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Immunology

Heat shock fusion proteins as vehicles for antigen delivery into the major histocompatibility complex class I presentation pathway

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ABSTRACT **Mice immunized with heat shock proteins (hsps) isolated from mouse tumor cells (donor cells) produce CD8 cytotoxic T lymphocytes (CTL) that recognize donor cell peptides in association with the major histocompatibility complex (MHC) class I proteins of the responding mouse. The CTL are induced apparently because peptides noncovalently associated with the isolated hsp molecules can enter the MHC class I antigen processing pathway of professional antigen-presenting cells. Using a recombinant heat shock fusion protein with a large fragment of ovalbumin covalently linked to mycobacterial hsp70, we show here that when the soluble fusion protein was injected without adjuvant into H-2^b mice, CTL were produced that recognized an ovalbumin-derived peptide, SIINFEKL, in association with K^b. The peptide is known to arise from natural processing of ovalbumin in H-2^b mouse cells, and CTL from the ovalbumin-hsp70-immunized mice and a highly effective CTL clone (4G3) raised against ovalbumin-expressing EL4 tumor cells (EG7-OVA) were equally effective in terms of the concentration of SIINFEKL required for half-maximal lysis in a CTL assay. The mice were also protected against lethal challenge with ovalbumin-expressing melanoma tumor cells. Because large protein fragments or whole proteins serving as fusion partners can be cleaved into short peptides in the MHC class I processing pathway, hsp fusion proteins of the type described here are promising candidates for vaccines aimed at eliciting CD8 CTL in populations of MHC-disparate individuals.**

The cytotoxic T lymphocytes (CTL) that play an important role in protective cellular immunity, including the destruction of virus-infected cells, are predominantly CD8 T cells (1, 2). Antigen-specific activation of these cells depends on their recognition of peptide–major histocompatibility complex (MHC) complexes, which normally arise within antigen-presenting cells by proteolytic cleavage of cytosolic proteins (3). Translocated into the endoplasmic reticulum, the resulting peptides bind to nascent class I MHC molecules for transport to the cell surface (4). Because intact proteins in the extracellular medium do not ordinarily penetrate into a cell's cytosol, soluble proteins typically fail to stimulate mice to produce CTL (5), although there are exceptions (6).

In comparison with other proteins, the soluble heat shock protein (hsp) termed gp96 is an unusually effective stimulator of CD8 CTL (7). Mice injected with gp96 isolated from tumor cells (donor cells) produce CTL that are specific for donor cell peptides in association with the responder mouse's class I MHC proteins (8, 9). Because donor peptides are bound noncovalently by the isolated hsp, the results suggest that the hsp molecules are capable of delivering noncovalently associated peptides to MHC class I proteins of other (recipient) cells, including antigen-presenting cells.

The noncovalently bound peptide–gp96 complexes that are purified from a tumor cell appear to represent a broad array of proteins expressed by the cell (10, 11). In contrast, recombinant hsp fusion proteins in which specific proteins of interest are covalently linked to the hsp would provide a well characterized polypeptide that would lack extraneous peptides. In addition, a large protein fragment covalently linked to the hsp would be an especially rich source of many different naturally processed peptides. Peptide mixtures of this kind, derived from specific antigens of interest, would be particularly suitable for forming intracellular peptide–MHC complexes with the highly diverse MHC proteins found in different individuals of genetically outbred populations.

We have accordingly taken advantage of a recombinant hsp70 protein expression vector that permits diverse proteins and peptides to be fused to the amino terminus of mycobacterial hsp70. We have previously shown that *Mycobacterium tuberculosis* hsp70 can be used as an adjuvant-free carrier to stimulate the humoral and cellular response to a full-length protein that is covalently linked to the hsp (12). The special properties of hsp70 prompted us to investigate whether soluble hsp70 fusion proteins could be utilized to elicit MHC class I-restricted CD8$^+$ CTL.

We show here that a soluble hsp70 fusion protein having a large fragment of chicken ovalbumin (ova) as fusion partner could, in the absence of adjuvants, stimulate H-2^b mice to produce ovalbumin-specific CD8 CTL. The CTL recognized an immunodominant ovalbumin octapeptide, SIINFEKL, known to be a naturally processed peptide derived from ovalbumin expressed in mouse cells (13), in the context of K^b. CTL from the immunized mice were as active cytolytically as a highly effective CTL clone (4G3) that had been raised against ovalbumin-expressing tumor cells, as both caused half-maximal lysis of K^{b+} target cells with the SIINFEKL peptide at about the same concentration (10^{-13} M). Our results thus indicate that the ova-hsp70 fusion protein, injected as a soluble protein into mice, can enter the MHC class I processing pathway in antigen-presenting cells and stimulate the production of CD8 CTL.

MATERIALS AND METHODS

Expression Vector Constructs. The DNA fragment containing the *M. tuberculosis* hsp70 coding sequence was synthesized by PCR using DNA purified from λgt11 clones Y3111 and Y3130 as a template (14). The complete coding sequence of

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Abbreviations: CTL, cytotoxic T lymphocytes; MHC, major histocompatibility complex; hsp, heat shock protein; ova, ovalbumin fragment; IFN-γ, interferon γ; E:T ratio, effector-to-target ratio.

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hsp70 was synthesized by using the upstream primer oKS63 (5′-GCCCGGGATCCATGGCTCGTGCGGTCGGGAT-3′) containing a *Bam*HI site immediately before the hsp70 coding sequence and the downstream primer oKS79 (5′-GCGGAATTCTCATCAGCCGAGCCGGGGT-3′) containing an *Eco*RI site immediately after the last coding sequence of hsp70. The DNA fragment containing the ovalbumin coding sequence was synthesized by PCR using plasmid pOv230 (15) as a template. The upstream primer oKS83 (5′-GCGGATCCATATGGTCCTTCAGCCAAGCTCCGTGG-3′) contained a *Nde*I site immediately before amino acid 161 of ovalbumin and the downstream primer oKS82 (5′-GCAGGATCCCTCTTCCATAACATTAGA-3′) contained a *Bam*HI site immediately after amino acid 276 of ovalbumin. Another downstream primer containing a *Bam*HI site, oKS80 (5′-GCTGAATTCTTACTCTTCCATAACATTAG-3′), included a translation stop codon immediately after amino acid 276 of ovalbumin.

Construction of the vector used to produce hsp70 alone, pKS74, has been previously described (12). The vector pKS11h was made by modifying the plasmid vector pET11 (16) with a histidine tag coding sequence and with the polylinker from pET17b. Plasmid pKS28 was made by subcloning the DNA encoding amino acids 161–276 of ovalbumin into the *Nde*I and *Bam*HI sites of pKS11h. Plasmid pKS76 was created by subcloning ovalbumin-(161–276) and hsp70 into the *Nde*I and *Bam*HI sites of pKS11h.

Protein Purification. Cultures of BL21(DE3)pLysS (16) were grown and induced with 0.5 mM isopropyl thiogalactoside (IPTG). hsp70 and ova-hsp70 proteins were both purified as inclusion bodies, refolded stepwise in guanidine, and subsequently purified by ATP affinity chromatography as previously described (12). Protein purity was verified by SDS/PAGE and protein fractions were pooled and dialyzed against PBS. Protein concentrations were determined by the bicinchoninic acid assay (BCA; Pierce).

Peptides. The peptides SIINFEKL (corresponding to ovalbumin amino acids 258–276) and RGYVYQGL (corresponding to the vesicular stomatitis virus nucleoprotein amino acids 324–332), were synthesized by the Biopolymers Facility at the Center for Cancer Research at the Massachusetts Institute of Technology. Peptides were stored as 1 mg/ml stock solutions in PBS.

Mice and Immunizations. Seven- to 8-week-old female C57BL/6 mice were obtained from The Jackson Laboratory and Taconic Farms. Mice were immunized i.p. on day 0 and s.c. on day 14 with 120 pmol of purified protein in PBS.

Cell Lines. EL4 (H-2^b) thymoma cells, from the American Type Culture Collection, were grown in RPMI medium 1640/10% fetal calf serum (FCS). E.G7-OVA cells (ovalbumin-transfected EL4 cells) (17) were cultured in RPMI 1640/10% FCS in the presence of 320 μg of G418 per ml. The human cell line T2, is a TAP-deficient, T-B lymphoblastoid fusion hybrid. The K^b-transfected clone, T2-K^b, a generous gift from P. Cresswell, was cultured in RPMI 1640/10% FCS in the presence of 320 μg of G418 per ml. The CTL clone 4G3 was maintained by weekly restimulation with irradiated E.G7-OVA cells in RPMI 1640/10% FCS/5% rat Con A supernatant (18). The C57BL/6-derived melanoma B16 and the ovalbumin-transfected B16 clone, MO5 (19), were generously provided by L. Rothstein and L. Sigal. The B16 cells were grown in RPMI 1640/10% FCS and the MO5 cells were grown in the presence of 2.0 mg of G418 and 40 μg of hygromycin per ml.

Interferon γ (IFN-γ) ELISA. Spleens were removed from mice 10 days after the last injection. The spleens from 3–10 mice in each treatment group were pooled. Single-cell suspensions were prepared by grinding tissue through a sterile nylon mesh. Erythrocytes were removed by suspending the cells in pH 7.2 lysis buffer (0.15 M NH_4Cl/1 M $KHCO_3$/0.1 mM Na_2EDTA) and rinsing the cells two times with RPMI medium 1640. Splenocytes were then cultured at 1×10^7 cells per ml in 96-well round-bottom microculture plates in RPMI medium 1640, supplemented with 10% FCS and 50 μM 2-mercaptoethanol at 37°C in 5% CO_2. The cells were stimulated with recombinant ovalbumin (10 μg/ml), SIINFEKL peptide (10 μg/ml), RGYVYQGL peptide (10 μg/ml), or Con A (5 μg/ml). Cell culture supernatants were removed at 72 h. A sandwich ELISA using paired monoclonal antibodies (Endogen, Cambridge, MA) was used to measure IFN-γ.

CTL Assay. Single-cell suspensions of splenocytes were prepared as above. Then 25×10^6 splenocytes were cultured with 5×10^6 irradiated (15,000 rads) E.G7-OVA cells in RPMI medium 1640 supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, and 100 μM nonessential amino acids. After 6–7 days in culture, splenocytes were purified by Ficoll-Paque (Pharmacia) density centrifugation and then utilized as effector cells.

Target cells were labeled with 100 μCi (1 μCi = 37 kBq) of sodium [^{51}Cr]chromate at 37°C for 1–2 h. For peptide sensitization of target cells, 50 μg of peptide was added to the target cells (300 μg/ml final peptide concentration) during the labeling period. The cells were then rinsed and 5,000 ^{51}Cr-labeled targets (T) and serial dilutions of effector cells (E) were incubated at various E:T ratios in 96-well U-bottom plates at 37°C. For peptide titration assays, the target cells were not pulsed with any peptide during the ^{51}Cr-labeling period and instead, the peptide was directly added to the 96-well U-bottom plate at final concentrations of 10^{-10} M to 10^{-14} M. Supernatants were harvested after 4–6 h and the radioactivity was measured in a γ counter. Percent specific lysis was calculated as equal to 100× [(release by CTL − spontaneous release)/(maximal release − spontaneous release)]. Maximal release was determined by addition of 1% Triton X-100 or by resuspending target cells.

***In Vitro* Depletion or Enrichment of Lymphocyte Subpopulations.** Splenocytes were cultured with irradiated E.G7-OVA cells and purified by Ficoll-Paque density centrifugation as described above. Cells were resuspended in cold PBS with 1% FCS and incubated with anti-mouse CD4 (L3T4) microbeads or with anti-mouse CD8a (Ly-2) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min at 4°C. For cell depletion, the cells were applied on to a Mini MACS column (Miltenyi Biotec) with an attached flow resistor. The cells from the flow-through were collected and used as effector cells in the cytolytic assay. For positive selection of CD8 cells, the cells were applied on to a Mini MACS column without a flow resistor. The column was rinsed and the cells adhering to the column were released by removing the column from the magnetic holder. The positively selected cells were then used as effector cells in the cytolytic assay. The effectiveness of positive and negative selection of cells was verified by flow cytometry using phycoerythrin-conjugated anti-mouse CD4 and fluorescein isothiocyanate-conjugated anti-mouse CD8a antibodies (PharMingen).

Tumor Protection Assay. C57BL/6 mice were injected i.p. with 120 pmol of ovalbumin or ova-hsp70 without adjuvant and boosted s.c. 2 weeks later. Ten days after the last immunization the mice were injected s.c. on the right flank with 1×10^5 MO5 tumor cells or with 1×10^5 B16 tumor cells. As a control, unimmunized mice were also inoculated with the tumor cells. Five to 10 mice were used for each experimental group. On the day of the tumor challenge, the B16 and MO5 cells were harvested by trypsinization and rinsed three times in PBS. The cells were resuspended in PBS and administered s.c. in a volume of 0.1 ml. Tumor growth was assessed by measuring the diameter of the tumor in millimeters (recorded as the average of two perpendicular diameter measurements). Mice that became moribund were sacrificed. Consistent results were observed in three separate experiments.

RESULTS

Purified Recombinant Proteins. A recombinant system developed to permit production of *M. tuberculosis* hsp70 fusion proteins in *Escherichia coli* (12) was utilized to attach amino acids 161–276 of ovalbumin to the N terminus of *M. tuberculosis* hsp70. A comparable recombinant ovalbumin protein (amino acids 161–276) was also produced. The selected portion of ovalbumin contains the immunodominant epitope SIINFEKL recognized by CTL in association with K^b (13, 20). The ova-hsp70 fusion protein and the ovalbumin (amino acids 161–276) protein were expressed at high levels in *E. coli* (Fig. 1*A*). These proteins were purified as inclusion bodies, refolded *in vitro*, and further purified by column chromatography. The purity of the recombinant proteins was assessed by SDS/PAGE (Fig. 1*A*). Examination of commercial preparations of crystallized and high-grade ovalbumin by SDS/PAGE and silver staining revealed that they are highly contaminated with low molecular weight polypeptides. For this reason, only the highly purified recombinant ovalbumin (amino acids 161–276) protein, referred to below simply as ovalbumin, was used in these studies.

Immunization of Mice with hsp70 Fusion Protein in PBS Elicits T Cell Responses Against the Attached Antigen. We investigated whether mice injected with soluble protein without adjuvant could be primed to produce anti-ovalbumin T cells (Fig. 1*B*). C57BL/6 mice were inoculated i.p. with 120 pmol of ovalbumin or with 120 pmol of ova-hsp70 fusion protein in PBS. A second equivalent dose was given s.c. at 2 weeks. A third group of mice was injected with 120 pmol of ova-p24 gag fusion protein, purified as described in ref. 12, to examine the immune responses elicited by administering ovalbumin covalently linked to a protein other than hsp70, in the absence of adjuvant. Splenocytes of immunized mice were removed 10 days after the s.c. immunization and cultured *in vitro* for 6 days with irradiated E.G7-OVA cells (syngeneic EL4 cells transfected with ovalbumin) (17). The cultured cells were then used as effector cells in CTL assays. Cells from naive mice (data not shown) and from mice injected with ovalbumin protein or with ova-p24 fusion protein were unable to lyse T2-K^b target cells or T2-K^b cells pulsed with SIINFEKL peptide. In contrast, effector cells from mice primed with ova-hsp70 fusion protein were able to lyse T2-K^b cells pulsed with SIINFEKL peptide (Fig. 1B).

Results obtained with other target cells also show that the anti-ovalbumin CTL recognized SIINFEKL in association with K^b. Splenocytes from ova-hsp70-immunized mice were able to lyse both E.G7-OVA target cells and EL4 cells pulsed with SIINFEKL peptide but were unable to lyse EL4 cells in the absence of peptide or EL4 cells pulsed with another K^b-binding peptide [RGYVYQGL, from vesicular stomatitis virus (21), data not shown].

To assess the effectiveness of the CTL from ova-hsp70-immunized mice, they were tested after 6 days in culture in cytolytic assays using T2-K^b as target cells and SIINFEKL at various concentrations. For purposes of comparison, the assay included a well characterized CTL clone (4G3) that recognizes the SIINFEKL-K^b complex. As shown in Fig. 1*C*, half-maximal lysis was obtained with both the CTL line and the 4G3 clone at about the same peptide concentration, approximately 5×10^{-13} M. Thus CTL from the ova-hsp70-immunized mice and the clone against the ovalbumin-expressing tumor (E.G7-OVA) were equally effective in terms of the SIINFEKL concentration required for half-maximal lysis. It may be noted that in Fig. 1*C* the ratio of 4G3 cells to target cells (E:T ratio) was 5:1, whereas for the CTL line this ratio was 80:1. The E:T ratio has a large impact on the maximal lysis of target cells at 4 hr, but changing this ratio over an 80-fold range (1:1 to 80:1) has a negligible effect on the peptide concentration required for half-maximal lysis (unpublished data).

We next verified that the cytolytic activity of the CTL line from ova-hsp70-immunized mice was due to $CD8^+$ T cells (Fig. 2). For this purpose we used a MACS column to separate the CTL line into T cell subsets (see *Materials and Methods*). CTL

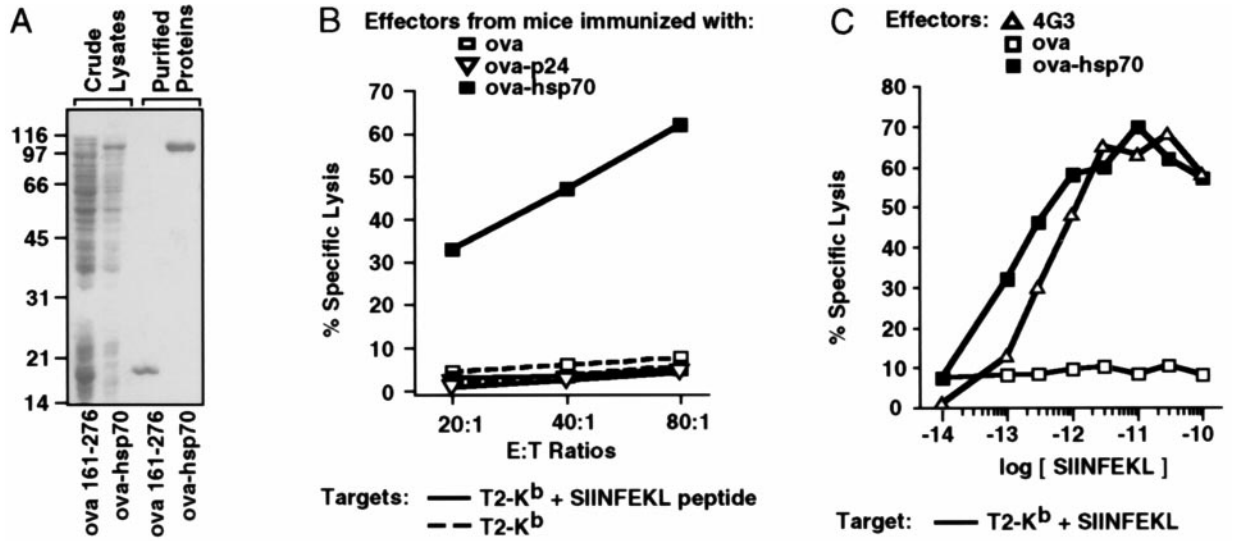

FIG. 1. A) Production of recombinant proteins. *E. coli* cell lysates and purified proteins were examined by SDS/PAGE and proteins were visualized by Coomassie staining. The gel contains crude extracts from isopropyl thiogalactoside (IPTG)-induced *E. coli* containing pKS28 (ova 161–276) and from IPTG-induced *E. coli* containing pKS76 (ova-hsp70), and the purified proteins ova 161–276 and ova-hsp70. Molecular weight markers ($\times 10^{-3}$) are at left. (*B*) Generation of ovalbumin-specific CTL by immunization with ova-hsp70 fusion protein in saline. Mice were injected i.p. with 120 pmol of recombinant ovalbumin, ova-p24, or ova-hsp70 protein without adjuvant. The injections were repeated s.c. 2 weeks later. Mice were sacrificed 10 days after the boost and for each mouse group, 5–10 spleens were pooled and splenocytes from immunized mice were incubated for 6 days in the presence of irradiated E.G7-OVA cells without added interleukins. The splenocyte cultures derived from mice immunized with ovalbumin □, ova-p24 ▽, or ova-hsp70 ■ were then used as effector (E) cells in a standard cytotoxicity assay. The following ^{51}Cr-labeled target (T) cells were used: T2-K^b cells (broken line) and T2-K^b pulsed with SIINFEKL peptide (solid line) at 300 μg/ml. (*C*) SIINFEKL peptide titration. T2-K^b cells were incubated with the indicated molar concentrations of SIINFEKL peptide for 45 min for use as target cells in a CTL assay. The effector cells primed with ovalbumin □ or ova-hsp70 ■ as described above were used at an E:T ratio of 80:1. The 4G3 CTL clone △ was used at an E:T of 5:1.

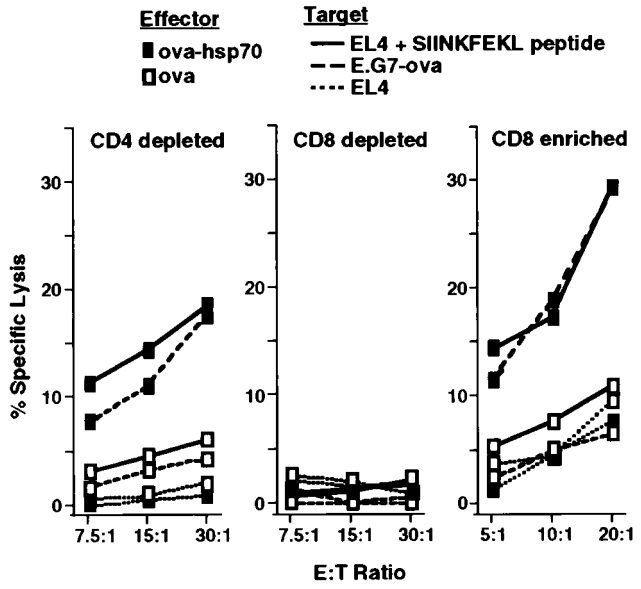

FIG. 2. Immunization with ova-hsp70 elicits ovalbumin-reactive $CD8^+$ T cells. C57BL/6 mice were injected i.p. with 120 pmol of ovalbumin or ova-hsp70 without adjuvant and boosted s.c. with the same amounts of these proteins 2 weeks later. Mice were sacrificed 10 days after the boost and for each mouse group, 5–10 spleens were pooled and splenocytes were incubated for 6 days in the presence of irradiated E.G7-OVA cells. Prior to the cytotoxicity assay, the effector cells were negatively or positively selected for $CD4^+$ cells or $CD8^+$ cells by using paramagnetic antibodies. Splenocyte cultures were depleted of $CD4^+$ cells ($CD4^-CD8^+$), were depleted of $CD8^+$ cells ($CD4^+$ $CD8^-$), or were enriched for $CD8^+$ cells ($CD8^+$).

activity was unaffected by removing $CD4^+$ cells, but it was completely abrogated by removing $CD8^+$ cells. Retrieval of the $CD8^+$ cells from the MACS column led to recovery of cytolytic activity. The results were the same when target cells were EL4 cells incubated with SIINFEKL or ovalbumin-expressing EL4 cells (E.G7-OVA). Thus, administration of ova-hsp70 fusion protein, but not ovalbumin alone, elicits $CD8^+$ CTL specific for SIINFEKL-K^b.

The lower level of cytolytic activity in Fig. 2 relative to Fig. 1 (*B* and *C*) reflects the different target cells used. T2-K^b cells (Fig. 1) and EL4 cells (Fig. 2) have approximately the same high level of cell surface K^b (roughly 100,000 molecules per cell, unpublished observations), but the peptide transporter (TAP) is defective in T2-K^b (22), and not in EL4. Hence, at a given free concentration of SIINFEKL the target cell epitope density (number of SIINFEKL-K^b complexes per cell) is much greater on T2-Kb than on EL4 cells.

hsp70 Must Be Covalently Coupled to Ovalbumin to Engender Anti-Ovalbumin T Cell Responses. We examined whether the covalent fusion of hsp70 to ovalbumin was necessary to elicit cellular responses to ovalbumin or whether the same results could be obtained if the two proteins were simply mixed but not covalently attached (Fig. 3). Mice were injected with 120 pmol of ova-hsp70 fusion protein, with 120 pmol of ovalbumin, or with 120 pmol of hsp70 mixed with 120 pmol of ovalbumin. The level of IFN-γ secreted by the splenocytes in response to restimulation with ovalbumin *in vitro* was measured by ELISA. Splenocytes from mice immunized with ovalbumin alone or with a mixture of ovalbumin and hsp70 proteins produced less than 6 ng/ml IFN-γ in response to stimulation with SIINFEKL peptide or ovalbumin (Fig. 3*A*). In contrast, splenocytes from mice injected with the ova-hsp70 fusion protein secreted substantially higher levels of IFN-γ when restimulated *in vitro* with SIINFEKL peptide or ovalbumin. The release of IFN-γ was ovalbumin-specific, because splenocytes cultured in medium alone or with control RGYVYQGL peptide secreted low levels of IFN-γ.

Similar results were obtained by cytolytic assays (Fig. 3*B*). Ovalbumin-specific CTL were produced by mice injected with the ova-hsp70 fusion protein but not by those injected with a mixture of ovalbumin with hsp70.

Immunization of Mice with ova-hsp70 Protein Without Adjuvant Engenders Protective Immunity to MO5 Tumor Challenge. The MO5 cell line, which is a B16 melanoma cell line transfected with ovalbumin-expressing DNA, presents the immunodominant SIINFEKL peptide in association with K^b

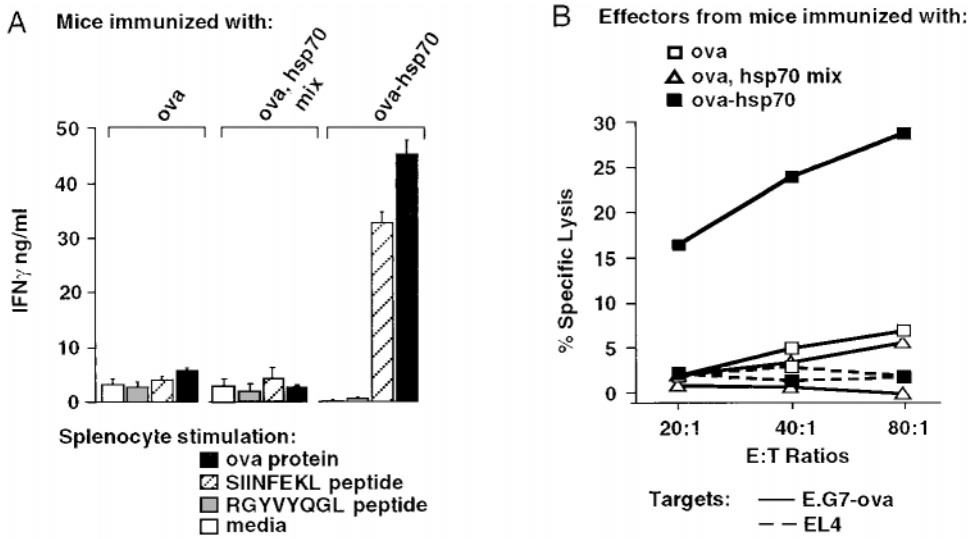

FIG. 3. Examination of ovalbumin-specific T cell responses in mice injected with a mixture of ovalbumin and hsp70. Mice were injected twice as described in the legend of Fig. 1 with 120 pmol of recombinant ovalbumin, with 120 pmol of ova-hsp70 fusion protein, or with 120 pmol each of ovalbumin and hsp70. Ten days after the boost 5–10 spleens from each mouse group were pooled and processed. (*A*) IFN-γ secretion by splenocytes stimulated 72 h *in vitro* with 5 μg/ml recombinant ovalbumin protein (filled bars), SIINFEKL peptide (hatched bars), RGYVYQGL peptide (shaded bars), or tissue culture medium alone (empty bars). All samples were examined in triplicate. (*B*) Generation of ovalbumin-specific CTL by immunization with ova-hsp70 fusion protein in saline. Splenocyte cultures from mice immunized with recombinant ova □, ova-hsp70 fusion protein ■, or a mixture of ovalbumin and hsp70 proteins △, were used as effector cells in a standard cytotoxicity assay. The following ^{51}Cr-labeled target cells were used: E.G7-OVA (solid line) and EL4 cells alone (broken line).

on the cell surface (19). Using this tumor, we could determine whether the immune response induced by ova-hsp70 fusion protein is sufficient to engender protective tumor immunity. Mice were injected i.p. with 120 pmol of ovalbumin or ova-hsp70 without adjuvant and boosted s.c. 2 weeks later. Ten days later the mice were injected s.c. on the right flank with 1×10^5 MO5 tumor cells or with 1×10^5 B16 tumor cells. As an additional control, naive mice were also inoculated with the tumor cells.

All mice challenged with tumor cells were monitored for tumor growth, and growth was recorded as the average tumor diameter in millimeters (Fig. 4*A*). Twenty-one days after the MO5 tumor challenge, the average tumor diameter in the control and the ovalbumin-immunized mice was greater than 15 mm. Because the control and ovalbumin-immunized mice began dying 21 days after the tumor challenge, tumor growth was not recorded beyond 21 days. In contrast to the control and the ovalbumin-immunized mice, no tumors were detected in the ova-hsp70 immunized mice 21 days after the tumor challenge. All groups of mice (control, ovalbumin-immunized, or ova-hsp70 immunized) which were challenged with the B16 tumor cells developed tumors (Fig. 4*A*) and became moribund by 21 days after the tumor challenge.

The survival of mice was recorded as the percentage of mice surviving after the tumor challenge (Fig. 4*B*). Mice that appeared moribund were sacrificed. Forty days after the MO5 tumor challenge, none of the control mice and only 10% of the ovalbumin-immunized mice had survived. In contrast, 80% of the ova-hsp70-immunized mice had survived. These experiments demonstrate that immunization of mice with the ova-hsp70 fusion protein, but not with the ovalbumin protein alone, induces ovalbumin-specific protective tumor immunity.

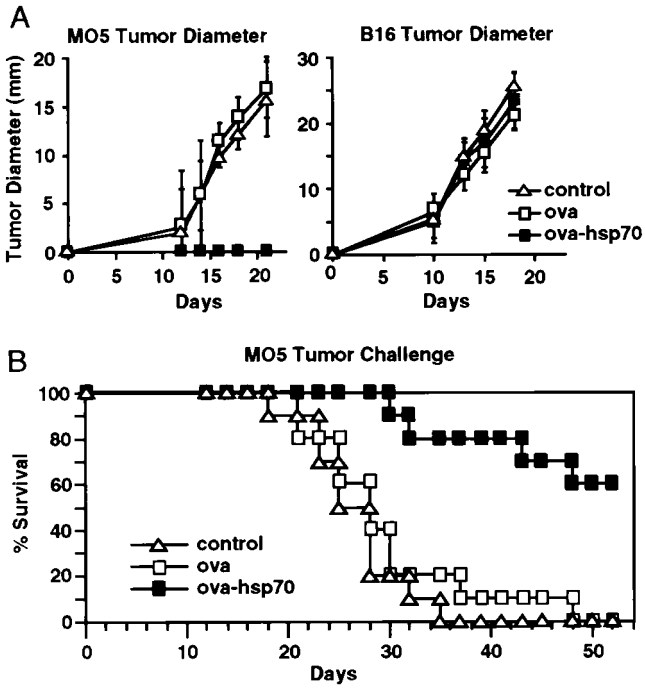

FIG. 4. Immunization of mice with ova-hsp70 protein without adjuvant engenders protective immunity to MO5 tumor challenge. Mice were injected i.p. with 120 pmol of ova or ova-hsp70 without adjuvant and boosted s.c. with the same amounts of these proteins 2 weeks later. Ten days after the last immunization the mice were injected s.c. on the right flank with 1×10^5 MO5 tumor cells. Each group contained at least 5 mice. (*A*) Following the MO5 and B16 tumor challenges, tumor growth was monitored in control mice and in ovalbumin- □ and ova-hsp70- ■ immunized mice. Growth was recorded as the average tumor diameter in millimeters. (*B*) The survival of mice was recorded as the percentage of mice surviving after the tumor challenge. Mice that appeared moribund were killed and scored as "not surviving."

DISCUSSION

The principal finding in this study is that injection of an hsp70-ovalbumin fusion protein into H-2^b mice stimulated the production of CD8 CTL that recognize the immunodominant ovalbumin octapeptide, SIINFEKL, in association with K^b. The immunized mice were protected against an otherwise lethal challenge with an ovalbumin-expressing melanoma tumor, and their CTL were as effective (see Fig. 1*C*) in recognizing the SIINFEKL-K^b complex as a CTL clone (4G3) that was raised against cells (EG7-OVA) in which ovalbumin is expressed and processed naturally for class I MHC presentation. These findings clearly imply that the covalently linked fusion partner of the injected hsp fusion protein was processed in the same way as ordinary cytosolic proteins for presentation with MHC class I proteins in antigen-presenting cells.

We previously reported that mice injected with an HIV-1 gag protein (p24) linked to hsp70 produced p24-specific T cells. Although the peptide-MHC complexes recognized by the T cells were not identified, the splenocytes from the fusion-protein-immunized mice exhibited p24 antigen-dependent production of IFN-γ, which implies the presence of Th1 helper T cells and CTL. The previous findings, taken in conjunction with the present results, suggest that hsp70 fusion proteins may prove to be generally useful as immunogens for stimulating CD8 CTL that are specific for peptides produced by natural proteolytic processing of the fusion partners within antigen-presenting cells.

The mechanisms by which hsp70 enables covalently linked polypeptide fusion partners to gain entry into the MHC class I processing pathway and elicit CD8 CTL could be based on: (*i*) hsp70's ability to assist protein folding (23, 24) and to facilitate the translocation of proteins into subcellular compartments (25, 26), (*ii*) hsp70's ability to facilitate the breakdown of intracellular proteins (27), and (*iii*) the high frequency of T cells directed against mycobacterial hsp70.

hsp70 is an integral component of the protein folding machinery (28–30) and functions through its ability to bind short linear peptide segments of folding intermediates. Detailed studies of the peptide-binding activity of hsp70 have shown that it has a clear preference for peptides with aliphatic hydrophobic side chains (24, 31). Thus hsp70 appears to transiently associate with hydrophobic protein regions and prevent protein aggregation. The kinetics of hsp70-substrate binding is governed by the ATP binding and ATPase activity of hsp70 (32). The combination of the peptide and ATP binding functions of hsp70 may be involved in the efficient transfer of antigenic peptides into the MHC class I antigen presentation pathway. hsp70 also associates with nascent polypeptide chains as they emerge from ribosomes and are involved in stabilizing nascent polypeptides prior to their translocation into various subcellular compartments (33, 34), including chloroplasts, the endoplasmic reticulum, lysosomes, mitochondria, the nucleus, and peroxisomes (25, 26). The present findings indicate that hsp70 also promotes delivery of covalently linked fusion polypeptides to the subcellular compartment(s) required for cell surface presentation of peptide–MHC-I complexes.

hsp70's role in intracellular protein breakdown may be especially relevant for the immunogenic effectiveness of its fusion partner. Experiments with yeast cell mutants and with mammalian cell extracts have shown that, in addition to its function in protein refolding, hsp70 serves an essential role in the degradation of certain abnormal polypeptides (27, 35). Thus, if hsp70 fails to refold a denatured protein, it can facilitate its degradation by the cell's proteolytic machinery. In eukaryotes, hsp70 is essential for the ubiquitination of certain

abnormal and regulatory proteins and thus in the breakdown of polyubiquitinated polypeptides by the 26S proteasome (27). The peptides generated by the proteasome in the cytosol appear to be the primary source of the peptides that are translocated into the endoplasmic reticulum for association with MHC class I molecules. Thus proteins that are linked to hsp70 may be ubiquitinated and processed especially well for presentation with MHC I proteins.

Immune responses to hsp70 have been detected after exposure to a broad spectrum of infectious agents (36–38). In addition, anti-hsp70 immune responses were induced in infants by the trivalent vaccine against tetanus, diphtheria, and pertussis (39). It seems that the immune system is routinely stimulated to respond to hsp70, and such stimulation may cause an expansion of hsp70-reactive cells. The cellular responses to mycobacterial hsps are profound; limiting dilution analysis indicates that 20% of the murine $CD4^+$ T lymphocytes that recognize mycobacterial antigens are directed against hsp60 alone (40). The high frequency with which human $CD4^+$ T cell clones directed against mycobacterial hsp70 and hsp60 have been detected suggests that these hsps are also major targets of the cellular response in humans (41). Thus, although soluble proteins administered in the absence of adjuvant do not typically elicit CD8 CTL, it is possible that the abundant hsp70-reactive helper T cells are involved in facilitating the unusually efficient CTL response against the soluble hsp70 fusion protein.

Another hsp, gp96, isolated from various tumors and tumor cell lines, has previously been shown to be a potent immunogen for eliciting CD8 CTL. The effectiveness of gp96 derives from (*i*) the many peptides that remain bound noncovalently to the protein when isolated from cells (10, 11), and (*ii*) its ability to facilitate the transfer of those peptides to MHC I proteins of ''professional'' antigen-presenting cells (42). Detailed studies of the peptide-binding activity of hsp70 has shown that it has a clear preference for peptides over 7 amino acids in length and those with aliphatic hydrophobic side chains (24, 31). Although gp96 can bind many different peptides (9, 43, 44), studies with hsp70, as well as general considerations, indicate that no protein can serve as a universal receptor for all peptides. Recombinant hsp70 fusion proteins, in contrast, are thus likely to provide a richer source of peptides available for binding to diverse MHC molecules.

Many different proteins can be linked to hsp70, and the fusion proteins studied so far are effective immunogens in the absence of adjuvants. hsp70 fusion proteins are thus attractive candidates for vaccines intended to stimulate CD8 CTL in humans.

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1. Byrne, J. A. & Oldstone, M. B. (1984) *J. Virol.* **51,** 682–686.
2. Nagler-Anderson, C., Verret, C. R., Firmenich, A. A., Berne, M. & Eisen, H. N. (1988) *J. Immunol.* **141,** 3299–3305.
3. Townsend, A. & Bodmer, H. (1989) *Annu. Rev. Immunol.* **7,** 601–624.
4. Heemels, M. T. & Ploegh, H. (1995) *Annu. Rev. Biochem.* **64,** 463–491.
5. Braciale, T. J., Morrison, L. A., Sweetser, M. T., Sambrook, J., Gething, M. J. & Braciale, V. L. (1987) *Immunol. Rev.* **98,** 95–114.
6. Jondal, M., Schirmbeck, R. & Reimann, J. (1996) *Immunity* **5,** 295–302.
7. Udono, H., Levey, D. L. & Srivastava, P. K. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3077–3081.
8. Udono, H. & Srivastava, P. K. (1994) *J. Immunol.* **152,** 5398–5403.
9. Arnold, D., Faath, S., Rammensee, H. & Schild, H. (1995) *J. Exp. Med.* **182,** 885–889.
10. Arnold, D., Wahl, C., Faath, S., Rammensee, H. & Schild, H. (1997) *J. Exp. Med.* **186,** 461–466.
11. Li, Z. & Srivastava, P. K. (1993) *EMBO J.* **12,** 3143–3151.
12. Suzue, K. & Young, R. A. (1996) *J. Immunol.* **156,** 873–879.
13. Rotzschke, O., Falk, K., Stevanovic, S., Jung, G., Walden, P. & Rammensee, H. G. (1991) *Eur. J. Immunol.* **21,** 2891–2894.
14. Young, D. B., Kent, L. & Young, R. A. (1987) *Infect. Immun.* **55,** 1421–1425.
15. McReynolds, L., O'Malley, B. W., Nisbet, A. D., Fothergill, J. E., Givol, D., Fields, S., Robertson, M. & Brownlee, G. G. (1978) *Nature (London)* **273,** 723–728.
16. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) *Methods Enzymol.* **185,** 60–89.
17. Moore, M. W., Carbone, F. R. & Bevan, M. J. (1988) *Cell* **54,** 777–785.
18. Walden, P. R. & Eisen, H. N. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9015–9019.
19. Falo, L., Jr., Kovacsovics-Bankowski, M., Thompson, K. & Rock, K. L. (1995) *Nat. Med.* **1,** 649–653.
20. Carbone, F. R. & Bevan, M. J. (1989) *J. Exp. Med.* **169,** 603–612.
21. Van Bleek, G. M. & Nathenson, S. G. (1990) *Nature (London)* **348,** 213–216.
22. Anderson, K. S., Alexander, J., Wei, M. & Cresswell, P. (1993) *J. Immunol.* **151,** 3407–3419.
23. Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M. E. & Hendrickson, W. A. (1996) *Science* **272,** 1606–1614.
24. Flynn, G. C., Pohl, J., Flocco, M. T. & Rothman, J. E. (1991) *Nature (London)* **353,** 726–730.
25. Cyr, D. M. & Neupert, W. (1996) in *Stress-Inducible Cellular Responses*, eds. Feige, U., Morimoto, R. I., Yahara, I. & Polla, B. S. (Birkhauser, Basel), Vol. 77, pp. 25–40.
26. Brodsky, J. L. (1996) *Trends Biochem. Sci.* **21,** 122–126.
27. Sherman, M. Y. & Goldberg, A. L. (1996) in *Stress-Inducible Cellular Responses*, eds. Feige, U., Morimoto, R. I., Yahara, I. & Polla, B. S. (Birkhauser, Basel), Vol. 77, pp. 57–78.
28. Hartl, F. U., Hlodan, R. & Langer, T. (1994) *Trends Biochem. Sci.* **19,** 20–25.
29. Hartl, F. U. (1996) *Nature (London)* **381,** 571–579.
30. Gething, M. J. & Sambrook, J. (1992) *Nature (London)* **355,** 33–45.
31. Rudiger, S., Germeroth, L., Schneider-Mergener, J. & Bukau, B. (1997) *EMBO J.* **16,** 1501–1507.
32. Flynn, G. C., Chappell, T. G. & Rothman, J. E. (1989) *Science* **245,** 385–390.
33. Beckmann, R. P., Mizzen, L. E. & Welch, W. J. (1990) *Science* **248,** 850–854.
34. Frydman, J., Nimmesgern, E., Ohtsuka, K. & Hartl, F. U. (1994) *Nature (London)* **370,** 111–117.
35. Nelson, R. J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M. & Craig, E. A. (1992) *Cell* **71,** 97–105.
36. Selkirk, M. E., Denham, D. A., Partono, F. & Maizels, R. M. (1989) *J. Immunol.* **143,** 299–308.
37. Hedstrom, R., Culpepper, J., Harrison, R. A., Agabian, N. & Newport, G. (1987) *J. Exp. Med.* **165,** 1430–1435.
38. Young, D., Lathigra, R., Hendrix, R., Sweetser, D. & Young, R. A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4267–4270.
39. Del Giudice, G., Gervaix, A., Costantino, P., Wyler, C. A., Tougne, C., de Graeff-Meeder, E. R., Van Embden, J., Van der Zee, R., Nencioni, L., Rappuoli, R., Suter, S. & Lambert, P. H. (1993) *J. Immunol.* **150,** 2025–2032.
40. Kaufmann, S. H., Vath, U., Thole, J. E., Van Embden, J. D. & Emmrich, F. (1987) *Eur J. Immunol.* **17,** 351–357.
41. Munk, M. E., Schoel, B. & Kaufmann, S. H. (1988) *Eur. J. Immunol.* **18,** 1835–1838.
42. Suto, R. & Srivastava, P. K. (1995) *Science* **269,** 1585–1588.
43. Udono, H. & Srivastava, P. K. (1993) *J. Exp. Med.* **178,** 1391–1396.
44. Nieland, T. J., Tan, M. C., Monne-van Muijen, M., Koning, F., Kruisbeek, A. M. & van Bleek, G. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6135–6139.