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Identification of HLA-A24-Restricted Epitopes with High Affinities to Hsp70 Using Peptide Arrays

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Heat shock protein 70 (Hsp70) family members are known as facilitators of immune responses by interacting with receptors on antigen-presenting cells leading to Hsp70-peptide uptake and antigen cross priming. Here, identification of human leukocyte antigen (HLA)-A24-restricted epitopes was achieved using peptide arrays for evaluation of their affinities to Hsp70 and HLA-A24 binding prediction tools. Using Hsp70 as the model antigen, the GYPVTNAVI and VFQHGKVEI peptides were identified as antigens. These peptides actually bound to HLA-A24 in the stabilization assay using T2-A*2402 cells, and induced a strong peptide-reactive cytotoxic T lymphocyte response in HLA-A24 transgenic mice after vaccination.

[**Key words:** Hsp70, peptide array, chaperone, HLA-A24 restricted epitopes, antitumor vaccines]

Heat shock proteins (Hsps) are molecular chaperones that participate in numerous normal cellular processes such as protein folding, transport, and peptide processing and trafficking. Recently, Hsps have been shown to serve as facilitators of immune responses in tumors *in vivo* and *in vitro* (1–8). Hsp70, Hsp90, gp96, calreticulin, and Hsp110 have been reported to form highly immunogenic Hsp-peptide complexes. Immunization with Hsp70 purified from tumor cells containing tumor-specific peptides induces a strong cytotoxic T lymphocyte (CTL) response against tumor cells after autologous vaccination (7–10). Hsp70 expressed under hyperthermia has also been reported to induce antitumor immunity by producing the Hsp70-immune complex via necrotic tumor cell death *in vivo* (11). Furthermore, synthetic peptides fused to Hsp70 *in vitro* induce MHC class I-restricted CTL responses (9).

Hsps facilitate induction of the immune responses by the following mechanisms. Hsp70 and several other molecular chaperones have the ability to bind to antigenic peptides stably in the cytosol of tumor cells during antigen processing. Hsps are efficiently internalized by antigen-presenting cells (APCs) via receptor-mediated endocytosis (12). Following internalization, these proteins are trafficked into different cellular compartments in which chaperoned peptides are released, processed, and made available for assembly into new major histocompatibility complex (MHC) molecules (13). The common receptor CD91 as well as other receptors mediate internalization of chaperone proteins and induce phenotypic and functional maturation in APCs (12, 14–17). Because Hsp-peptide complexes exhibit these qualities that are conducive to evoking an immune response, effective tumor vaccines can be designed from peptides that have high affinities to Hsps and human leukocyte antigen (HLA) molecules.

In this study, we developed a methodology for identification of peptide antigens from highly expressed proteins in tumors using peptide arrays and HLA binding prediction tools. To meet the demand for a design of order-made tumor vaccines, it is necessary to develop a method for the identification and design of HLA epitopes that can efficiently be internalized and processed for assembly into HLA molecules. Here, Hsp70, which is the major stress responsive protein overexpressed frequently in human tumors of various origins but not in most physiological normal tissues, was selected as a model antigen, and the affinities of the Hsp70-derived peptides to the Hsp70 chaperone was investigated using peptide arrays. Because Hsp70 upregulated in tumor cells or Hsp70-rich cell lysates by heat- or drug-induced stress or gene transfection has been reported to increase tumor immunogenicity (6, 18, 19), Hsp70-derived peptides can be used as multivalent, autologous vaccines for tumor cells. The HLA-A*0201-restricted CTL epitope peptide at $Hsp70_{391–399}$ (LLLLDVAPL) (20) has already been reported as a valuable target for CTL in broad spectrum cancer immunotherapy. A peptide array is synthesized on a cellulose membrane support using the SPOT technology by Fmoc solid-phase peptide synthesis (21). It enables the synthesis of a variety of peptides from the C-terminus to the N-terminus in a parallel manner on one membrane. This peptide array

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can be a versatile tool for molecular recognition and screening of biologically active peptides (22–25). *In vitro* assessment of the affinity of a peptide to Hsp70 would be useful for determining the ability to be presented by MHC molecules as the antigen peptide.

MATERIALS AND METHODS

Preparation of peptide arrays covering the amino acid sequence of Hsp70 A peptide library covering the entire Hsp70 sequence was prepared by automated spot synthesis (21). Peptide arrays covering Hsp70 sequence (9-mer peptide fragments with a two-residue shift, resulting in 282 spots) were constructed in triplicate. Reference spots used to define a positive affinity to Hsp70 were ALLLSAPRR (26), IPGLPLSL (27, 28), and VYDFFVWL (9). The negative control sequence of AAAAAAAAA was used. Standard Fmoc chemistry was used in accordance with the manufacturer's instructions (Intavis, Köln, Germany) using a peptide autospotter (model ASP222; Intavis) as described previously with some modifications (23, 24). The concentration of activated amino acids was 0.5 M. The side-chain protecting group was removed by 3 h incubation in a mixed solvent of trifluoroacetic acid, tioanisol, ethandiol, and *m*-cresol (at a ratio of 40:6:3:1).

Hsp70 binding assay using peptide arrays Hsp70 binding assay using peptide arrays was performed as described by Rüdinger *et al.* (29). A peptide array was soaked in blocking buffer (Tris-buffered saline containing 0.05% Tween 20 [TBS-T] and 0.4% block-ace [Dainippon Sumitomo Pharma, Osaka]) for 1 h. Then, human recombinant Hsp70 (150 nM, no. NSP-555; Stressgen, Ann Arbor, MI, USA) was incubated with the peptides on the peptide array in TBS-T for 2 h at room temperature with gentle shaking. Unbound Hsp70 was removed by washing the array four times with TBS-T. The peptide-bound Hsp70 was electrotransferred onto polyvinylene difluoride (PVDF) membranes. After blotting, the PVDF membranes were washed and then labeled with mouse anti-human Hsp70 (SPA-810; Stressgen Bioreagents, British Columbia, Canada) and HRP-labeled goat anti-mouse IgG, and chemiluminescence was detected using an ECL advance western blotting kit (RPN2135; Invitrogen, Japan K.K., Tokyo).

Peptide binding affinity to HLA-A24 Estimation of HLA-A24-restricted epitopes was performed using the prediction tool BIMAS (http://www-bimas.cit.nih.gov). Peptides predicted to have high binding affinities to HLA-A24 were then subjected to HLA-A24 stabilization assay using T2-A*2402 cells. HLA-A*2402 is a common and widely distributed allele. T2-A*2402 cells, which are the transporter associated with antigen processing (TAP)-deficient human T2 cells transfected with an MHC class I cDNA derived from the human HLA-A*2402 molecule. After incubation of cells in culture medium at 26°C for 18 h, the cells (2×10^5) were suspended in 1 ml of MEM containing 3 mg/ml β2-microglobulin in the presence or absence of a peptide (100 μg). The cells were then incubated at 26°C for 3 h and then at 37°C for 3 h. After washing with PBS, the cells were incubated with anti-HLA-A24 mAb (c7709A2.6, kindly provided by Dr. P. G. Coulie, Ludwig Institute for Cancer Research, Brussels Branch) at 4°C for 30 min. Then, the cells were incubated with FITC-conjugated goat anti-mouse IgG at 4°C for 30 min. The cells were suspended in 1 ml of PBS containing 1% formaldehyde and analyzed using FACSVantage (Becton Dickinson, Franklin Lakes, NJ, USA). Binding affinity was evaluated by comparing the mean fluorescence intensity of HLA-A24 in the presence of peptide pulsation with that of no peptide. Positive control peptides for HLA-A24 were the survivin-2B peptide (AYACNTSTL) and HIV peptide (SFHSLHLLF). The negative control peptide was the vesicular stomatitis virus (VSV)-8 peptide (RGYVYQGL), which binds to mouse H-2K^b.

Vaccination and induction of CTL RMA-S-A*2402 cells were RMA-S transfected with the gene encoding HLA-A*2402 (provided by Dr. H. Takasu, Dainippon Sumitomo Pharmaceutical, Osaka). The mouse thymoma cell line (E.G7 cells; EL-4 cells transfected with cDNA encoding OVA) was obtained from American Type Culture Collection (ATCC). HLA-A*2402/K^b transgenic mice were purchased from SLC Japan. The mice were kept in a specific pathogen-free mouse facility. Each HLA-A*2402/K^b transgenic mouse was immunized with one of the Hsp70-derived peptides (50 μg) with incomplete Freunds adjuvant (IFA) subcutaneously at the base of the tail four times at 1 week intervals. One week after the last immunization, spleen cells were removed, and cultured *in vitro* with irradiated (100 Gy) and candidate peptide-pulsed spleen cells for 5 days. Subsequently, the generation of peptide-specific CTLs was evaluated by ^{51}Cr release assay. The specificity of CTLs induced in an individual HLA-A*2402/K^b transgenic mouse was evaluated using RMA-S/A*2402 cells as the target in the presence or absence of the candidate peptide.

^{51}Cr release assay The cytolytic activity of the induced CTL was determined by a standard 4-h ^{51}Cr release assay. Target cells were labeled with 50 μCi ^{51}Cr-labeled sodium chromate in RPMI with 10% FCS for 1 h at 37°C and then washed twice with RPMI. Target cells (5×10^3) were added to a titrated CTL effectors in 96-well round-bottom plates with a final volume of 200 μl of RPMI with 10% FCS. The cells were centrifuged to promote cell contact and then incubated at 37°C for 4 h. The supernatant (100 μl) from each well was harvested manually. Radioactivity released into the supernatant was measured using a gamma counter, and percent specific release was calculated from the mean of duplicate cultures using the following formula:

$$\text{Percent specific release} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximal release} - \text{spontaneous release})} \times 100$$

For peptide-pulsed target cells, RMA-S/A*2402 cells were incubated with 1 μg/ml peptide at room temperature for 1 h before the assay.

RESULTS

Hsp70 binding assay on peptide array The design of a universal tumor vaccine requires the identification of tumor antigens expressed in the majority of tumors. Because Hsps have recently been shown to participate in the generation of anti-tumor immunity, the binding affinity of Hsp70 to an Hsp70-derived 9-mer peptide was investigated. Figure 1 shows the typical results of the Hsp70 binding assay obtained by dot-blot analysis using peptide arrays. The average relative binding intensity of the negative control peptide was below 1000. This value was calculated from three different cellulose membranes with six spots. In contrast, the average relative binding intensities of the positive control sequences from three spots on four different cellulose membranes were 17,620, 13,300, and 18,630 for ALLLSAPRR, IPGLPLSL, and VYDFFVWL, respectively. Because these sequences were reported to bind DnaK or Hsp70 (25), screening of Hsp70-binding peptides using peptide arrays appeared feasible. The binding of Hsp70 to cellulose-bound peptides was also examined using ATP or ADP. The Hsp70-binding patterns were similar with and without the addition of ADP, and the bound Hsp70 was released following the addition of 2 mM ATP (Fig. 2). Therefore, the release of Hsp70-chaper-