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 $\alpha 1$ 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, FKBPs and CyP40.

T cell responses triggered by polymorphic differences between MHC class I molecules of recipient and donor tissue are the major barrier to successful transplant engraftment. Despite advances in immunosuppressive therapy, acute graft rejection and failure to achieve long-lasting graft acceptance still persist. Thus, the ultimate goal in transplant biology is to achieve long-lasting, antigen-specific unresponsiveness (immunological tolerance). The beneficial effect of blood transfusion on the survival of organ allografts has been recognized for years (1–3). Although the mechanisms underlying the enhancing effect of blood transfusion are undoubtedly complex, the contribution of soluble HLA class I molecules to induce nonresponsiveness to some allografts is well documented (2, 4, 5). However, studies using soluble HLA have been hindered by the difficulties in purifying sufficient quantities of material. We and others began to investigate the immunomodulatory activi-

ties of soluble HLA using synthetic peptides corresponding to regions of the HLA class I heavy chain that appeared to be functionally important. Peptides corresponding to polymorphic sequences of the $\alpha 1$ and $\alpha 2$ domains of HLA class I molecules were found to inhibit lysis by CTL (6, 7). The inhibition was allele specific in that a given peptide could affect lysis only of CTL specific for the HLA molecule from which it was derived. For clinical application these peptides would be of limited value, as each donor–recipient combination would require a different cocktail of allele-specific peptides. Synthetic peptides corresponding to the conserved region of the $\alpha 3$ domain of HLA class I molecules, which is involved in the interaction of HLA class I with its CD8 coreceptor, prevented the differentiation of precursor T cells into mature CTL, but they failed to inhibit cytolysis of mature CTL (8, 9).

Recent studies using peptides corresponding to more

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 antisera 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 nonallele 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)[1] (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

[1] *Abbreviations used in this paper:* BiP; immunoglobulin binding protein; B-LCL, B lymphoblastoid cell line; CsA, cyclosporin A; DSG, deoxyspergualin; grp, glucose-regulated protein; HSP, heat–shock protein; HSC70, constitutively expressed member of the heat–shock protein 70 family; HSP70, heat-inducible member of the heat–shock protein 70 family; NHS, N-hydroxy-succinimidyl-ester; TNEN, NP40, Tris-HCl, NaCl, EDTA.

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 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 B2702.75-84/75-84 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, B2702.84-75/75-84T, 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): 1B5 (rat IgG1a) 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 (RPN1197, 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 Biomedical Inc., Costa Mesa, CA) supplemented with 5% dialyzed FCS, 2 mM L-glutamine, and 1.4 mM sodium pyruvate for 1 h.

Then [35S]methionine/cysteine (50 μCi/ml of ProMix; Amersham Corp.) was added to the culture and the cells further incubated for 4 h. After radiolabeling, cells were washed twice in ice-cold PBS.

Preparation of Peptide Affinity-matrix. Saturating amounts of biotinylated peptide were incubated with Streptavidin-agarose beads (Pierce Chemical Co.) for 2 h at room temperature. Before use in precipitation assays, unbound peptide was removed by extensive washing in PBS.

Precipitation of Peptide Binding Proteins. For each precipitation, 5–7 × 10⁶ radiolabeled CTL were used. Cell pellets were lysed in 500 μl of 0.6% CHAPS (3-[(cholamidopropyl)-dimethylammonio]-1 propanesulfonate)/PBS, pH 7.4, containing protease inhibitors, pepstatin (1 μg/ml), leupeptin (10 μg/ml), and PMSF (0.2 mM) on ice for 30 min. Cell debris was spun out by microcentrifugation for 10 min at 4°C. Peptide binding proteins were isolated by incubating cell lysates with peptide/Streptavidin–agarose beads for 1.5 h at 4°C. Where indicated, 5 mM of either ATP or γS-ATP (Boehringer Mannheim, Indianapolis, IN) was added simultaneously with the peptide matrix. After incubation, Streptavidin-agarose beads were pelleted from the lysate and washed sequentially in 1 ml each of: 0.6% CHAPS/PBS; TNEN (0.5% NP40, 20 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 10 mM EDTA) diluted 1:10 in dH₂O and supplemented with 0.05% deoxycholic acid, 0.01% SDS; and a 1:10 dilution of TNEN containing 0.5 M NaCl.

Precipitation of Stress Proteins. Radiolabeled cell lysates were incubated with 5 μl of normal rabbit serum and 250 μl of a 10% solution of protein A positive *Staphylococcus aureus* cells (Boehringer Mannheim) for 2–4 h at 4°C (preclearing). *S. aureus* cells were spun out and the precleared lysates immunoprecipitated with 5 μg of purified monoclonal antibody or 10 μl of rabbit antiserum or ascites fluid and 50 μl of packed protein G–Sepharose beads (Pharmacia Biotech Inc., Alameda, CA) for 1.5 h at 4°C. Immune complexes were collected by centrifugation and washed sequentially in 1 ml each of: TNEN supplemented with SDS (0.1%) and deoxycholic acid (0.5%); TNEN, diluted 1:10 in dH₂O and supplemented with 0.5 M NaCl.

Electrophoresis. Precipitated proteins were separated by reducing SDS-PAGE (25) or IEF (26).

Western Blot Analysis. Peptide binding proteins were precipitated from unlabeled cell lysates as described above. After separation on SDS-PAGE, proteins were transferred to polyvinyldifluoride (PVDF) membrane (Millipore Corp., Bedford, MA). Immunodetection using enhanced chemiluminescence (ECL; Amersham Corp.) was performed according to the manufacturer's instruction. The membrane was probed first with anti-HSP70 antibody (C92, 1:2,000) and horseradish peroxidase–conjugated anti–mouse antibody (1:5,000). After ECL detection, the membrane was stripped of bound antibodies by incubating it in a solution of

100 mM β-mercaptoethanol/2% SDS/62.5 mM Tris-HCl, pH 6.8, for 30 min at 50°C. Then the membrane was reprobed using anti-HSC70 antibody (1B5, 1:5,000) and horseradish peroxidase–conjugated anti–rat antibody (1:5,000).

One-dimensional Peptide Mapping with V8 Endoproteinase Digestion. Radiolabeled protein bands were eluted from nonfixed, dried SDS-PAGE gels in PBS/1% SDS over 2 d. After acetone precipitation, proteins were solubilized in 40 μl of V8 digestion buffer (0.125 mM Tris-HCl, pH 6.8, containing 10% glycerol, 1 mM EDTA, 0.1% SDS, and 0.001% bromophenol blue) and loaded onto 15% SDS-PAGE. Digestion was performed "in gel" (27, 28) by overlaying the protein solution with 0.5 μg of *S. aureus* V8 endoproteinase (Boehringer Mannheim) and running the sample through the stacking gel slowly. At the stacking/separating gel interface, the gel run was interrupted for 30 min to allow further digestion. Then, electrophoresis was performed as usual (25).

Cell-mediated Cytotoxicity Assay. Cell killing was measured using a standard 4-h ⁵¹chromium radioisotope release assay (24). Briefly, CTL lines specific for allogeneic HLA class I molecules were cultured as described above. B-LCLs expressing the HLA class I molecule recognized by the CTL line were labeled with ⁵¹chromium (Amersham Corp.) for 1 h and added to the CTL at an effector to target cell ratio such that the percent specific lysis in 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 kD. 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 are of particular interest in transplant therapy as they can potentially be used for all donor–recipient combinations. Peptides with sequences corresponding to the Bw4a epitope 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	60									Amino acid residues															84
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	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											R	E	S	L	R	N	L	R	G	Y
B2702.75-84	02											R	E	N	L	R	I	A	L	R	Y
B2705.75-84	05											R	E	D	L	R	T	L	L	R	Y
B0701.84-75/75-84	B7/B7	Y	G	R	L	N	R	L	S	E	R	R	E	S	L	R	N	L	R	G	Y
B2702.84-75/75-84	02/02	Y	R	L	A	I	R	L	N	E	R	R	E	N	L	R	I	A	L	R	Y
B2702.84-75T/75-84	02T/02	Y	R	L	A	T	R	L	N	E	R	R	E	N	L	R	I	A	L	R	Y
B2702.84-75/75-84T	02/02T	Y	R	L	A	I	R	L	N	E	R	R	E	N	L	R	T	A	L	R	Y
B2702.84-75T/75-84T	02/02T	Y	R	L	A	T	R	L	N	E	R	R	E	N	L	R	T	A	L	R	Y
B2702.75-84/75-84	02/02d	R	E	N	L	R	I	A	L	R	Y	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.
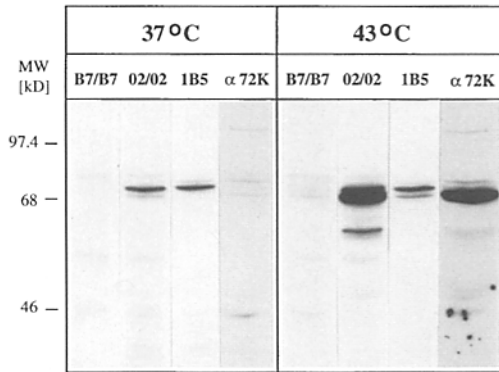
To elucidate the mechanism through which these HLA-derived peptides mediate their T cell inhibitory effect, peptide binding proteins were isolated from radiolabeled T cell lysates using Streptavidin-agarose beads conjugated with biotinylated peptides. Precipitations were performed with the inverted repeat 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-
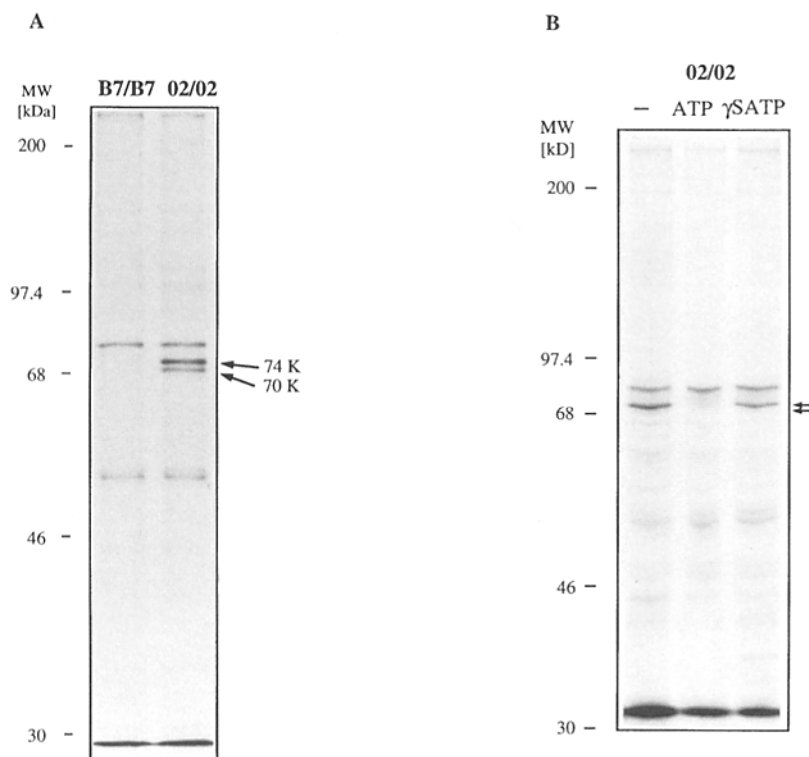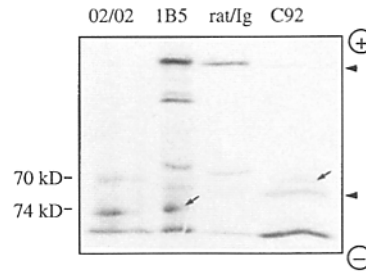

Figure 1. T cell inhibitory peptide, B2702, binds to 74- and 70-kD proteins in an ATP-dependent manner. Biosynthetically labeled CTL lysates were incubated with biotinylated inverted repeat dimers of the B0701 (*B7/B7*) and B2702 (*02/02*) peptide bound to Streptavidin-agarose beads. Peptide binding proteins were analyzed on 8% SDS-PAGE (*A*). Incubation in the presence of ATP (5 mM) abrogated precipitation of the 74- and 70-kD proteins by the B2702 peptide, whereas γS-ATP had no effect (*B*).

## A 7.5 - 12.5 % SDS-PAGE

B Isoelectric focusing

Figure 2. 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, 1B5 (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*, *1B5*) and HSP70 (lane *4*, *C92*). (Lane *3*) The precipitation pattern obtained with the rat IgG1 antibody (isotype control for *1B5*). The comparison of precipitation patterns obtained with 1B5 and isotype control antibody helps to segregate nonspecific from specific bands for the 1B5 antibody. (◄) Bands present in precipitations with 1B5, C92, and the isotype control antibody, rat/Ig. The nature of these bands is unknown.

tory activity in CTL assays suggesting that peptide length may be important for both binding to the 74- and 70-kD proteins and inhibitory function (our unpublished observation; see also Materials and Methods). When the lysis buffer was supplemented with ATP, the B2702 peptides no longer bound to the 74- and 70-kD proteins (Fig. 1 *B*). The nonhydrolysable ATP derivative, γS-ATP, had no effect, suggesting that ATP hydrolysis abrogates the binding of the B2702 peptide to the 74- and 70-kD proteins.

Heat–shock treatment of cells before metabolic labeling resulted in a dramatic increase in intensity for the 70-kD band and a modest increase for the 74-kD band (Fig. 2 *A*). The weak signal observed for the 70-kD protein under normal growth conditions, therefore, reflects lower quantities of the radiolabeled 70-kD protein present in cells grown under normal conditions, rather than lower affinity of the B2702 peptide for the 70-kD protein compared with the 74-kD protein.

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 HS̲C70) 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-

A 8% SDS-PAGE
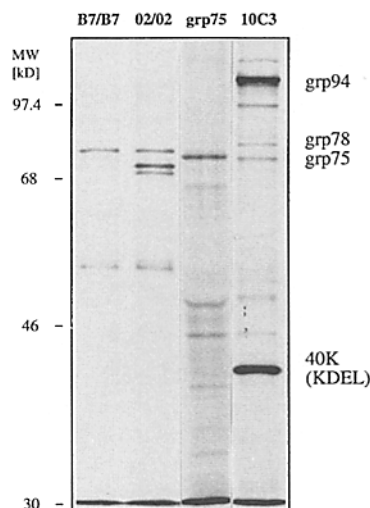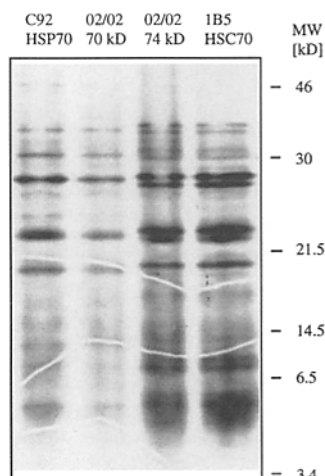

B Isoelectric focusing
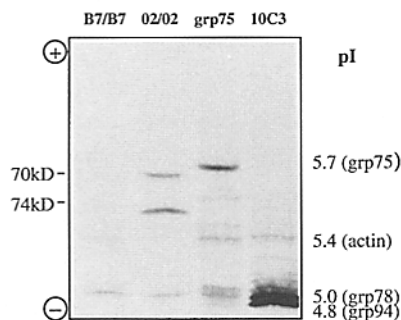
Figure 3. Peptide binding proteins are not identical to grp75 and grp78/BiP. Peptide binding proteins and grp75 and grp78/BiP proteins were precipitated from metabolically labeled long-term CTL lysates using biotinylated inverted repeat dimers of the B0701 (*B7/ B7*) or B2702 (*02/02*) peptide and antibodies to grp75 and grp78/BiP (*10C3*), respectively. Each precipitation was split into two aliquots and analyzed on 8% reducing SDS-PAGE (*A*) or IEF (*B*).
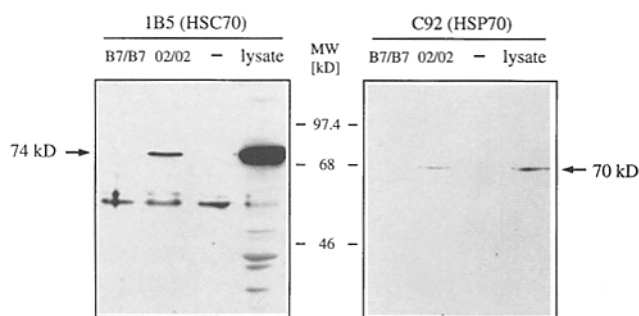
Figure 4. Peptide binding proteins are recognized by anti-HSP antibodies on Western blot. Peptide binding proteins were precipitated from unlabeled cellular extracts of long-term CTL using Streptavidin-agarose beads with inverted repeat dimers of the B0701 and B2702 peptides, or Streptavidin-agarose 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).

posure to high temperature. The 74- and 70-kD peptide binding proteins closely resemble the HSC70 and HSP70 proteins in electrophoretic mobility on SDS-PAGE (Fig. 2 A) and IEF (Fig. 2 B) and display identical response to heat (Fig. 2 A). Furthermore, in Western blot analysis the 74-kD peptide binding protein was recognized by 1B5 (specific for HSC70) and the 70-kD peptide binding protein was recognized by C92 (specific for HSP70) (Fig. 4). Thus, the 74- and 70-kD peptide binding proteins appeared to correspond to HSC70 and HSP70 proteins, respectively. This hypothesis was confirmed by digestion with V8 endoproteinase, which created identical patterns for the 74-kD protein and HSC70 and the 70-kD protein and HSP70 (Fig. 5).
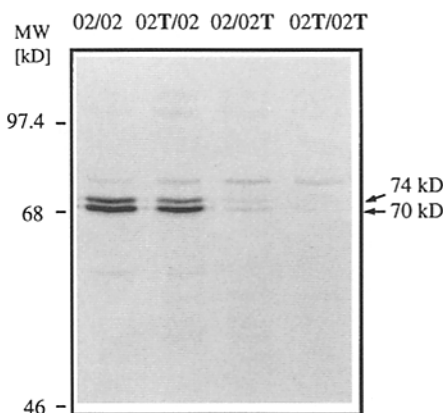
Peptide Binding to HSC/HSP70 Is Sequence Specific and Correlates with the Peptide Effects on T Lymphocyte Function. HSP70 family members are known to bind peptides and the binding motifs have been described recently (29). Alternating hydrophobic or aromatic residues with relative

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, RENLRIALRY (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, RESLNRLRGY (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 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,


Figure 5. V8 digestion creates identical peptide patterns for the 74- and 70-kD peptide binding proteins and HSC70 and HSP70, respectively. Proteins were precipitated from metabolically labeled CTL lysates and separated on 8% SDS-PAGE. After detection by autoradiography, bands corresponding to peptide binding proteins, 74 and 70 kD, HSC70 (precipitated with antibody 1B5) and HSP70 (precipitated with antibody C92) were excised, eluted, and reelectrophoresed on 15% SDS-PAGE in the presence of 0.5 μg V8 endoproteinase.

Figure 6. Substitution of isoleucine for threonine abrogates peptide binding to HSC70 and HSP70. Inverted repeat dimers of the B2702 peptide and its single and double threonine-substituted derivatives, 02/02T, 02T/02, and 02T/02T, were used to precipitate peptide binding proteins from metabolically labeled long-term CTL total cell extracts.
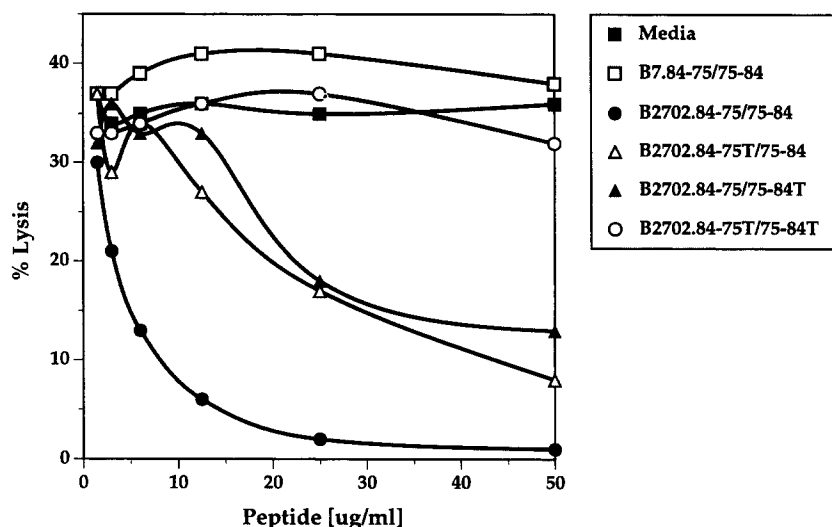
Figure 7. Amino acid isoleucine is critical for the inhibition of cytolysis by the B2702 peptide. An HLA-A2 restricted CD8^{+} CTL line was tested for its ability to lyse [51]chromium-labeled HLA-A2 expressing B-LCL, JY. The E/T cell ratio was 2:1 and resulted in a specific lysis of ~35% for the untreated 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.

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 petpide.

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

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	−	−

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. We proposed that HLA-derived peptides function intracellularly much like other immunosuppressive reagents, such as CsA and FK506, with a role for HSC/HSP70 similar to immunophilins, FKBP and CyP40 (see below). 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.

HSPs and Immune Suppression. The role of HSPs in antigen presentation and tumor immunity is currently an area of intense investigation (33, 35–38). Of particular interest in this context is the observation that an immunosuppressive drug, deoxyspergualin (DSG), was found to bind to HSC70 (39). Recently, it has been demonstrated that DSG exerts its immunosuppressive activity at the level of the monocytes, apparently by interfering with their antigen presentation function (40). Therefore, the DSG system

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 cytolysis. 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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