Differential Acquisition of Antigenic Peptides by Hsp70 and Hsc70 under Oxidative Conditions*

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Hsp70 and Hsc70 are two chaperones of high homology expressed under contrasting situations. Hsc70 is constitutively expressed and poorly stress-inducible, whereas Hsp70 is unabundant in normal physiological situations and strongly induced under oxidative stress. In the present study we show that the chaperoning activity of purified Hsp70 and Hsc70 is minimal under reducing conditions and increases in environments that mimic oxidative stress. Association with peptides is more pronounced for Hsp70 than for Hsc70 in every condition tested and is accompanied with a gradual change in secondary structure during oxidation. The binding of peptides to Hsp70 and Hsc70 under oxidative conditions is not reversible by treatment with a reducing agent, confirming that other chaperone-associated factors are required for substrate release. These findings support the idea that formation of HSP70-peptide complexes and possibly their immunogenicity is enhanced in conditions of stress.

The Heat shock proteins (HSPs)1 are a family of molecular chaperones that have recently been placed in the center of a new immunological paradigm. They have been shown to chaperone MHC class I-restricted peptides and/or their elongated precursors and to deliver them to the immune system (1–3). The mechanism by which purified HSP70s immunize is relatively well understood due to (i) the discovery of CD91, its receptor on antigen-presenting cells (4, 5), (ii) the identification of HSP70 peptide binding site and their ability to release their substrate upon ATP treatment (6–8), and (iii) the physical isolation of antigenic peptides associated with Hsp70 (3, 9). The current view is that HSP70-peptide complexes, the antigen-presenting cells migrate to the lymphatic organs where they prime CD8+ T lymphocytes. Importantly, HSPs that chaperone non-immunogenic peptides and “empty” HSPs cannot immunize (11). Therefore, the biochemical mechanism by which immunogenic peptides associate with HSPs is of primary importance for the initiation of a specific immune response (12).

The general chaperoning features of HSPs have been studied in detail, mainly in terms of their ability to release their polypeptide substrate (7, 13). However, the initial trigger for substrate binding has not been clearly established. Additionally, because some HSPs are stress-inducible, like Hsp70, whereas some are constitutively expressed, like Hsc70, one may ask if their respective chaperoning functions are identical and whether they are influenced by the biochemical status of the stressed cell (14). Remarkably, the common denominator in all the Hsp70-inducing stresses is oxidation of the cytosol (12, 14). The dramatic change in the redox status of the cytosol is characterized by an increase in permeability of the mitochondrial inner membrane (15), oxidation and depletion of non-oxidized glutathione, NAD(P)H2, and hyperproduction of reactive oxygen species (16). Direct chemically induced oxidative stress can also act as a signal for the induction of Hsp70 through oxidation of protein thiols, formation of a non-native disulfide bond, and glutathione depletion (17, 18). Inversely, dithiothreitol (DTT), among other reducing agents, inhibits the heat-induced synthesis of Hsp70, whereas oxidized DTT is ineffective (19). Altogether, these reports illustrate that the raison d’être of the Hsp70 is strongly linked to an environment more oxidative than the normally reducing cytosol.

We previously showed that tumor immunogenicity co-segregates with the expression of Hsp70 but not Hsc70 (14, 20). The immunological importance of the inducible Hsp70 has also been shown by hyperthermia (21) and transfection (22, 23). However, the molecular mechanism by which expression of Hsp70 but not Hsc70 induces tumor immunogenicity has not yet been unraveled. In this study, we have tested the hypothesis that Hsp70 and Hsc70 acquire immunogenic peptides differently during oxidative stress. We have shown that the peptide binding capacities of both chaperones are minimal under reducing conditions but optimal under moderately oxidative conditions. Moreover, Hsp70 is quantitatively a better peptide binder than Hsc70 through out all the conditions tested. The
binding of peptides to HSP70s is limited in reducing conditions; however, once the HSP70-peptide complexes are formed, they are poorly dissociated by reducing agents.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of HSP70s—Mouse Hsp70 and Hsc70** were purified as previously described (24). Briefly, *Escherichia coli* (BL21LDE3 LysS, Stratagene, La Jolla, CA) transformed with full-length mouse Hsp70 cDNA and full-length mouse Hsc70 cDNA into pM70–1 vector or BALB/c mouse liver tissues were homogenized and a 100,000 × g supernatant was obtained. The samples were applied to an ADP-agarose column (Sigma), washed and eluted with buffer D (20 mM Tris acetate, 20 mM NaCl, 3 mM MgCl₂, pH 7.5) containing either 10 mM ADP or ATP. Eluted fractions were applied on a DEAE column and washed and eluted with 20 mM sodium phosphate and 150 mM NaCl, pH 7.0. The bacterial Hsp70, DnaK, eluting at NaCl concentrations higher than 150 mM, did not contaminate the preparation. Purity and homogeneity of Hsp70 and Hsc70 preparations were assessed by SDS-PAGE and immunoblot analysis. Mouse Hsp70 purified from mouse liver tissue and unmodified full-length recombinant mouse Hsp70 purified from *E. coli* were compared and found identical in term of DNA sequence and biochemical properties including apparent molecular size, secondary structure defined by circular dichroism, peptide binding activity, ATPase activity, and foldase activity (data not shown).

**SDS-PAGE and Autoradiography—**Proteins were submitted to reducing or oxidative treatment as indicated in the figures legends, mixed with non-reducing SDS-PAGE sample buffer and resolved on 10% SDS-PAGE. After electrophoresis, polyacrylamide gels were Coomassie Blue-stained, dried, and exposed for autoradiography.

**Complexing of HSP with Radiolabeled Peptides—**The MHC-I epitopes, HA518 (NH₂IYSTVASSLCOOH, H₂-K* restricted) and AH1 (NH₂SPSYYYHQFCOOh, H₂-L* restricted) were purchased from Genemed Synthesis Inc. (South San Francisco, CA), iodinated as described before (25), mixed with the Hsc70, Hsp70, or transferrin (Sigma) in buffer E (20 mM phosphate, 20 mM NaCl, pH 7.0) alone or in the presence of reducing or oxidative agents, and incubated for 30 min at 37 °C as described elsewhere (24).

**HSP70s Peptide Elution and Circular Dichroism—**Purified HSP70s were incubated for 16 h at 37 °C in plain buffer E, in buffer E with 10 mM ATP, and in buffer E with 10 mM DTT. The peptides were recovered by filtration on biomax-10 (10 kDa exclusion limit) (Millipore, Bedford, MA), loaded on a C18 column (the Nest Group, Southborough, MA) in MeOH, and eluted by a linear acetonitrile gradient as described previously (26). Hsp70 and Hsc70 (10 μM) were analyzed by circular dichroism on JASCO J-175 at room temperature in CD buffer (5 mM phosphate, 50 mM NaF, pH 7.0) under concentrations of DTT varying from 0 to 1 mM.

**RESULTS**

**Hsp70 Is a Better Peptide Binder than Hsc70—**We have investigated the ability of Hsp70 and Hsc70 to bind peptides by incubating purified proteins with a molar excess of radiolabeled peptides (protein:peptide ratio = 1:20) and traced the presence of radioactivity associated with 70-kDa proteins by gel electrophoresis as previously described (24, 27). We observed by autoradiography that tissue-derived and recombinant Hsc70 and Hsp70 associated with peptides, whereas a non-chaperone protein of similar molecular weight, transferrin, did not (Fig. 1). Quantification of the radiolabeled peptides migrating in the area of 70-kDa indicates that Hsp70 reproducibly binds more peptide than Hsc70 irrespectively of the buffer used for the complexing reaction (data not shown). Surprisingly, association of peptides with both Hsp70 and Hsc70 is promoted in oxidative buffer and considerably reduced by addition of 0.1 mM of reducing agents, such as DTT (Fig. 1A, lanes 5–8) and 2-mercaptoethanol (2ME) (Fig. 1A, lanes 9–11), in the reaction mixture. In addition, tissue-derived and recombinant Hsc70s peptide binding capacities follow the same patterns in the different conditions tested (Fig. 1A, lanes 1–2, 5–6, and 9–10). We took advantage of the presence of peptides bound to Hsp70/Hsc70 after ADP purification and their absence after ATP purification (24) to analyze the influence of endogenous peptides bound to HSP70s on their peptide binding capacities in the presence of an excess of exogenous radiolabeled peptides under various conditions (Fig. 1B). We observed identical binding patterns between ADP- and ATP-purified HSP70s, with the difference that empty ATP-purified Hsp70/Hsc70 bind more peptides than the ADP-purified ones in every condition tested. We have determined the quantity of peptide bound per mole of Hsp70 and Hsc70 by cutting the 70-kDa band out of the gels and counting in a γ-counter. Knowing the specific radioactivity of the peptides (cpm/mol of peptide) and assuming that the stoichiometry of the peptide is only one peptide per molecule of HSP70 as suggested by crystallographic study of Hsp70 peptide binding domain (8), we have calculated that a maximum of 25% of chaperones are loaded with exogenous peptides (ATP-purified Hsp70 in oxidative conditions, Fig. 1C). However, the same sample treated with DTT binds only 13% of peptides, confirming that Hsp70 binds peptide more efficiently under oxidative conditions (p < 0.005; probability associated with a Student’s paired t-test). Additionally, we measured that, as shown in Fig. 1, A and B, Hsp70 binds more peptides than Hsc70 under the same conditions (13% instead of 25%, p < 0.01) and that this percentage decreases in reducing milieu from 13 to 5% (p < 0.01) (Fig. 1C). The binding of peptides to HSP70s is probably not optimum under our experimental conditions, likely due to the non-physiological parameters of the experiment, the lack of co-chaperones, and the stringency of the SDS-PAGE analysis. Similar binding has been reported using different loading conditions and peptides with high affinity for Hsp70 (10).

**Peptide Binding to Hsp70 and Hsc70 Is Improved under Oxidative Environment—**The ability of Hsp70 and Hsc70 to bind peptides under a broad range of redox conditions has been tested using the two immunogenic peptides AH1, NH₂SPSYYYHQFCOOh, and HA518, NH₂IYSTVASSLCOOH. We first observed that under highly reducing conditions (1 mM DTT, Fig. 2A, lane 1) Hsp70-peptide complex formation was weak but clearly detectable. On the contrary, in the same conditions, no peptide could be detected in association with Hsc70. By extending the comparison between Hsp70 and Hsc70 under increasingly oxidative conditions, we observed a similar but shifted pattern of binding. For every concentration of DTT varying on a logarithmic scale from 1 to 10⁻³ mM, Hsp70 binds more peptide than Hsc70 (Fig. 2A, lanes 2–5). A comparable shifted pattern was observed with another reducing agent, 2-mercaptoethanol (data not shown). Additionally, we observed that the binding of peptides under increased oxidative conditions in the presence of hydrogen peroxide was not more pronounced than in plain (non-reducing) buffer (Fig. 2A, lanes 5–6). To avoid a misrepresentation of the radioactivity intensity, the autoradiographies were exposed for the same length of time such that the maximum nonspecific binding ever observed with the control protein transferrin was of similar intensity on every autoradiogram. A third peptide, precursor of the H₂-K* restricted immunodominant epitope of the vesicular stomatitis virus nucleoprotein (NH₂SLSDLRGYVQGLKSGNVS COOH) was also tested and showed an identical binding pattern under reducing and oxidative conditions compared with AH1 and HA518 (data not shown). The observation that three peptides of unrelated sequences, sizes, and charges behave similarly argues in favor of a structural difference between Hsp70 and Hsc70 in redox conditions rather than a substrate-directed phenomenon.

Hsp70 and Hsc70 Modulate Differently Their Secondary Structure under Different Redox Conditions—In the previous section, we observed that Hsp70 and Hsc70 chaperoning was...
Peptides Bound to Hsp70s under Oxidative Conditions Are Poorly Dissociated by Reducing Treatment—By analogy with the required role of ATP for the release of peptides bound to HSP70s and based on our observation that HSP70s do not chaperone peptides under a strongly reducing environment, we investigated whether a reducing agent may dissociate HSP70-peptide complexes. In a previous study we demonstrated that iodinated synthetic peptides complexed in vitro with HSP70s (using the same method as in the present work) can be dissociated by purification on ATP-agarose but not ADP-agarose chromatography, confirming the ATP-dependent release of HSP70s substrate (24). In Fig. 3A, we have tested the reversibility of peptide binding by a reducing agent. The experiment was designed such that the oxidative buffer used for a first incubation became reducing for a second successive complexing reaction by addition of an excess of DTT. By doing so, we could compare the sequence of oxidation/reduction in multi-step complexing reactions ending with identical buffers. We observed that the Hsc70-peptide complexes formed during a first oxidative incubation (1 mM H2O2) were not dissociated by a further incubation in the presence of an excess of reducing agent (10 mM DTT) (Fig. 3A, lane 2). As a control, we showed that peptides incubated with Hsc70 in a reducing buffer (1 mM H2O2 + 10 mM DTT) did not associate with the chaperone protein (Fig. 3A, lane 1). Note that the final buffer composition of these two reactions was identical and that the net redox potential was minimal under reducing conditions and progressively increased as the milieu became more oxidative (Fig. 2A). Therefore, we investigated possible changes in Hsp70 and Hsc70 secondary structures that may support the increased acquisition of peptides under oxidative stress. We visualized highly purified Hsp70 and Hsc70 (see inset in Fig. 2B) by circular dichroism (CD) in conditions varying from reducing (i.e., high DTT concentration), possibly mimicking a resting cytosol, to more oxidative (i.e., low DTT concentration) that may reflect an oxidative stress (see “Discussion”). The absolute molar ellipticity, i.e., the percentage of amino acids participating in the formation of α-helices and β-sheets, was similar but minimal for Hsc70 and Hsp70 in presence of 1 mM DTT (Fig. 2B, left panel) probably due to a reversible and mild denaturation. In a more oxidative milieu (0.01 and 0.001 mM DTT), we reproducibly observed a more dramatic gain of secondary structure for Hsp70 than for Hsc70 (Fig. 2B, middle and right panels). Identical results have been obtained with unmodified full-length Hsp70 purified from E. Coli and with Hsc70 purified from mouse liver. The minimal absolute molar ellipticity of Hsp70 and Hsc70 under highly reducing conditions suggests that they adopted a conformation with less structured domains that may not easily accept peptides. Conversely, in the presence of more oxidative buffer the proteins may adopt a more structured conformation favorable to association with peptides. In addition, the higher gain in secondary structure of Hsp70 over Hsc70 during oxidation supports the notion that Hsp70 is a chaperone more responsive to environmental stresses than Hsc70.
reducing. A complete parallel observation was made for the inducible Hsp70, although the intensity of binding was superior than for the Hsc70, and some peptides remained associated with Hsp70, even in reducing conditions (Fig. 3A, lanes 4 and 5). This experiment demonstrated that the initial binding of peptides to Hsp70 and Hsc70 could not be easily reversed in the presence of reducing buffer. Additionally, the lack of binding of peptides in 1 mM DTT (as shown in Fig. 2A) is reversible by incubation with an excess of oxidative agent (10 mM H$_2$O$_2$) (Fig. 3A, lanes 3 and 6). Therefore, the lack of association of peptides...
to Hsp/Hsc70 in reducing conditions is not due to an irreversible denaturation of their peptide binding sites. Moreover, the lack of dissociation of peptides from Hsp70 and Hsc70 upon addition of reducing agent argues also against intrachain disulfide bound formation that would define a domain holding the peptides (see “Discussion”).

We then considered the status of naturally bound peptide-HSP70 complexes. We tested this phenomenon with mouse tissue-derived Hsc70s isolated with their naturally bound peptides as demonstrated previously (11). Hsc70 treated for 16 h at 37 °C in ATP-containing buffer released a heterogeneous population of peptides that were visualized at 214 nm. On the contrary, treatment with 10 mM DTT for 16 h at 37 °C induced a marginal release of peptides (Fig. 3B, top right panel). Comparison of the chromatogram areas indicated that Hsc70 incubated with ATP released between 4.5× more peptide (6.7× more in a second independent experiment) than if incubated with DTT (peptide release was measured as area between buffer and treated sample). It is noteworthy that Hsc70 incubated in oxidative buffer overnight did not release peptides at all (Fig. 3B, oxidative treatment) confirming the stability of the chaperoning in such environment. This suggests that Hsc70 adopts a conformation favorable for a slow release of their substrates in reducing conditions, whereas the peptides remained tightly bound to HSP70s in an oxidative milieu.

**DISCUSSION**

In this study we have tested the hypothesis that cellular stress involving oxidative conditions influences binding of immunogenic peptides to Hsp70 and Hsc70. Our experiments show that acquisition of peptides by HSP70s is enhanced under oxidative environment and limited under reducing conditions. This phenomenon has been tested for two HSPs, Hsp70 and Hsc70, through a broad range of redox conditions and with three peptides of unrelated sequences. Second, we have shown that Hsp70 is a better peptide binder than Hsc70 in every condition tested. Third, we have demonstrated that peptides bound in oxidative conditions are not released by addition of reducing buffer. These observations have important biochemical and immunological implications in that they link oxidation to immunogenicity.

The partial loss of secondary structure of the chaperones in reducing conditions, as shown by circular dichroism, is likely to alter the accessibility of the substrates to the peptide binding site of HSP70s. In a reducing milieu, such as the resting cytosol, HSP70s possibly adopt a conformation in which the peptide binding site of the chaperones is poorly accessible to the substrate. Conversely, upon oxidation of the milieu due to an exogenous stress, the gain of the HSP70s secondary structure is translated into a better accessibility of peptides to the peptide-binding pocket and therefore to a more effective chaperoning. This view is compatible with the general concept that HSP70s associate with polypeptides that unfold during oxidative stress, helping them to regain a functional structure or directing them to a degradation pathway (28). This general biochemical mechanism will have important immunological consequences. First, the oxidative stress induces Hsp70 expression, increasing the net quantity of chaperones that can associate immunogenic peptides. Additionally, upon oxidation both Hsp70 and Hsc70 will carry a larger population of peptides. The high stability of the HSP70-peptide complexes formed under oxidation is compatible with their efficient delivery to the antigen, presenting cells that will prime CD8+ T lymphocytes. It has also been proposed and indirectly shown that HSP70s participate in the processing of the endogenous antigen to the MHC-I pathway (29). Therefore, an increase in the quantity of peptides chaperoned by HSP70s under oxidative stress may affect direct antigen presentation by MHC I molecules.

The constitutive Hsc70 and inducible Hsp70 are generally considered, with rare exceptions (31), to be equivalent proteins exhibiting functional similarities. However, although the overall structures of these two chaperones are almost identical, they do differ in some specific areas (12, 14): Their carboxyl-
terminal domains (amino acid 510–641) differ considerably in structure (“only” 69% amino acid identity). This domain may stabilize the peptide-binding pocket more efficiently in Hsp70 than in Hsc70 and therefore possibly regulates differently the access of the substrates to the peptide-binding pocket. By analogy with the activation of Hsp33 through oxidation of its cysteines (32–34), it is also possible that oxidized Hsp70 forms intrachain disulfide bonds. We have, in another publication (12, 14), calculated the theoretical disulfide bond-forming potentials of the cysteines of HSP70s and reported that one cysteine (position 603) is hyper-reactive in Hsp70, whereas none are in Hsc70. In theory, Cys603 of Hsp70 could easily form a disulfide bond with another reactive cysteine on Hsp70 or on another protein. However, no evidence of Hsp70 intrachain and interchain disulfide bonds have been detected. Nevertheless, it cannot be excluded that under oxidative stress, Cys603 is involved into the overall stabilization of Hsp70 by forming a labile interaction with a poorly reactive site localized on another domain of the protein. At this point, genetic manipulations of Hsp70 are required to determine the amino acids involved in its reactivity to the oxidating milieu and its improved substrate binding ability by comparison with Hsc70.

How do the changes in structure measured by CD relate to the physiological state? The redox potentials of Hsp70 and Hsc70 are unknown at this time. Hence, we can compare the previous example of Hsp33, a chaperone activated under oxidative stress that set a precedent for understanding the physiology of this phenomenon. It has been reported that under stress, the redox potential increases from −250/280 mV to about −150 mV, showing that even under oxidative stress the environment stays globally reducing (35). The reported redox potential of Hsp33, −170 mV, is close to an oxidative cytosol, explaining the very low activity of Hsp33 under resting cellular condition (33). Our prediction would be that, like for Hsp33 their values lie closer to the redox of a stressed than a resting cytosol. Because our circular dichroism data indicates that Hsp70 is more reactive to oxidative conditions than Hsc70 we anticipate that the redox potential of Hsp70 would be higher than that of Hsc70.

Our previous observation that immunogenicity of tumor cells co-segregate with the expression of Hsp70 but not with Hsc70 was surprising due to their high structural homology (12, 14, 20), implying that subtle differences between these two HSPs may have dramatic biological consequences. The observation that Hsp70 associates with immunogenic peptides quantitatively and qualitatively better than Hsc70 in all of the conditions tested brings a new element in trying to explain the different immunological properties of these two chaperones. It suggests that Hsp70 could be a chaperone designed to preferentially capture and deliver polypeptidic information to the immune system under stress.

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