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Isolation of MHC Class I-Restricted Tumor Antigen Peptide and Its Precursors Associated with Heat Shock Proteins hsp70, hsp90, and gp96

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We have previously demonstrated that vaccination with heat shock proteins hsp70, hsp90, and gp96 elicits specific immunity against the tumor from which the hsps were purified. Although the association of tumor Ag peptides with these hsps have been suggested, the identification of the peptides or their precursors stripped from the hsps remained to be resolved. We show in this report that an L1-restricted cytotoxic T lymphocyte epitope of a mouse leukemia RL1 is associated with its precursors. The gp96 was associated with the octamer and one of the two precursors. Thus, each of the hsps bound a distinct set of peptides. Our results have implications for the first time that the hsps associate not only with final sized tumor Ag peptide but also with its precursors. The implication of this evidence is also discussed in terms of the roles of hsps in MHC class I Ag processing/presentation.

Materials and Methods

Animals and cell lines

RL1 and Meth A cells were expanded in ascitic form in BALB/c mice. The mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in pathogen-free condition at the animal facility of Okayama University Medical School (Okayama, Japan). RL1-specific CTL clone Y-15 was maintained by weekly stimulation with irradiated RL1 cells and feeder cells as described previously (16).

Purification of gp96, hsp90, hsp70, and L4 molecules

The gp96 and hsp90 were purified simultaneously as described previously (17). Briefly, a 10-ml cell pellet (~10^10 cells) of ascitic RL1 cells was homogenized in 40 ml of a hypotonic buffer (30 mM NaHCO3, and 0.5 mM PMSF (pH 7.1)), and its 100,000 × g supernatant was applied to a Con A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) column followed by elution with 10% a-methyl-mannopyranoside. For isolation of gp96, Con A-bound material was resolved on a Mono Q FPLC.
Materials

1000 × g supernatant of the RL1 cell homogenate was dialyzed against 20 mM of sodium phosphate (pH 7.4), 1 mM EDTA, and 15 mM 2-ME. The proteins were resolved by 6% native PAGE and visualized by staining with Coomassie Brilliant Blue R-250. The bands of the stained hsp70-lactalbumin complex were quantitated by densitometry.

Dissociation of the endogenous peptides from gp96, hsp90, and hsp70

Approximately 2 mg of the purified hsp70 preparations in a 1-ml volume were exposed to 10 mM ATP and 3 mM MgCl2 at 37°C for 1 h. Purified gp96 and hsp90 preparations (2 mg each) and immunofinity-purified Ld were recovered from 1.5–2 mg of gp96, 2–2.5 mg of hsp70, and 4–6 mg of hsp90.

Cytotoxicity assay

The fractions collected by reverse phase HPLC were completely evaporated by the Speed-Vac and dissolved in 150 μl of 0.1% TFA. P-815 target cells were labeled by incubating 2 × 106 cells with 2 MBq of Na251CrO4 in 0.3 ml of RPMI (with 10% FCS) for 1 h at 37°C under 5% CO2 in air. The cells were washed and cultured in 100 μl of medium at 5 × 103 cells/well in a 96-well round-bottom plate with sensitization by the peptides containing each 10 μl of each HPLC fractions for 1 h. Then the RL1-specific CTL clone 3Y-15 cells were added and cultured for 4 h. Finally the supernatants (100 μl) were removed, and their radioactivities were measured. The percent specific 51Cr release was calculated by the following equation: [(a – b)(c – b)] / a × 100, where a is the radioactivity in the supernatant of target cells mixed with effector cells, b is the spontaneous release, and c is the maximum release after lysis of target cells with 1% Nonidet P-40.

Peptide synthesis

Peptides were synthesized by standard solid phase methods using F-moc chemistry in a peptide synthesizer (model 430A, Applied Biosystems, Foster City, CA). The peptides were purified by reverse phase HPLC on a preparative C18 column (10 × 100 mm, 20-mm particle size; Applied Biosystems) in 0.1% TFA with an acetonitrile gradient and freeze-dried for stock.

Mass spectrometry analysis

The masses of the peptides were determined on-line by a tandem quadrupole mass spectrometer (TSQ 700, Finnigan MAT, San Jose, CA) equipped with an electrospray ion source. The peptides were identified by their molecular mass as the m/z peaks of single charged ion.

Competition assay by pRL1a and pRL1b of complex formation between hsp70 and lactalbumin

Peptide pRL1a and pRL1b binding to hsp70 was evaluated by their competition of complex formation between hsp70 and the unfolded form of lactalbumin as described previously (20). Four micrograms of hsp70 (final concentration, 2.8 mM) and 11 μg of lactalbumin (final concentration, 40 mM) suspended in PBS, pH 7.2 were incubated at 37°C for 30 min with or without gradient doses of pRL1a and PRL1b as indicated in Fig. 3, then resolved by 6% native PAGE and visualized by staining with Coomassie Brilliant Blue R-250. The bands of the stained hsp70-lactalbumin complex were quantitated by densitometry.
Results

**Dissociation of the endogenous peptides from gp96, hsp90, and hsp70 purified from RL\(\beta\)1 and fractionation of the peptides by reverse phase HPLC**

The CTL epitope of the RL\(\beta\)1 mouse leukemia has been identified previously as the octamer epitope (pRL1a), derived from a mutated akt gene product (13). A putative precursor of pRL1a, an amino terminal-extended 10 mer peptide (pRL1b), was also identified in this previous study. Association of pRL1a, pRL1b, and other possible precursors with the chaperones hsp70, hsp90, and gp96 was investigated. Fig. 1A shows apparently homogeneous preparations of gp96 (lane a), hsp90 (lane b), and hsp70 (lane c) purified from RL\(\beta\)1 cells and examined by SDS-PAGE and silver staining. In our preparation, another member of the hsp70 family, grp78 was not detected. Immunoblotting of preparations by anti-grp94, anti-hsp90, and anti-hsp70 mAbs was used to confirm their identity and the lack of cross-contamination of the preparations (Fig. 1B). Peptides associated with three hsps were isolated as described in Materials and Methods and were separated in a C\(_{18}\) reverse phase column. The chromatograms of peptides dissociated from gp96, hsp90, and hsp70 are shown in Fig. 2, a–c. A distinct profile was observed for the peptides eluted from each hsp's preparation. The synthetic 8 mer peptide pRL1a and its putative 10 mer precursor pRL1b were also analyzed, and the chromatograms were shown to elute in fractions 39 and 46, respectively (Fig. 2, d and e).

**Sensitization of P815 cells with endogenous peptides dissociated from hsp70, hsp90, gp96, and L\(^d\) molecules, for lysis by CTL clone**

All peptide fractions from each of the hsps, L\(^d\) molecules, and synthetic peptides pRL1a and pRL1b were used to pulse P815 cells and were tested for recognition by CTL clone Y-15, which recognizes the 8 mer peptide, pRL1a, in association with L\(^d\) molecules. We gave careful attention to avoid the carried over peptides with each other; thus, a blank run before running the biological samples was performed in each experiment and confirmed there were no carried over peptides in each preparation. Among RL\(\beta\)1-derived, hsp70-associated peptides eluted by treatment with ATP (Fig. 3A-a) or TFA (Fig. 3A-b), significant sensitization activity was observed in fraction 39, which corresponds to the precisely eluted position of L\(^d\)-associated or the synthetic 8 mer pRL1a (Fig. 3, A-e and A-f). A molecule with a molecular mass (m/z) of 809, which is precisely the mass of pRL1a, was identified in fraction 39 of the hsp70-associated peptides (Fig. 3, B-I and B-II). As shown in Fig. 3A-c, hsp90 was found associated with three different peptides eluted in fractions 32, 39, and 46, which were able to sensitize P815 cells for lysis by the CTL clone Y-15. The position of fraction 39 has been shown to be identical with that of pRL1a, while fraction 46 contains the synthetic putative precursor 10 mer pRL1b (Fig. 3A-g). A molecule with an m/z value of 1008, precisely equal to that of pRL1b, was identified in fraction 46 of the hsp90-associated peptides from RL\(\beta\)1 (Fig. 3, B-III and B-IV). Thus, hsp90 was associated with the final sized CTL epitope as well as with two other putative precursor peptides, one of which is pRL1b and the other of which was unknown. The size of the precursor peptide eluted in fraction 32 was deduced to be larger than a 10 mer on the basis of the following observations. Peptides shorter than 8 mer pRL1a had no sensitizing activity (data not shown), while the 8 mer pRL1a and 10 mer pRL1b eluted in fractions 39 and 46, respectively. The 9 mer intermediate between pRL1a and pRL1b was observed to elute in fraction 36 or 37 (data not shown). Thus, the sensitizing activity in fraction 32 was >10 mer. The chaperone gp96 was found associated with both pRL1a and pRL1b eluted in fractions 39 and 46, respectively (Fig. 3A-d). No sensitizing activity was detected in fraction 32 or any other fraction among the peptides eluted from gp96. No positive fractions were identified in any peptide fractions eluted from hsp70, hsp90, and gp96 isolated from altered akt-negative BALB/c fibrosarcoma Meth A (Fig. 3A-h).
Requirement of serum proteases for hsp90- and gp96-associated precursor peptides to sensitize P815 cells for lysis by the CTL clone

The pRL1a, but not the pRL1b, was detected in TFA-treated immunoaffinity-purified L<sup>d</sup> preparations (Fig. 3A-c) (13), indicating that this putative precursor peptide pRL1b does not bind L<sup>d</sup>. It was presumed that the 10-mer pRL1b and the longer precursor in hsp90-eluted fraction 32 are able to sensitize P815 cells for lysis by the CTL clone Y-15 because of an exopeptidase activity in the serum-containing medium in which the assay was performed. In fact, the synthetic peptide pRL1b was shown to lose its sensitizing activity of P815 cells for lysis by CTLs in the serum-free plain RPMI medium (18). This premise, especially in the case of natural peptides associated with the hsps, was tested directly by carrying out the assay in medium with or without serum. It was observed that (Table I) synthetic peptide pRL1a and peptides in fraction 39 derived from hsp70, hsp90, and gp96 were not impaired in this medium, indicating that the putative precursor peptides are real
precursors and must be trimmed at the N-terminus to bind Ld molecules in the medium containing FCS. We cannot determine whether C-terminal trimming of the peptide in fraction 32 from the hsp90 preparation is also necessary.

**Binding of pRL1a peptide to hsp70 does not occur after cell lysis**

The 8-mer pRL1a was added to a suspension of Meth A cells, which do not express this Ag, and hsp70 preparations were derived from the lysate of such Meth A cells. The concentration of peptide added to Meth A (320 pM final concentration) was severalfold higher than an abundant estimate of the natural peptide in RL.α 1. If the association of pRL1a peptide with hsp70 can occur after cell lysis, hsp70 preparations derived from the Meth A cell lysate, to which large quantities of pRL1a have been added, should be found associated with pRL1a. Peptides eluted from such an hsp70 preparation were used to pulse P815 cells, which were then tested for recognition of pRL1a by CTL clone Y-15. No recognition was observed (Table II). In contrast, peptides eluted from hsp70 preparations derived from the RL.α 1 could sensitize P815 cells for lysis by CTL clone Y-15 successfully (Table II). Similar studies with hsp90 and gp96 are unnecessary, as association of these hsps with peptides in vitro requires harsh conditions, such as high temperatures or the use of denaturants (17), which were not used in their purification here (see Materials and Methods).

**Table II. Binding of peptides to hsp70 does not occur after cell lysis**

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>No Peptide</th>
<th>pRL1a</th>
<th>pRL1b</th>
<th>Synthetic peptides (P815 in AIM-V, serum-free medium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P815 in medium with fetal calf serum</td>
<td>4.9</td>
<td>66.9</td>
<td>73.8</td>
<td>Hsp70, fraction 39</td>
</tr>
<tr>
<td>P815 in AIM-V, serum-free medium</td>
<td>11.4</td>
<td>70.5</td>
<td>23.9</td>
<td>Hsp90, fraction 39</td>
</tr>
<tr>
<td>(% inhibition)</td>
<td>(0)</td>
<td>(0)</td>
<td>(67.6)</td>
<td>FCS</td>
</tr>
<tr>
<td></td>
<td>(0)</td>
<td>(67.9)</td>
<td>(20.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(12.1)</td>
<td>(64.3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table I. *Hsp90 and gp96 are associated with precursor peptides, whose ability to sensitize p815 cells for lysis by CTL clone Y-15 requires their trimming by serum proteases*.

- Sensitization of P815 cells for Y-15 with the synthetic peptide pRL1a, pRL1b, and hsp-associated natural peptides (fraction 39 eluted from hsp 70; fractions 32, 39, and 46 from hsp90; fractions 39 and 46 from gp96). 31Cr-release assay was performed in the presence or absence of FCS. In the absence of FCS, AIM-V serum free medium shows the results at the concentration of 6.2×10−8 M to 2.3×10−7 M. The table shows the results at the concentration of 6.2×10−8 M of pRL1a and pRL1b. E:T ratio was 20:1.

The specificity of the observed association of pRL1a to the hsp70 was tested in another manner. Although hsp70 was found in association with pRL1a alone (Fig. 3, A-a and A-b), studies in vitro indicate that hsp70 can bind equally effectively with both pRL1a and pRL1b; this was demonstrated by competition studies in which the abilities of the two peptides to inhibit formation of the hsp70-lactalbumin complex was tested quantitatively as described in Materials and Methods (20). It was observed that generation of the hsp70-lactalbumin complex, as judged by native gel electrophoresis, was inhibited equally in a dose-dependent manner by pRL1a and pRL1b (Fig. 4). Thus, the observed association of pRL1a, but not pRL1b, with hsp70 is significant and occurs in vivo; if the observation were the result of artifactual association of peptides with hsp70 after cell lysis, pRL1a and pRL1b would both be found in association with hsp70 molecules. This is not the case. Thus, pRL1b is not available to hsp70 in vivo.

**Discussion**

Our studies show that a tumor-specific CTL epitope and its precursors are associated with cytosolic hsp90 and hsp70 and with ER luminal hsps gp96. Hsp90 was found associated with the final sized 8 mer epitope as well as two other precursor peptides, while hsp70 was associated with only the 8 mer epitope, and gp96 was associated with the 8 mer epitope and the 10 mer precursor peptide. The association of hsps with peptides, as reported here, occurs in vivo and does not reflect a random association of peptides with hsp after cell lysis. Thus, exogenously added pRL1a did not associate with endogenous hsp70 (Table II), and pRL1b, although capable of binding hsp70 in vitro, was not found in association with it (Fig. 4). In other studies, it has been demonstrated that addition of a broad array of labeled peptides of diverse sizes and sequences to cell suspensions before lysis does not lead to their association with gp96 (A. Menoret and P. K. Srivastava, manuscript in preparation). Further, as observed here, the patterns of association of peptides were distinct and specific for each hsps; while hsp70 was found associated solely with pRL1a, gp96 is observed associated with both pRL1a and pRL1b, and hsp90 associated with pRL1a, pRL1b, and the larger precursors. In three complete sets of experiments repeated, no deviation from this pattern was detected.

Another issue that needs to be addressed has to do with the possibility that the exact octamer or the putative precursor peptides found associated with the three hsps could have dissociated from
The association of hsps with the exact-sized MHC I ligand and its precursors might be implicated as the roles of the hsps in MHC I Ag processing/presentation. Peptides associated with the MHC I molecules are eight or nine amino acids long (21), and peptides with different motifs bind specific MHC I alleles (22). The peptides or their precursors originate in the cytosol (23–25) and are transported into the ER, where the association with MHC I molecules occurs (26). The events between the generation of peptides in the cytosol and their final association with MHC I molecules are not fully understood. The peptides are clearly not present in the cytosol in a freely diffusible manner; it has been proposed that molecular chaperones of the hsps family, such as hsp90 and hsp70 in the cytosol and gp96 in the lumen of the ER, constitute a molecular relay line that chaperones the peptides from their generation in the cytosol to their binding to MHC I in the ER (27).

Very recently, Shimbara et al. have shown that pRL1b precursor peptide, but not pRL1a, was produced from synthetic peptide by proteasome in the presence of the IFN-γ-inducible activator PA28 (28). To date, pRL1b is the true precursor peptide and needs to be trimmed to become Ld ligand-pRL1a by aminopeptidases. In this context, our data may suggest that the precursor peptide pRL1b (and presumably larger precursors, such as those in fraction 32 of hsp90-associated peptides) are received by hsp90 first, followed by N-terminal trimming of some of the precursor molecules through as yet uncharacterized mechanisms in the cytosol. This idea is consistent with recent reports that demonstrate physical association of hsp90 molecules with the proteasomes (29, 30). However, not all the precursor molecules delivered to hsp90 from the proteasome are completely processed, as the ER luminal hsps gp96 is still observed associated with the pRL1b precursor. In addition, a proportion of precursor peptides may bypass hsp90 altogether and thus go on to associate with gp96. Interestingly, hsp70 molecules are not observed associated with precursor peptides. In light of the reported proteolytic activity of hsp70 (31), this observation might suggest that any precursors that come to associate with hsp70 are rapidly cleaved to the final product. If the transport of pRL1a, pRL1b, and other precursor peptides into the ER is TAP dependent, pRL1b would be expected to be transported at a higher efficiency than pRL1a because of the inhibitory presence of proline at position 2 (32) in the octamer but not in the 10-mer pRL1b. Nevertheless, it is conceivable that the constraints on the ability of TAP molecules to transport peptides do not reflect the situation in vivo, as they have been deduced by monitoring the transport of exogenously added free peptides to TAP-containing vesicles or permeabilized cells. Thus, while free peptides with proline at position 2 may not be transported by TAP molecules, such peptides chaperoned by hsps may be transported by the same TAP molecules more efficiently. The recent observations of Lammert et al. (19, 33), Spee and Neefjes (34), and Marusina et al. (35) are in accord with our present observation and previous speculation that a relay line of hsps chaperones peptides from the point of their generation in the cytosol to their association with MHC I-β2m molecules in the ER. These authors have demonstrated that peptides associate with a number of peptide-binding proteins in the cytosol (gp100) and in the lumen of the ER (gp96, protein disulfide isomerase, gp120, and gp170). Our studies suggest that hsps are in a position to play an important role in the transport of antigenic peptides to MHC I molecules.

Regardless of the precise mechanisms by which the precursor peptides are transported into the ER, our results are the first evidence showing that hsps localized in distinct intracellular compartments are associated with different sets of precursors of MHC I-binding tumor Ag peptide.
Acknowledgments

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References