 represent inverted repeat dimers of amino acids 75-84 of the respective HLA class I molecules and peptide B2702.75-84/74-85 is a direct repeat of amino acids 75-84 (Table 2). Direct and inverted repeat dimeric peptides performed similarly in T cell assays with slightly enhanced activities over the monomeric versions (not shown). B2702.84-75T/75-84, B2702.84-75T/75-84T, and B2702.84-75T/75-84T contain single or double threonine substitutions (Table 2).

For biochemical analyses, a biotin group was attached to the NH2-terminal amino acid using N-hydroxy-succinimidyl-ester (NHS) activated biotin (NHS-LC-Biotin II; Pierce Chemical Co., Rockford, IL). Attachment of the biotin group had no effect on the T cell immunomodulatory activities of the peptides (not shown). Direct and inverted repeat dimeric peptides performed similarly in biochemical analyses with slightly enhanced activities over the monomeric versions (not shown) thus correlating with the results of the in vitro T cell assays. The studies presented in this article were performed with the inverted repeat dimeric peptides, B2702.84-75/75-84 and B0701.84-75/75-84, abbreviated as 02/02 and B7/B7.

Cells and Antibodies. PBL were isolated from the venous blood of healthy donors via Ficoll-Hypaque density centrifugation. Cytotoxic T cell lines (CTL) were established from PBL by stimulation with irradiated B-lymphoblastoid cell lines (B-LCL) (allogeneic stimulation) (23). Long-term CTL cultures were carried in T cell conditioned medium (24) by weekly stimulation with irradiated B-LCL. Antibodies specific for various stress proteins were purchased from StressGen (Victoria, BC, Canada): IBS-5 (rat IgG1) recognizes the constitutively expressed (HSC70) member of the HSP70 family, C92 (mouse IgG1), is specific for the heat-inducible (HSP70) member of the HSP70 family and N27 (mouse IgG2), which recognizes both HSC70 and HSP70. 10C3 (mouse IgG2a) recognizes the glucose-regulated proteins grp78/immunoglobulin binding protein (BiP) and grp94. The anti-grp75 rabbit-polyclonal antiserum (specific for mitochondrial grp75) was a gift from Dr. W. Welch (San Francisco General Hospital, University of California, San Francisco, CA). Anti-Hsp72 is a mouse ascites (RPNI1197, IgG1) (Amersham Corp., Arlington Heights, IL) with identical specificity to C92. Isotype-matched antibodies (Caltag Laboratories, South San Francisco, CA) were used as controls in immune precipitation assays.

Heat-Shock Treatment. Cells were incubated at 43°C for 1 h and allowed to recover for 3 h at 37°C before metabolic labeling or precipitations were performed.

Metabolic Radiolabeling. For radiolabeling, CTL were used on day 4 or 5 after allogeneic stimulation. 3 × 10^6 cells/ml were preincubated in methionine/cysteine-free RPMI 1640 (ICN Biomed Inc., Costa Mesa, CA) supplemented with 5% dialyzed FCS, 2 mM 1-glutamine, and 1.4 mM sodium pyruvate for 1 h.
mouse antibody (1:5,000). After ECL detection, the membrane was stripped of bound antibodies by incubating it in a solution of peptide 60 Amino acid residues 84 washed sequentially in 1 ml each of 0.6% CHAPS/PBS; TNEN incubated with 5 bL of normal rabbit serum and 250 I*V of a 10% immuneprecipitated with 5 µg of purified monoclonal antibody or 10 µL of rabbit antiserum to biotinylated peptides (0-50 µg/ml) were added to the CTL simultaneously with the untreated samples was between 30 and 50%. HLA-derived peptides (0-50 µg/ml) were added to the CTL simultaneously with the target cells and remained present during the 4-h incubation. All assays were performed in triplicate. The percent specific lysis was calculated by the formula: 100 × [(experimental cpm - spontaneous cpm)/(total cpm - spontaneous cpm)]. Nonbiotinylated and biotinylated peptides gave identical results.

Results and Discussion

The T Cell Inhibitory Peptide B2702 Binds to Proteins with Apparent Molecular Masses of 74 and 70 kDa. Synthetic peptides corresponding to amino acid sequences of the α1 helix of HLA class I molecules, which overlap the public epitope Bw4/Bw6, were found to inhibit T lymphocyte function in vitro (10, 17, 18) and to result in allograft tolerance in transplantation models (19-21). The peptide effects were not allele specific. Therefore, these HLA-derived peptides had the most potent effects in that they inhibited the differentiation of T cell precursors into mature CTL and also inhibited target cell lysis by mature CTL (17, 18).

Table 1. Amino Acid Sequences of Synthetic Peptides Encompassing Residues 60–84 of the α1 Helix of the HLA-B0701 and HLA-B2702 Molecules, Respectively

| HLA-derived peptide | Amino acid residues |
|----------------------|---------------------|
| B0701.60-84          | W D R N T Q I Y K A Q A Q T D R E S L R N L L R G Y |
| B2702.60-84          | W D R E T Q I C K A K A Q T D R E N L R I A L R Y |

Underlined are amino acid residues that comprise the public epitopes Bw4a (B2702) and Bw6a (B0701), respectively.
Table 2. Derivatives of HLA-derived Peptides with Sequences Corresponding to Residues 75–84 of the α1 Helix of Respective HLA Class I Molecules

| HLA-derived peptide | Abbreviation | Amino acid residues position: 75–84 |
|---------------------|--------------|-------------------------------------|
| B0701.75-84         | B7           | RES LR NL RG Y                      |
| B2702.75-84         | 02           | REN LR IA LR Y                      |
| B2705.75-84         | 05           | RED LR TL LR Y                      |
| B0701.84-75/75-84   | B7/B7        | YGR LN RS LR RY                     |
| B2702.84-75/75-84   | 02/02        | YRL AI RN ER REN LR IA LR Y         |
| B2702.84-75T/75-84  | 02T/02       | YRL AT RN ER REN LR TAL RY          |
| B2702.84-75/75-84T  | 02/02T       | YRL AT RN ER REN LR TAL RY          |
| B2702.84-75T/75-84T | 02/02T       | YRL AT RN ER REN LR TAL RY          |
| B2702.75-84/75-84   | 02/02d       | REN LR IA LR Y                      |

Underlined are amino acid residues that comprise the public epitopes Bw4a (B2702) and Bw6a (B0701), respectively.

To elucidate the mechanism through which these HLA-derived peptides mediate their T cell inhibitory effect, peptide binding proteins were isolated from radiolabeled T cell lysates using streptavidin-agarose beads conjugated with biotinylated peptides. Precipitations were performed with the inverted repeat dimeric peptides, B2702.84-75/75-84 and B0701.84-75/75-84, in parallel. Two proteins with molecular masses of 70 and 74 kD were identified that bound to the inhibitory peptide B2702, but not to the noninhibitory peptide B0701 (Fig. 1 A). Additional bands of 78 and 50 kD were found with both the B2702 and B0701 peptide and also with streptavidin-agarose in the absence of any peptide (not shown), indicating that their binding is not peptide dependent. The precipitation of the 74- and 70-kD proteins was also observed with the monomeric peptides, B2702.60-84 and B2702.75-84, and the direct repeats of the B2702 sequence, B2702.75-84/75-84 (not shown). Reproducibly, lower intensity for both protein bands was observed with the monomeric peptide, B2702.75-84. This correlated with slightly reduced inhibi-

![Figure 1](image-url)
Peptide binding proteins correspond to HSC70 and HSP70. Peptide binding proteins and HSPs (HSC70 and HSP70) were precipitated from metabolically labeled long-term CTL lysates using inverted repeat dimers of the B0701 and B2702 peptide, and antibodies, IB5 (specific for HSC70) and anti-72K (specific for HSP70). Before metabolic labeling, half of the culture received heat treatment (43°C). Proteins were analyzed on SDS-PAGE (A) and IEF (B). (B+) Bands corresponding to HSC70 (lane 2, IB5) and HSP70 (lane 4, C92). (Lane 3) The precipitation pattern obtained with the rat IgG1 antibody (isotype control for IB5). The comparison of precipitation patterns obtained with IB5 and isotype control antibody helps to segregate nonspecific from specific bands for the IB5 antibody. (●) Bands present in precipitations with IB5, C92, and the isotype control antibody, rat/ Ig. The nature of these bands is unknown.

The 74- and 70-kD Peptide Binding Proteins Correspond to the Constitutively Expressed (HSC70) and Heat-inducible (HSP70) Members of the HSP70 Family. Molecular mass, regulation of binding by ATP, and the upregulation upon heat-stress suggested that the B2702 peptide binding proteins are members of the HSP70 family. Different members of the HSP70 family were precipitated with respective antibodies and compared to the B2702 peptide binding proteins on SDS-PAGE and IEF. Two glucose-regulated family members, grp78 and grp75, were tested and found to differ in molecular mass and isoelectric point from the 74- and 70-kD peptide binding proteins (Fig. 3). HSC70 and HSP70 are two highly related family members that differ in their expression pattern. HSC70 is expressed constitutively (therefore called HSC70) in all cells and only moderately upregulated upon heat-shock. HSP70, also called the inducible family member, is expressed constitutively only in some human cells, but its expression is highly enhanced upon ex-
Peptide binding proteins are recognized by anti-HSP antibodies on Western blot. Peptide binding proteins were precipitated from unlabeled cellular extracts of long-term CTL using Streptavidin-agarose beads with inverted repeat dimers of the B0701 and B2702 peptides, or Streptavidin-agarose beads without any peptide. In addition, total cell lysate was separated on 8% SDS-PAGE under reducing conditions. After transfer to PVDF membrane, the membrane was sequentially probed, first with anti-HSP70 (C92) and second with anti-HSC70 (1B5). (Arrows) Positions of the 75-kD peptide binding protein (recognized by antibody 1B5) and the 70-kD peptide binding protein (recognized by antibody C92).

Figure 4. Peptide binding proteins are recognized by anti-HSP antibodies on Western blot. Peptide binding proteins were precipitated from unlabeled cellular extracts of long-term CTL using Streptavidin-agarose beads with inverted repeat dimers of the B0701 and B2702 peptides, or Streptavidin-agarose beads without any peptide. In addition, total cell lysate was separated on 8% SDS-PAGE under reducing conditions. After transfer to PVDF membrane, the membrane was sequentially probed, first with anti-HSP70 (C92) and second with anti-HSC70 (1B5). (Arrows) Positions of the 75-kD peptide binding protein (recognized by antibody 1B5) and the 70-kD peptide binding protein (recognized by antibody C92).

Peptide Binding to HSC/HSP70 Is Sequence Specific and Correlates with the Peptide Effects on T Lymphocyte Function.

HSP70 family members are known to bind peptides and the binding motifs have been described recently (29). Alternating hydrophobic or aromatic residues with relative positions 2, 4, and 6 (P2, P4, P6) in the peptide sequence were defined as a motif. In addition, HSC70 favors the binding of peptides with positively charged residues. The B2702 peptide sequence, RENRLRALRY (Table 2), fulfills these binding requirements precisely. The amino acid residues underlined (L, I, L) correspond to the defined hydrophobic anchors, P2, P4, and P6, of the binding motif. The B0701 peptide sequence, RESRNRLRGY (Table 2), does not match the binding motif. Furthermore, Fourie et al. (29) showed that amino acid substitutions at position 4 (P4) in the peptide sequence in particular resulted in significantly reduced binding affinity, indicating position 4 as the main anchor position. Aligning the B2702 sequence to fit the binding motif, the isoleucine residue corresponds to position P4 and is likely to be the main anchor residue for the B2702 peptide. This hypothesis was confirmed by binding studies. Exchanging the first isoleucines for threonine in the B2702.84-75/75-84 inverted repeat peptide resulted in slightly reduced binding to HSC/HSP70. Significantly reduced binding was observed when the second isoleucine was replaced by threonine and exchanging both isoleucines resulted in a peptide, B2702T/T, which no longer bound to HSC70 or HSP70 (Fig. 6). Thus, the binding motif provides an explanation for the failure of the B0701 and B2702T/T peptides to precipitate the 74- and 70-kD molecules.

Most interesting, however, is that the importance of isoleucine in the peptide sequence had previously been defined functionally by comparing peptides derived from closely related HLA-B27 allotype sequences (17). HLA-B2702 and HLA-B2705 are closely related proteins that differ by only three amino acids in the α1 helix (Table 2). Synthetic peptides corresponding to that region were investigated in vitro T cell assays and the B2705 peptide was found ineffective whereas the B2702 peptide inhibited T cell function (17). When the B2705 amino acid residues were singly introduced into the B2702 peptide sequence,
only the substitution of isoleucine for threonine at position 80 caused a loss of the functional effects. The effect of substituting the isoleucine residue was further analyzed using the inverted repeat peptide, B2702.84-75/75-84. The inhibitory effect was reduced when one or the other of the isoleucines was replaced for threonine and exchanging both the isoleucines resulted in a complete loss of function (Fig. 7). This loss of function correlated strictly with the loss of binding to the HSC/HSP70 proteins (Fig. 6 and Table 3).

The binding of B2702 peptides and not B0701 or B2702T/T peptides to HSC/HSP70 proteins can be explained on the basis of the defined HSC70 binding motif. Most intriguing, however, is the correlation between the functional effects and the binding to HSC/HSP70. Without exception to date, peptides with T cell inhibitory effects bind to HSC/HSP70, whereas noninhibitory peptides do not bind. This correlation suggests that the binding to HSC/HSP70 might be of physiological relevance and may be involved in mediating the T cell immunomodulatory effect of the HLA-derived B2702 peptide.

**Peptide-mediated Immunomodulation and the Potential Roles for HSPs.** Consistent with the broad expression of HSC/HSP70 proteins, the 74/70-kD peptide binding proteins were found in whole cell lysates of all cell types studied, including B-LCL, T cell tumor lines, HeLa, a preerythroid cell line (K562), and an endothelial cell line (SK-HEP1) (not shown). The T cell–specific peptide effect must, therefore, be due to an additional mechanism not found in other cell types. Indicative of such a T cell–specific mechanism is the observation that, when intact cells were incubated with the B2702 peptides, HSC/HSP70 proteins were isolated only from T lymphocytes and CTL. One possible explanation for this finding is that HSC/HSP70 are expressed at the surface of the T cells and that the binding of HLA-derived peptides to these surface HSPs initiates signals that inhibit T cell function. Surface expression of HSP70 has been reported for antigen-presenting cells (B cells and monocytes) and some tumor cells, but not for T cells (30–34). Similarly, we find no evidence for surface expression of HSC70 or HSP70 on the affected T cells using cytofluorimetric analyses and monoclonal antibodies specific for HSP70 (C92, N27, anti-Hsp72) or HSC70 (1B5, N27) (not shown). Based upon the lack of evidence for cell surface expression of HSP70 on T cells, we do not favor a model in which HLA-derived peptides mediate their T cell inhibitory activity by signaling through cell surface HSPs. Within such a model, the observation that HSC/HSP70 binding was only observed for T cells when intact cells were used might indicate T cell–specific uptake mechanisms that promote peptide access to the cytosolic HSPs.

**Table 3. Peptide Binding to HSC70/HSP70 Correlates with Peptide Function**

| Peptide | Inhibition of T cell function | Binding to HSC70/HSP70 |
|---------|--------------------------------|------------------------|
| 02/02   | + + +                          | + + +                  |
| 02T/02  | + +                            | + +                    |
| 02/02T  | +                              | +/-                    |
| 02T/02T | –                              | –                      |

**Figure 7.** Amino acid isoleucine is critical for the inhibition of cytolysis by the B2702 peptide. An HLA-A2 restricted CD8+ CTL line was tested for its ability to lyse chromium-labeled HLA-A2 expressing B-LCL, JY. The E/T cell ratio was 2:1 and resulted in a specific lysis of ~35% for the untreated sample. Inverted repeat dimers of the B0701 and B2702 peptides, as well as the single and double threonine-substituted derivatives of the B2702 peptide were added at the indicated concentrations at the beginning of the 4-h assay. Each time point represents the mean of triplicate. This experiment is a representative of five independent assays using HLA-A2, -B43, -B27, or -Cw4 specific CTL lines.
provides substantiating evidence for the hypothesis previously proposed by Srivastava et al. (38) implicating HSP70 in antigen presentation pathways.

DSG and the HLA-derived B2702 peptide, although apparently binding to the same ligand HSC70, differ in their immunomodulatory activity. First, the HLA-derived peptide inhibits allospecific cytotoxic T cells and inhibits T cell proliferation (our unpublished observation), whereas DSG does not inhibit cytosis. Second, the effects of HLA-derived peptides are exerted at the level of the T cell, not at the level of the antigen-presenting cell as demonstrated for DSG (40). Combined, these differences suggest that HLA-derived peptides mediate immunosuppression through a mechanism different than that described for DSG, and that the role of HSC/HSP70 in the peptides' immunomodulation does not involve antigen presentation.

Other immunosuppressive compounds, such as FK506, CsA, and rapamycin, mediate immune suppression by interfering with signaling events required for T cell activation (41). CsA and FK506 both target an intracellular phosphatase, calcineurin, a key enzyme in the signaling cascade resulting in IL-2 transcription and T cell proliferation (42-44). Inhibition of calcineurin activity and hence immunosuppression is dependent upon the formation of a complex between the drug and an immunosuppressant binding protein. Two classes of immunosuppressant binding proteins, FKBPs (for FK506 binding proteins) and cyclophilins (for CsA binding proteins), collectively called immunophilins, have been identified. They are abundantly expressed proteins that are conserved throughout evolution. They are found in different isoforms and are localized to various subcellular compartments. In addition, both exhibit rotamase activity and are postulated to be involved in protein folding, reduction of protein aggregates, and protein translocation (45). These characteristics also apply to the HSP70 family (46). Combined, the biochemical similarities between immunophilins and HSP70 proteins and the finding that HSP70 family members bind immunosuppressive compounds, DSG (39) and the HLA-derived B2702 peptide, indicate that HSP70 family members may represent a third class of immunophilins. Therefore, we propose a function for the HLA-derived B2702 peptide analogous to the mode of action of CsA and FK506 with a role for HSC/HSP70 similar to that of immunophilins, such as CyP40 and FKBP. The definition of an intracellular target protein for the peptide-HSC70 complex, analogous to calcineurin, will be necessary to provide substantiating evidence for the proposed model.

The analysis of immunosuppressive compounds like CsA and FK506 has substantially increased our understanding of signal pathways involved in T lymphocyte activation (41). Investigations on the mechanism by which the HLA-derived peptides mediate their immunomodulatory effects will further extend our knowledge about T cell activation and the regulation of T cell effector function. In addition, the results of these studies may provide new insights into the development of novel immunotherapeutics capable of inducing immunologic tolerance.

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