Heat Shock Protein-chaperoned Peptides but Not Free Peptides Introduced into the Cytosol Are Presented Efficiently by Major Histocompatibility Complex I Molecules*

Received for publication, December 21, 2000, and in revised form, February 20, 2001

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The studies reported here bear on the events in the cytosol that lead to trafficking of peptides during antigen processing and presentation by major histocompatibility complex (MHC) I molecules. We have introduced free antigenic peptides or antigenic peptides bound to serum albumin or to cytosolic heat shock proteins hsp90 (and its endoplasmic reticulum homologue gp96) or hsp70 into the cytosol of living cells and have monitored the presentation of the peptides by appropriate MHC I molecules. The experiments show that (i) free peptides or serum albumin-bound peptides, introduced into the cytosol, become ligands of MHC I molecules at a far lower efficiency than peptides chaperoned by any of the heat shock proteins tested and (ii) treatment of cells with deoxyspergualin, a drug that binds hsp70 and hsp90 with apparent specificity, abrogates the ability of cells to present antigenic peptides through MHC I molecules, and introduction of additional hsp70 into the cytosol overcomes this abrogation. These results suggest for the first time a functional role for cytosolic chaperones in antigen processing.

Cellular proteins undergo degradation in the cytosol, and the resulting peptides are transported into the endoplasmic reticulum (ER), generally through transporter associated with antigen processing (TAP). Within the ER, the peptides are charged onto MHC I molecules. One of the key unresolved questions in this scheme pertains to the mechanism through which peptides are channeled to the TAP or other transporters.

Although peptides are generated in the cytosol, there is little evidence that the cytosol harbors free peptides. It has been proposed that the peptides exist in association with peptide-binding proteins in the cytosol and the ER (1, 2). Because heat shock proteins (HSPs) are known to chaperone a wide array of molecules (3) and because immunological and structural evidence exists that HSPs chaperone antigenic peptides (see Ref. 4 for review), it was suggested that HSPs are the peptide-binding proteins that transport peptides (1, 2). This view has received little formal attention in the form of support or rejection, although no alternative mechanisms of peptide traffic have been suggested. Nonetheless, evidence has continued to accumulate that (a) HSPs are associated with peptides from a wide spectrum of antigens, including tumor antigens (5, 6), viral antigens (7), model antigens (8–10), and minor H antigens (8), and that (b) the repertoire of peptides associated with the HSP of the ER is dependent upon the functional status of TAP (9).

In this report, we address the issue functionally and ask if the chaperoning of peptides in the cytosol by HSPs confers on the HSP-chaperoned peptides any advantage not available to unchaperoned peptides in terms of their presentability by MHC I molecules.

EXPERIMENTAL PROCEDURES

Brefeldin A Treatment—EL4 cells were treated with brefeldin A (BFA) at two different concentrations in succession to respectively block the MHC I pathway of antigen presentation (6.0 μg/ml for 3 h) and to maintain the block (0.6 μg/ml for up to 12 h). Maintenance of the BFA block did not affect CTL function during the CTL assay. EL4 cells, untreated or treated with BFA at these concentrations, were analyzed by FACScan to show maximal decreases (40%) in surface expression of MHC I after 20 h (data not shown). BFA-treated cells were loaded with protein and used as targets in the CTL assay, as described, in the presence of BFA.

Cell Lines, Mice, and Reagents—The T-Ag-transformed cell lines SVB6 and PS-C5H were obtained from Prof. S. S. Tevethia and have been previously described (11) The VSVPN-transfected EL4 cell line, N1, was obtained from Dr. Lynn Puddington and has been previously described (12). EL4 cells, the TAP-dysfunctional cell line, RMA-S, were obtained from Prof. S. Nathenson. The RMA cell line has been previously described (13).

All chemicals were purchased from Sigma Chemical Co. unless otherwise specified. HL-1 and RPMI media, together with pyruvate, glutamine, penicillin-streptomycin, and non-essential amino acids were purchased from Life Technologies, Inc. RPMI containing 5% fetal calf serum (Intergen) and 1% each of pyruvate, glutamine, penicillin-streptomycin, and non-essential amino acids was subsequently referred to as complete RPMI.

Antibodies—HSPs were detected by immunoblotting with specific antibodies: gp96 (rat monoclonal antibody SPA-550, clone 9G10); cytosolic hsp70 (mouse monoclonal antibody SPA-820, clone N27F3-4 recognizes constitutive hsp73 and inducible hsp72); hsp90 (rat monoclonal antibody SPA-845, clone 12R2D2p90). All these antibodies were purchased from StressGen Biotechnologies Corp., Victoria, Canada. Anti-
K<sup>+</sup>, anti-D<sup>-</sup>, anti-D<sup>+</sup>, or anti-LFA1 (clones AF6-88-5, KH95, 34-2-12, and 2D7, respectively)-fluorescein-conjugated monoclonal antibodies were obtained from PharMingen (San Diego, CA).

**Cellular Loading of Proteins or Peptides—** To prepare proteins (gp96, hsp70, hsp90, or SA; complexed or not for loading, the indicated amount of protein was incubated with DOTAP (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium methosulfate (C<sub>2}H<sub>4}NO<sub>3}<sub>)(Roche Molecular Biochemicals) at a 3:2 ratio (microgram amounts) for 15 min at room temperature. In all loading experiments, 1.5 x 10<sup>5</sup> cells (EL4, RMA, or RMA-S) were washed three times with serum-free HL-1 media and then incubated in 1 ml of HL-1 media with a protein:DOTAP combination for 4-4.5 h at 37°C. Controls were either by incubating 1.5 x 10<sup>6</sup> cells in the same amount of DOTAP alone or were incubated with protein alone in the absence of DOTAP (pulsed cells). After loading (or mock loading for controls), cells were washed three times with HL-1 media and once with complete RPMI. Where indicated, loaded, mock-loaded, or pulsed cells were used as targets in CTL assays. Loading efficiencies of gp96, hsp70, hsp90, or serum albumin alone were the same, and inter-experimental values did not vary significantly. Free peptides were loaded into cells using the same protocol.

**CTL Assays—** CTL assays were carried out as follows. Briefly 2 x 10<sup>3</sup> (supplied as Na<sub>2</sub>CrO<sub>4</sub>; ICN) labeled target cells in 100 μl of complete RPMI were added to various dilutions of T-Ag or VSV8-specific CTL in 100 μl of complete RPMI. Maximum and spontaneous releases were measured by culturing 2 x 10<sup>3</sup> labeled target cells in lysis buffer (0.5% Nonidet P-40, 10 mM Tris, 1 mM EDTA, 150 mM NaCl) and complete RPMI, respectively, for 4 h. VSV8-specific CTL were obtained by dual immunizations of C57BL/6 mice, 1 week apart, with N1 cells. Spleen cells were harvested 1 week after the second immunization, restimulated in culture with irradiated N1 cells, and cloned by limiting dilution (14). The specificity of the CTL clone was tested by cold target inhibition and antibody blocking experiments. This CTL clone was shown to be specific for the VSV8 peptide (NH<sub>2</sub>-RGYYQQL-COOH) bound to K<sup>a</sup> molecules. A similar strategy, with chemically synthesized peptides, was used to obtain the T-Ag specific CTL clone. This clone was shown to be specific for the 9-mer peptide (NH<sub>2</sub>-AINNYAQKL-COOH), previously named epitope 1 (11).

**Flow Cytometry Analysis of DSG-treated Cells—** N1 cells were irradiated (5000 rads) and allowed to recover in AIM V medium with or without DSG for 48 h at 37°C or 25°C. Half of the cells incubating at 25°C were then plated at 37°C for an additional 8 h. One group of the cells was treated with DSG but incubated at 25°C for 48 h was plated at 37°C in the presence of DSG for 8 h. Cells (1 x 10<sup>6</sup>) were incubated with anti-α5β1 (lanes 1 and 2) and anti-αvβ3 (lane 3) antibodies, respectively. The cells were then stained with fluorescein-conjugated secondary antibody and analyzed on a FACScan flow cytometer purchased from Becton Dickinson (San Jose, CA).

**Immunofluorescence—** Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin and probed with anti-VSV glycoprotein Cy3-coupled antibody (Sigma). Cells were visualized using a Zeiss LSM confocal microscope.

**Infection of Cells with Vesicular Stomatitis Virus—** Vesicular stomatitis virus (VSV) was obtained from Advanced Biotechnology (Columbus, MD). Meth A or EL4 cells were incubated with 10 plaque forming units of VSV per cell for 1 h at 37°C in plain RPMI and allowed to recover in RPMI with 10% fetal calf serum for 4 h. Cells were washed three times in PBS (10 mM phosphate buffer, 150 mM NaCl, 2.7 mM KCl, pH 7.4). gp96 was then purified from these cells as described below.

**In Vitro Reconstitution of Protein-Peptide Complexes—** The following peptides were used (underlined sequences represent the precise MHC I binding epitope): unextended MHC binding 9-mer, NH2-AINNYAQKL-COOH; T-Ag 20-mer (N terminus extended), NH2-AINNYAQKL-COOH; T-Ag 20-mer (C terminus extended), NH2-AINNYAQKL-TFSFL-COOH; T-Ag 20-mer (C terminus extended), NH2-AINNYAQKL-TFSFLGKCV-COOH.

Peptides were synthesized by Genemed with a purity greater than 95% as determined by high pressure liquid chromatography. The unextended MHC I binding 9-mer peptide is identical to epitope 1 of the T-Ag (11). The T-Ag 9-mer stabilized high-molecular weight complexes on RMA-S cells and sensitized targets for lysis by the T-Ag-specific CTL. All three T-Ag 20-mer peptides failed to bind MHC H-2D<sup>D</sup> as determined by their inability to stabilize empty MHC molecules on the surface of RMA-S cells and their inability to sensitize target cells for lysis by T-Ag-specific CTL.

**Treatment of MLTC and N1 Cells with 15-Deoxyspergualin—** 15-Deoxyspergualin (DSG) was a gift from Dr. S. Nadler at Bristol-Myers Squibb Co. (Wallington, CT). Lyophilized DSG was dissolved in PBS and stored in aliquots at a concentration of 10 mg/ml at -130°C. Twenty micrograms per ml of DSG, with or without peptide (final concentration of 1 μM), was added to the MLTC of VSV CTL clones. After a 5-day incubation at 37°C, each well of the MLTC was harvested and tested for its ability to lyse 51Cr-labeled N1 and EL4 cells in a 4-h 51Cr release assay.

**RESULTS**

**Demonstration of the Experimental System to Introduce Molecules into the Cytosol—** The cationic liposome, DOTAP, was used to introduce HSP-peptide complexes or free peptides into the cytosol. Distinct properties of the detergents Nonidet P-40 and Saponin were used to demonstrate that DOTAP-loaded gp96 enters the soluble, non-vesicular, cytosolic compartment of the cells (Fig. 1). Although the cytosolic HSPs are of primary interest in this study, gp96 was used as a test case, because its distinct non-cytosolic localization (in the ER) permitted determination of the compartment into which the HSP-peptide complexes were being introduced, as will become clear from the following. The gp96/DOTAP-loaded cells were lysed with each of two detergents. Lysis of liver cells with 0.5% Nonidet P-40 leads to solubilization of all non-nuclear membranes, whereas lysis with 0.01% Saponin results in solubilization of plasma membranes but not internal membranes (19). The lysates were centrifuged to obtain the solubilized components, which were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (data not shown). The efficiency of gp96, hsp70, hsp90, or SA to complex peptides was comparable.

**Inhibition of Proteasome Function—** EL4 cells (10<sup>5</sup>) in complete RPMI were treated for 2 h with 100 μM of the proteasome inhibitor N-acetyl-l-leucinyl-l-leucinal-l-norleucinal (LlNLl) in Me<sub>SO</sub> or with 0.002% Me<sub>SO</sub> alone. In other experiments, EL4 cells were treated with 100 μM lactacystin dissolved in Me<sub>SO</sub> for 1 h. In these cases, the treated cells were constantly in the presence of the inhibitor during loading with protein. FACScan analysis of inhibitor-treated cells showed greater than 35% decrease in cell surface MHC I expression after 20 h confirming inhibition of MHC I trafficking.

**Purification and Identification of HSPs—** hsp70 and gp96 were purified from cells according to previously described methods (16, 17). hsp90 was purified according to the protocol of Denis (18) with minor modifications. Briefly, 100,000 × g supernatants were obtained from N1 lysates and applied to a Mono Q column (Mono Q HR 16/10, purchased from Amersham Pharmacia Biotech and attached to the BIOCAD, Perseptive Biosystems).

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Saponin-solubilized cells is if it has been introduced along with DOTAP into cells (lane 4), i.e. from an exogenous source. As additional controls, all samples tested predictably positive for the cytosolic chaperone hsp70 (Fig. 1, bottom panel, lanes 1–4). Thus, DOTAP-mediated delivery of gp96 (and by deduction other proteins) into cells introduces them into the cytosolic compartment. Similar results were obtained with introduction of labeled peptides by DOTAP. Quantitative analysis of exogenously introduced radiolabeled proteins through DOTAP indicated that ~5% of the DOTAP-loaded protein is introduced into the cytosol and that >96% of this 5% is detected in a soluble, non-vesicular, cytosolic compartment of the cells (data not shown).

**HSP-chaperoned Peptides Introduced into the Cytosol Become Ligands for MHC I—**As discussed in the previous section, the cytosolic chaperones hsp90 and hsp70 are of primary interest for the studies described all through this report. However, the ER chaperone gp96 was also used in all studies, primarily because (i) gp96 was used for the demonstration that DOTAP introduces proteins into the cytosol, (ii) gp96 is highly homologous (protein sequence homology of 50%) (see Ref. 1) to the cytosolic chaperone hsp90, and (iii) considerable immunological and structural information on gp96-peptide interaction is already available (see Ref. 4).

gp96, purified from the T-Ag-transformed cell line SVB6, and chaperoning T-Ag-derived peptides was loaded into EL4 cells by DOTAP. Presentation of T antigen-derived peptides by MHC I molecules of EL4 cells was monitored by specific lysis of EL4 cells pulsed with HSPs without DOTAP. Cells were used as targets for T-Ag-specific CTL clones in a 51Cr release assay. **Fig. 2A** shows that lysis of EL4 cells pulsed with gp96 or hsp70 was detected when either gp96, hsp70, or hsp90 purified from the T-Ag-transformed cell line SVB6 were loaded into EL4 cells at different doses as indicated. Closed crosses indicate EL4 cells pulsed with HSPs without DOTAP. Cells were used as targets for T-Ag-specific CTL clones in a 51Cr release assay. **Fig. 2B** shows that lysis of EL4 cells pulsed with HSPs without DOTAP was detected when either gp96, hsp70, or hsp90 was loaded into EL4 cells at different doses as indicated. Open crosses indicate EL4 cells pulsed with HSPs without DOTAP. ATP treated hsp70 indicates the N1-derived hsp70 preparation treated with ATP to remove peptides. Loaded cells were used as targets for VSVNP-specific CTL clones in a 51Cr release assay.

A second, well characterized antigenic system, the Vesicular Stomatitis Virus (VSV) system, was used to test the generality of the observation in the T-Ag system. VSV nucleoprotein (VNP) derived peptides chaperoned by gp96 or hsp70 (purified from the VSVNP-transfected cell line N1 (12) were effectively re-presented and recognized by VSVNP-specific CTL after the respective HSPs are introduced into the cytosol of EL4 cells by DOTAP (Fig. 2B). To demonstrate that lysis by VSVNP-specific
CTL, of cells loaded with HSPs, is peptide-dependent, equivalent amounts of peptide-free hsp70, obtained by ATP treatment of N1-derived hsp70 preparations (15), were delivered into EL4 cells. No lysis of EL4 cells loaded with peptide-depleted hsp70 preparations was observed (Fig. 2B). Furthermore, HSP preparations not carrying VSVNP-derived peptides (EL4-derived HSPs) (Fig. 2B), did not render loaded cells susceptible to VSVNP-specific CTLs, with any amount of HSP loaded. The results imply that presentation and consequent cell lysis are both peptide-dependent and -specific and require intracellular processing of the HSP peptide complexes.

**HSP-chaperoned Peptides Are Presented >100-fold More Efficiently Than Free Peptides**—It is difficult to monitor and quantify presentation of specific antigenic peptides in naturally derived HSP-peptide complexes. To quantitate the efficiency of re-presentation of specific HSP-chaperoned peptides, HSPs reconstituted in vitro with known quantity of antigenic peptides or their extended versions were used. The D8-restricted 9-mer epitope I of the SV40 T-Ag protein (NH2-AINNYAQKL-COOH), or 20-mer peptides extended on the NH2 terminus, COOH terminus, or both termini (Fig. 3A) were complexed to HSPs gp96, hsp90, or hsp70, or a control peptide-binding protein serum albumin (SA) (15 and “Experimental Procedures”). Peptides thus complexed (~10^{-6} M with respect to peptide concentration) or free peptides (10^{-8} or 10^{-4} M) were loaded into EL4 cells with DOTAP. In parallel, experiments using radiolabeled HSPs, SA, and each of the peptides were used to determine how much of each moiety administered with DOTAP could be recovered in the cytosol of the cells. This exercise demonstrated that 6–8% of the quantity of each moiety introduced in the cells by DOTAP could be recovered from the cytosol (data not shown). The constancy of this number allows for valid comparisons among the results with each antigenic moiety. The cells into which the HSPs, SA, or peptides were introduced were then monitored for lysis by T-Ag-specific CTLs (Fig. 3B). It was observed that (i) a concentration of 10^{-4} M free peptide was required for loading to observe lysis of the EL4 cells comparable to that observed for 10^{-8} M concentration of peptide when
chaperoned by HSPs, (ii) peptides chaperoned by SA, which binds peptides efficiently (“Experimental Procedures”), were not re-presented by MHC I molecules, suggesting that HSPs play a role different from simply carrying the peptides, and (iii) MHC I epitopes are generated from peptides chaperoned by HSPs regardless of whether they are extended on the NH₂, COOH, or both termini.

Re-presentation of HSP-chaperoned Peptides Requires Functional Proteasomes, Is TAP-dependent, and Is Brefeldin A-sensitive—The cytosolic proteasomes have been implicated as the primary producers of peptide ligands for MHC I molecules (for review see Refs. 20, 21). Because DOTAP-mediated loading of cells with the HSP-peptide complexes results in presentation the peptides by MHC I, we tested the requirement for proteosomal activity for re-presentation of HSP-chaperoned peptides. Because HSPs are purified from cells after the peptides have been generated through protease activity and also have been shown to chaperone precise MHC I peptide epitopes (6, 7, 10), we expected that re-presentation of HSP-chaperoned peptides would not require further proteosomal action. EL4 cells were treated with the proteasome inhibitor, N-acetyl-Leu-Leu-norleucinal (LLnL) for 1 h prior to and during loading with either the endoplasmic reticulum (ER) chaperone gp96 or the cytosolic chaperone hsp70 derived from N1 cells. Surprisingly, re-presentation of VSVNP peptides chaperoned by gp96 or hsp70 was inhibited by LLnL (Fig. 4A), suggesting that re-presentation of HSP-chaperoned peptides requires functional protease activity. Control, LLnL-untreated EL4 cells loaded with gp96 or hsp70 in an identical manner were able to re-present VSVNP-derived peptides.

Because LLnL has been shown to have inhibitory effects on proteases other than the proteasome (22), we replaced LLnL with the proteasome-specific inhibitor, lactacystin. To examine the proteasome dependence of HSP-chaperoned peptide re-presentation more precisely, we used HSP-peptide complexes reconstituted in vitro instead of the naturally derived complexes. The four T-Ag-derived peptides used earlier (Fig. 3A) were complexed separately to gp96, hsp70, or hsp90. HSP-peptide complexes, reconstituted in vitro, were loaded independently but identically, into EL4 cells, not treated or treated with lactacystin prior to loading. It was observed (Table I) that (i) treatment with lactacystin inhibited re-presentation of all the extended peptides, (ii) surprisingly, treatment of cells with lactacystin inhibited re-presentation of even the precise unextended MHC I binding peptides when chaperoned by hsp70 or hsp90; (iii) in another surprise, re-presentation of the precise MHC I binding peptide complexed to gp96 was not inhibited by lactacystin. These observations suggest that, during re-presentation, proteasomes may contribute function(s) other than proteolytic degradation of extended peptides. They also suggest that peptides chaperoned by the ER HSP, gp96, are processed by a different mechanism from that of peptides chaperoned by the cytosolic hsp70 and hsp90. The structural basis for this difference is not yet clear.

Peptides generated in the cytosol are transported predominantly by TAP into the endoplasmic reticulum for association with MHC I molecules (23–27). The requirement for TAP in re-presentation of HSP-chaperoned peptides was tested by comparing peptide re-presentation in TAP-expressing cells (RMA) and in TAP-dysfunctional cells (RMA-S). RMA-S cells were
not lysed by VSVNP-specific CTL after being loaded with N1-derived gp96 or hsp70 at any dose of HSP used (Fig. 4B). In comparison, RMA cells, expressing functional TAP molecules, did re-present the HSP-chaperoned peptides as measured by the effective lysis of HSP-loaded RMA cells (Fig. 4B). RMA cells pulsed with HSPs in the absence of DOTAP were not susceptible to lysis, indicating that lysis was not due to extracellular exchange of peptides.

A requirement for TAP for presentation of VSVNP may appear inconsistent with the earlier findings of Bevan and colleagues (28) who showed that VSVNP can be presented by MHC I in the absence of functional TAP2 molecules in RMA-S cells. However, a closer scrutiny of the previous data and our results shows that the differences are not inconsistent. Essentially, TAP2-negative cells such as RMA-S can still re-present VSVNP, whereas TAP1-negative cells cannot, thus suggesting that TAP1 homodimers may still be able to transport peptides into the ER. It is conceivable that, under limited quantities of antigenic peptides, such as those created by introduction of HSP-VSVNP complexes, the relative efficiencies of the TAP1/ TAP2 heterodimer vis-à-vis the TAP1 homodimer, become more evident. A second possibility may be envisaged where the VSVNP peptides generated in N1 cells are transported by anomalous TAP-independent means, whereas direct introduction of the same peptides with HSPs introduces them into the classical TAP-dependent pathway.

After MHC I molecules are loaded with peptides in the ER, they are transported to the cell surface via the Golgi by vesicular traffic. Brefeldin A (BFA) is a known inhibitor of post-ER vesicular traffic (29). EL4 cells were not treated or treated with BFA for 1 h prior to and during DOTAP-mediated loading of SVB6-derived gp96, hsp70, or hsp90. The loaded cells were then tested for lysis by T-Ag-specific CTLs. It was observed that BFA completely inhibited re-presentation of peptides chaperoned by gp96, hsp70, and hsp90 (Fig. 4C). This inhibition was reversible by incubating BFA-treated EL4 cells in the absence of BFA for 3 h prior to loading with HSP-peptide complexes (Fig. 4C).

Sequestration of Endogenous Cytosolic HSPs Abrogates Presentation of Antigenic Peptides by MHC I Molecules—Deoxyspergualin (DSG) is a small molecular weight immunosuppressive drug shown to interact specifically with hsp70 and hsp90 (30, 31). The drug can enter cells and interact with endogenous hsp70. We sought to exploit the HSP-binding property of DSG to test whether binding of DSG will lead to sequestration of hsp70 and hsp90, which will now be unable to chaperone the newly generated antigenic peptides into the endogenous presentation pathway. This idea was tested in a series of experiments. As a first measure, DSG was added to mixed lymphocyte tumor cultures (MLTC) of N1 cells (EL4 cells transfected with the gene encoding VSV NP (12)) and anti-N1 CTL clones. The MLTCs generated in the presence of DSG were tested in a cytotoxicity assay for activation and proliferation of antigen-specific CTLs. Treatment with DSG was observed to inhibit dramatically the activation/proliferation of VSV NP-specific CTLs (Fig. 5A). However, this inhibition could be reversed completely if VSV NP-derived peptide VSV8 were added to the MLTC. Addition of an irrelevant peptide (corresponding to an epitope from SV40 T antigen with the same restriction element as the VSV epitope), did not reverse the inhibition. These data indicate that treatment with DSG resulted in a limitation in the quantity of the VSV epitope on N1 cells. To determine if DSG was acting at the level of the CTLs or the antigen-presenting cell, the CTLs were purified and were cultured in medium with or without DSG and were tested for their ability to lyse N1 cells. DSG treatment for as long as 100 h had no discernible effect on the CTLs (Fig. 5B).

The effect of DSG on N1 cells was monitored directly. As N1 cells already contain a population of specific MHC I peptide complexes, which have a certain half-life, and because even a very small number of MHC I peptide complexes are capable of stimulating activated CTLs (32), a system was sought where no preformed specific MHC I peptide complexes exist. EL4 cells were treated (or not treated) with DSG for 24 h so as to allow sequestration of hsp70 and hsp90 molecules. Cells were then infected with VSV. The virus-infected and viral antigen-expressing cells, which had or had not been exposed to DSG pre-infection, were used to stimulate anti-VSV NP CTLs, as described in a previous experiment (see Fig. 5A). The DSG-treated cells were observed to be unable to stimulate the CTLs at all, whereas the control cells stimulated them as expected (Fig. 6A). As an additional control in these studies, EL4 cells,

TABLE I

| Chaperone | Peptides          | N-extended, 20-mer | N+C-extended, 20-mer | C-extended, 20-mer | D<sup>8</sup>-binding, 9-mer | %  |
|-----------|------------------|--------------------|----------------------|--------------------|-----------------------------|----|
| gp96      |                  | 60.1               | 96.0                 | 81.0               | 71.1                        |    |
| hsp90     |                  | 61.2               | 98.0                 | 98.2               | 65.3                        |    |
| hsp70     |                  | 90.1               | 98.3                 | 65.3               | 46.1                        |    |

FIG. 5. hsp70 is involved in the transport of antigenic peptides to MHC I molecules. Treatment of cells with DSG reduces their capacity to stimulate CTLs. A, CTL clone against VSVNP and feeder cells were incubated in media without (open symbols) or with 20 μg/ml DSG (solid symbols) for 5 days in the presence of N1 (squares) or EL4 pulsed with VSV K<sup>b</sup> epitope (circles) or T-antigen (triangles) peptides. The CTLs recovered were tested for their ability to lyse N1 cells in a 4-h ⁵¹Cr release assay. B, cytotoxic activity of CTLs is not affected by treatment with DSG. CTLs incubated for 5 days without (open square) or with 20 μg/ml of DSG (closed square) were tested for cytotoxicity against ⁵¹Cr-labeled N1 cells.
with or without prior treatment with DSG, were pulsed with VSV8, and these were tested for the ability to stimulate CTLs. Treatment with DSG was found to have no effect on the antigen-presenting ability of VSV8 pulsed cells (Fig. 6A). Prior treatment of cells with DSG had no effect on viral infection and expression of viral proteins as determined by staining of infected cells with anti-G protein antibody coupled to a photochrome (Fig. 6B). These results show clearly that treatment with DSG interferes with a step in the antigen-presenting cell, which is required for generation of the specific MHC I peptide complex, although the block is not in generation of MHC I molecules per se.

Although DSG has been shown to interact specifically with hsp70 and hsp90 (30), the possibility that the effects observed are not due to the HSPs but due to interaction of DSG with an unknown intracellular pathway, the role of hsp70 was tested more directly. Experiments shown in some of the previous figures (Figs. 1–3) demonstrate how it is possible to introduce molecules into the cytosol of living cell with the help of DOTAP. This method was now used to introduce hsp70, or as a control, SA (which has been shown previously to bind peptides efficiently (15)), into the DSG-treated cells 1 h after infection with VSV. The cells were used to stimulate the anti-VSV CTLs as before. The experiment showed (Fig. 7) that introduction of hsp70 but not SA could completely relieve the inhibition in antigen-presenting ability in DSG-treated cells.

The ability of DSG to block the trafficking of peptides destined for loading the cell surface MHC I molecules was tested by an independent assay. MHC I-β2 microglobulin complexes devoid of peptides are unstable on the cell surface at 37 °C but are stable at 25 °C (33). The MHC I-peptide complexes can also be detected by conformation and Kβ-specific antibodies. These tools were used to examine the presence of stable MHC I molecules on the cell surface of DSG-treated and untreated cells. It was observed that treatment of EL4 cells with DSG at 37 °C lead to a nearly 5-fold reduction in the number of Kβ-peptide complexes as determined by the specific antibody Y3 (Fig. 8). At 25 °C, no such inhibition was observed. Interestingly, if the EL4 cells kept at 25 °C were now shifted to 37 °C, the DSG-treated cells showed a nearly 5-fold less quantity of Kβ moieties than the DSG-untreated cells, indicating that a large proportion of Kβ molecules of DSG-treated cells at 25 °C were devoid of peptides and hence labile in the DSG-treated cells. EL4 cells also express the LFA molecule whose expression in DSG-treated and untreated cells was monitored and found to be unaffected by treatment with DSG, indicating that DSG was not affecting the secretory pathway per se, as also indicated by the experiment carried out at 25 °C.

These observations provide strong support for the idea that HSPs are necessary for transport of antigenic peptides in the cytosol and that DSG interferes with this step.

**DISCUSSION**

The studies reported here shed light on trafficking of peptides in the cytosol, leading to presentation of peptides by the MHC I molecules. First, free peptides introduced into the cytosol are presented quite inefficiently as compared with HSP-chaperoned peptides. This observation supports the idea that the trafficking of peptides in the cytosol does not occur by passive diffusion but by active mechanisms, including chaperoning (1, 2, 4). This is particularly relevant, because of quantitative considerations. The quantity of peptides available naturally in a cell (in the order of sub-femtograms/cell for an epitope derived from a moderately expressed protein) is too low to allow presentation by MHC I molecules, if the peptide were to diffuse passively. The same quantity of peptide has a significantly higher likelihood of getting presented if it were chaperoned by an HSP molecule, as shown here. Second, chaperoning (i.e. being carried by a larger molecule) is necessary but insufficient for peptide re-presentation, because SA-chaperoned peptides are not presented any more efficiently than free peptides. The structural rules that define the requirement for and efficiency of chaperoning in presentation for each HSP, such as binding affinities, number of peptide binding sites, peptide-dissociation rates, association with other peptide-recipient proteins are not yet known; however, our studies provide an assay through which they could be divined. These structural rules may account, at least partially, for the differences in proteosome requirements observed for hsp90 and hsp70 versus gp96 (Table I). In addition, because hsp70 and hsp90 are cyto-
Fig. 7. Recovery of antigen presentation in DSG-treated cells by introduction of hsp70. EL4 cells were irradiated and incubated without (open symbols) or with 40 μg/ml DSG (closed symbols) for 24 h. EL4 cells were washed and infected with VSV for 1 h prior to introduction of PBS (circles), 50 μg of hsp70 (squares), or 50 μg of mouse serum albumin (triangles) by the transfection reagent DOTAP. Cells were then tested for their ability to be recognized by VSV CTLs in a 4-h ⁵¹Cr release assay.

Fig. 8. Treatment of cells with DSG affects detection of folded MHC I peptide complexes on the cell surface. EL4 cells were incubated with or without DSG at 25 °C or 37 °C for 48 h. One group of cell, as indicated, was changed at 40 h from 25 °C to 37 °C for 8 additional hours. Cells were then stained with fluorescein-conjugated anti-Kb, anti-D, or anti-LFA antibodies and analyzed by flow cytometry.

Acknowledgment—We thank Dr. Sreyashi Basu of our laboratory for critically reading the manuscript.
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