CD40, an extracellular receptor for binding and uptake of Hsp70–peptide complexes

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Tumor and viral antigens elicit a potent immune response by heat shock protein–dependent uptake of antigenic peptide with subsequent presentation by MHC I. Receptors on antigen-presenting cells that specifically bind and internalize a heat shock protein–peptide complex have not yet been identified. Here, we show that cells expressing CD40, a cell surface protein crucial for B cell function and autoimmunity, specifically bind and internalize human Hsp70 with bound peptide. Binding of Hsp70–peptide complex to the exoplasmic domain of CD40 is mediated by the NH2-terminal nucleotide–binding domain of Hsp70 in its ADP state. The Hsp70 cochaperone Hip, but not the bacterial Hsp70 homologue DnaK, competes formation of the Hsp70–CD40 complex. Binding of Hsp70-ADP to CD40 is strongly increased in the presence of Hsp70 peptide substrate, and induces signaling via p38. We suggest that CD40 is a cochaperone-like receptor mediating the uptake of exogenous Hsp70–peptide complexes by macrophages and dendritic cells.

Introduction

Heat shock proteins and molecular chaperones, including Hsp70 and glucose-regulated protein 94 (Grp94),1,2 have been established in recent years as immune adjuvants for cross priming with antigenic peptides (Srivastava et al., 1994). In this process, antigen-presenting cells (APCs) internalize exogenously administered heat shock proteins with bound peptides by receptor-mediated endocytosis (Arnold-Schild et al., 1999), resulting in antigen presentation via MHC I (Castellino et al., 2000). Tumor-bearing mice injected with Hsp70 (or Grp94) isolated from the tumor or with a complex of recombinant Hsp70 with bound, tumor-specific antigenic peptide have been shown to mount a potent CD8+ T cell response that can reduce or eliminate tumor progression (Srivastava et al., 1994). However, despite intensive research, the mechanistic details and the components involved in this pathway have remained obscure.

Various cell surface proteins on immune cells are thought to play a role in the induction of cellular responses to heat shock proteins. One group of proteins, including the toll-like receptors 2 and 4 with their cofactor CD14 (Asea et al., 2002), and CD36, are reported to induce cytokine secretion in an Hsp70-dependent manner. So far, only one surface protein (CD91) has been implicated in eliciting a CD8+ T cell response upon administration of peptide bound to heat shock protein (Grp94; Binder et al., 2000), but an essential role of CD91 in this process has been questioned (Berwin et al., 2002). A recent report by Wang et al. (2001) described a direct interaction between bacterial Hsp70 (DnaK) with CD40. However, this interaction is mediated by the substrate-binding domain of Hsp70, making a role in uptake of antigenic peptides unlikely.

The list of heat shock proteins and molecular chaperones implicated as immune adjuvants includes Grp94, Hsp60, calreticulin, and mammalian cytosolic Hsp90 and Hsp70 (Srivastava et al., 1986; Udono and Srivastava, 1993; Basu and Srivastava, 1999; Kol et al., 2000), as well as DnaK (Wang et al., 2001). In general, these chaperones bind to peptide segments of nonnative polypeptides either during synthesis or in conditions of cellular stress, preventing protein aggregation and mediating proper folding (Hartl and Hayer-Hartl, 2002). The highly conserved members of the Hsp70 family are the best studied among this class of components (Bukau and Horwich, 1998). Hsp70 consists of an NH2-terminal nucleotide-binding (ATPase) domain of ~44 kD and a COOH-terminal 25-kD domain that binds peptide or polypeptide substrate. In its ATP-bound state,
Hsp70 binds and releases peptide rapidly, whereas after hydrolysis, in the ADP state, bound peptide is held tightly (Flynn et al., 1989). Hsp70 recognizes heptapeptide segments with a broad specificity but with a preference for hydrophobic residues, such as leucine or isoleucine (Flynn et al., 1989; Blond-Elguindi et al., 1993; Rudiger et al., 1997). Based on this broad range of peptides recognized, Hsp70 would be especially suited to serve as a carrier of antigenic peptides for cross priming. Hsp70–peptide complexes may reach the extracellular space from necrotic cells or on viral cell lysis (Basu et al., 2000; Berwin and Nicchitta, 2001).

Here, we show that binding of Hsp70 to ANA-1 macrophages is markedly increased after stimulation with bacterial lipopolysaccharide (LPS). LPS treatment results in increased expression of CD40 (Tone et al., 2001), a member of the TNF receptor family with a crucial role in B cell function and autoimmunity (Bodmer et al., 2002). We find that human Hsp70 binds to the exoplasmic domain of CD40. Interestingly, this interaction is mediated by the NH2-terminal ATPase domain of human Hsp70 in its ADP-bound state. It is strongly enhanced by the presence of substrate peptide in the COOH-terminal domain of Hsp70 (C70) and is inhibited by Hip, a co-chaperone known to stabilize the Hsp70 ATPase domain in the ADP state. These surprising mechanistic features explain why the binding of human Hsp70 has remained undetected in a recent report describing the interaction of the COOH-terminal chaperone domain of bacterial Hsp70 (DnaK) with CD40 (Wang et al., 2001). We show further that binding of human Hsp70–peptide complex to cells that express CD40 leads to peptide uptake and induction of signaling via p38. Thus, CD40 is an extracellular receptor for peptide-loaded human Hsp70 and mediates the internalization of Hsp70-bound peptides.

**Results**

**LPS stimulation increases expression of CD40 and binding of Hsp70 to ANA-1 macrophages**

To determine the molecular basis for the binding of human Hsp70 to ANA-1 cells (Sondermann et al., 2001), cells were incubated with LPS or mock-treated as outlined in Materials and methods. (A) Cells were incubated either with biotinylated Hsp70, Hsp70 loaded with biotinylated peptide C, or with biotinylated GST as a control. After 30 min at 4°C, cells were washed, incubated with TRITC-labeled streptavidin, washed again, and processed for fluorescence microscopy. (top) mock-treated ANA-1 cells; (bottom) ANA-1 cells after treatment with LPS. Note that Hsp70 carries on average five biotins, whereas peptide C contains a single biotin. (B) Cells were harvested, lysed, and centrifuged to obtain a total membrane pellet. Identical protein amounts of the samples were analyzed by immunoblotting with an antibody directed against CD40. (Lanes 1 and 4) total cell lysates; (lanes 2 and 5) membrane fractions; (lanes 3 and 6) supernatants.
bound proteins were adsorbed to glutathione-Sepharose, of GST-CD40 were incubated with the lysates and fusion protein and used to study its interaction with heat action of CD40 with mammalian Hsp70s.

used as a negative control, did not bind Hsc70/Hsp70. CD40 is enhanced in the presence of ADP. GST alone, as will be shown later, the interaction of Hsp70 with concentration-dependent manner, whereas Hsp90 did not interact. As shown in Fig. 3 A, Hsc70 and Hsp70 bound to GST-CD40 in a concentration-dependent manner, whereas Hsp90 did not interact. As will be shown later, the interaction of Hsp70 with CD40 is enhanced in the presence of ADP. GST alone, used as a negative control, did not bind Hsc70/Hsp70. These experiments demonstrate a direct and specific interaction of CD40 with mammalian Hsp70s.

Mock-treated and LPS-stimulated ANA-1 cells were analyzed for their expression of CD40. As shown in Fig. 1 B, LPS-stimulated cells, but not control cells, express high levels of membrane-bound CD40, as detected by immunoblotting (Tone et al., 2001). To assess the possibility that CD40 itself may serve as an Hsp70 receptor, Cos-7 cells, which do not express CD40, were transfected with a cDNA construct containing the cDNA for human CD40, followed by an internal ribosomal entry site and the cDNA for EGFP. Transfected cells were identified by virtue of their GFP fluorescence, and were analyzed for their ability to bind biotin-labeled Hsp70 or Hsp70–biotin–peptide complex (Fig. 2). Indeed, Hsp70 and its peptide complex bound to the surface of transfected cells, but not to untransfected cells, whereas no binding above background was detected with biotinylated GST or biotinylated peptide alone (Fig. 2).

Binding experiments were performed in vitro to determine whether CD40 and Hsp70 interact directly. To this end, the exoplasmic domain of human CD40 (aa residues 20–212) was expressed in *Escherichia coli* as a soluble GST-fusion protein and used to study its interaction with heat shock proteins in HeLa cell lysates. Increasing amounts of GST-CD40 were incubated with the lysates and bound proteins were adsorbed to glutathione-Sepharose, followed by immunoblotting with antibodies directed against Hsc70, Hsp70, and Hsp90. As shown in Fig. 3 A, Hsc70 and Hsp70 bound to GST-CD40 in a concentration-dependent manner, whereas Hsp90 did not interact. As will be shown later, the interaction of Hsp70 with CD40 is enhanced in the presence of ADP. GST alone, used as a negative control, did not bind Hsc70/Hsp70. These experiments demonstrate a direct and specific interaction of CD40 with mammalian Hsp70s.

Human Hsp70 binds to CD40 via its ATPase domain

Next, we investigated the biochemical requirements for CD40–Hsp70 complex formation. Specifically, we addressed the question whether Hsp70 binds to CD40 via its COOH-terminal substrate-binding domain or via its NH₂-terminal nucleotide-binding domain. In this context, it was of interest whether the association depended on a specific nucleotide-bound state of Hsp70. Recombinant Hsp70 was incubated with GST-CD40 in the presence of ADP or ATP, with or without substrate peptide (Fig. 3 B). Hsp70 binding to CD40 was barely detectable in the presence of ATP, and was only seen in the presence of ADP. Notably, addition of a molar excess of peptide C over Hsp70 did not compete with the interaction, as might have been expected if the COOH-terminal Hsp70 domain were to mediate binding of Hsp70 to CD40. Only background signals were observed when GST alone was analyzed as a control. Recombinant, His6-tagged Hsp70 also interacted, in an ADP-dependent manner, with endogenous CD40 in extracts of LPS-stimulated ANA-1 cells, as detected by adsorption of the complex to Ni-NTA agarose and immunoblotting with CD40 antibody (Fig. 3 C). Together, these results suggested that Hsp70 binds to CD40 via its ATPase domain.

In light of recent findings that the bacterial Hsp70 homologue DnaK associates with CD40 via the COOH-terminal peptide–binding domain (Wang et al., 2001), experiments were performed to directly determine the specificity of CD40 for the domains of human Hsp70. Recombinant, full-length Hsp70 was incubated in the presence of ADP and a fivefold molar excess of either recombinant Hsp70 NH₂-terminal domain (residues 1–381; Sondermann et al., 2001) or COOH-terminal domain (residues 382–641; Scheufler et al., 2000), or of recombinant, full-length DnaK. The NH₂-terminal domain of Hsp70 (N70) efficiently competed for the binding of full-length Hsp70 to CD40, as detected with an antibody against the NH₂-terminal His6 tag on recombi-
nant Hsp70 (Fig. 4 A). The ATPase domain itself bound to CD40 in the presence of ADP, but not ATP (Fig. 4 C). In contrast, neither the COOH-terminal Hsp70 domain (C70) nor DnaK had a significant effect on this binding (Fig. 4 A).

To analyze whether under these conditions DnaK binds directly to CD40, a pulldown experiment was performed with DnaK. As shown in Fig. 4 B, DnaK indeed binds to CD40, and this interaction is enhanced in the presence of ADP when compared with ATP. This establishes that Hsp70 and DnaK bind to different sites on CD40, and confirms that DnaK binds via its COOH-terminal domain as reported earlier (Wang et al., 2001). This binding strongly suggested that it is the substrate-binding site of DnaK that mediates the interaction with CD40. To address this possibility, a competition experiment was performed with an excess of peptide C. As shown in Fig. 4 B, a 10-fold molar excess of the peptide almost completely abolishes the binding of DnaK to CD40. Thus, it is the substrate binding of DnaK that interacts with CD40. In summary, Hsp70 interacts with CD40 dependent on ADP, and via its ATPase domain, as shown with the endogenous proteins in cell extracts and the recombinant proteins in vitro. In contrast, DnaK binds to CD40 via its substrate binding site proper.

Hsp70 binding to CD40 is mediated by the NH2-terminal ATPase domain and is competed by Hip. (A) Human His6-tagged Hsp70, its NH2- or COOH-terminal domains, or recombinant bacterial DnaK was incubated either with GST-CD40 or with GST. (B) Recombinant DnaK was incubated with ADP, ATP, or an excess of peptide C, followed by addition of GST-CD40 or GST alone. (C) His6-tagged N70 was incubated in the presence of ADP or ATP, followed by incubation with a 10-fold molar excess of Hsp70 in the presence of ADP or ATP. (D) Recombinant human His6-tagged Hsp70 protein was incubated with a fivefold molar excess of either recombinant Hip protein or Bag-1, and with GST-CD40 or GST as a control. Bound protein was analyzed after affinity purification on glutathione-sepharose by immunoblotting with an antibody directed against the NH2-terminal His6 tags, or with an antibody directed against DnaK.

Hsp70 binding to CD40 is competed by the Hsp70 cochaperone Hip, and is enhanced by peptide substrate N70 is known to specifically interact with the regulatory cochaperone proteins Hip and Bag-1. Although Hip stabilizes the ADP state of Hsp70, which binds substrate tightly (Höhfeld et al., 1995), Bag-1 functions as an ADP–ATP exchange factor and causes substrate release from Hsp70 (Höhfeld and Jentsch, 1997; Sondermann et al., 2001). In light of the observed ADP dependence, it seemed possible that CD40 binding to Hsp70 has certain features in common with the interaction between Hip and Hsp70. Binding of Hsp70 to CD40 was analyzed in the presence of a fivefold molar excess of recombinant Hip or Bag-1 over Hsp70 (Fig. 4 D). Hip acted as an effective competitor of the interaction, in contrast to Bag-1, which interacts only relatively weakly
with the ADP state of Hsp70 (Höhfeld, 1998; Sondermann et al., 2001). Thus, CD40 and Hip may share similar binding regions on the ATPase domain of Hsp70.

The known cochaperone function of Hip in stabilizing Hsp70 in its substrate-bound ADP state (Höhfeld et al., 1995) raised the interesting possibility that the interaction between CD40 and Hsp70 may not only be ADP-regulated, but may also depend on Hsp70 substrate. Strikingly, binding of Hsp70-ADP to CD40 increased dramatically in the presence of peptide C (Fig. 5, A and B). The effect of peptide was saturable (k_s = 30 µM) in a range corresponding to the affinity of peptide C for Hsp70 (5–10 µM; Greene et al., 1995). These results demonstrate that it is the peptide- and ADP-bound state of Hsp70 that is recognized preferentially by CD40 and suggest that, similar to Hip, CD40 has a regulatory function in stabilizing the Hsp70-substrate complex. This effect would ensure that CD40 binds Hsp70 preferentially when it is in complex with peptide.

The striking increase in CD40 binding of ADP-loaded Hsp70 observed in the presence of peptide substrate raised the question of whether the free C70 has an inhibitory effect on the ATPase domain, reducing the ability of the latter to recognize CD40. This possibility was addressed by comparing the binding efficiency of substrate-saturated Hsp70 to that of the NH_2-terminal domain, both in their ADP states. As shown in Fig. 5 C, substrate-loaded Hsp70 and the NH_2-terminal domain bound to CD40 with similar efficiencies, indicating that the peptide-free C70 masks the NH_2-domain for binding to CD40, either directly or by causing an allosteric conformational change.

**Binding of Hsp70–peptide complex to CD40 results in intracellular signaling and peptide uptake**

Binding of CD40 ligand to CD40 induces signal transduction via phosphorylation of p38, a component of the signal cascade between activated CD40 and NFkB, which eventually results in the release of TNFα and subsequent secretion of interferon-γ (Pullen et al., 1999). Binding of the COOH-terminal domain of DnaK to CD40 was reported to have a similar effect (Wang et al., 2001). Therefore, we investigated whether binding of human Hsp70–peptide complex to CD40 also stimulates this signaling pathway. These experiments were performed in HEK293T cells stably transfected with human CD40 cDNA. After incubation with either Hsp70–peptide complex, recombinant Hsp70 domains, or DnaK, in the presence of ADP or the nonhydrolysable ATP analogue AMPPNP, cells were lysed and lysates were analyzed by immunoblotting with an antibody directed against active (i.e., phosphorylated) p38. Indeed, human Hsp70 and its ATPase domain caused an increase in phosphorylated p38 to an extent comparable to that observed with DnaK (Fig. 6 A, top panel). Although modest when compared with the signal induced by the same molar concentration of CD40 ligand, activation of p38 by Hsp70 was significant, and depended on the presence of ADP. As a control, HEK293T cells stably transfected with the same vector, but containing the cDNA for an unrelated membrane protein, murine cationic amino acid transporter (MCAT), did not show a detectable response to the various stimuli (Fig. 6 A, bottom panel). Thus, Hsp70–peptide complex and the

![Figure 6](image)

**Figure 6. Binding of Hsp70 complex to CD40-expressing HEK293T cells induces signaling via p38 and causes peptide uptake.** (A) HEK293T cells, stably transfected with cDNA encoding human CD40 or the unrelated membrane protein (MCAT) were incubated for 20 min at 37°C with CD40L or with Hsp70, N70, C70, or bacterial DnaK in the presence of peptide C and ADP or AMPPNP, or buffer alone. Thereafter, cells were washed, solubilized in SDS-sample buffer, and analyzed by immunoblotting with antibodies directed against phosphorylated (active) p38. Blots were also developed with an antibody against tubulin in order to control for equal loading. (B) Cells were incubated at 0°C for 30 min with recombinant human Hsp70, N70, or C70, all in the presence FITC-labeled peptide, or with the FITC-labeled peptide alone. Thereafter, cells were washed, incubated for 15 min at 37°C, and processed for fluorescence microscopy. (1) Hsp70, (2) FITC-labeled peptide alone, (3) C70, and (4) N70. Only cells stably transfected with CD40 are shown. Cell boundaries are emphasized with broken lines. MCAT-expressing control cells did not show fluorescence above background.

Hsp70 ATPase domain activate signaling via CD40, dependent on the presence of ADP and in a manner comparable to the effect of DnaK, although the latter binds to CD40 via its COOH-terminal domain (Wang et al., 2001).

Next, we examined whether binding of Hsp70–peptide complex to CD40 results in the uptake of peptide. To this end, HEK293T-CD40 cells and HEK293T-MCAT cells (not expressing CD40) were incubated with a fluorescent (FITC) derivative of peptide C for 30 min at 0°C in the presence of ADP, with or without Hsp70 or its two domains. After removal of excess material, the cells were incubated at 37°C for 15 min, fixed, and then analyzed by fluorescence microscopy. Representative images are presented in Fig. 6 B. After
mediates the uptake of peptide. Specific interaction of CD40 with Hsp70–peptide complex conditions tested (unpublished data). We conclude that the control cells did not give rise to a signal under any of the background (Fig. 6 B, panels 3 and 4). HEK293T-MCAT N70 and peptide did not result in a peptide signal above HEK293T-CD40 cells with either recombinant C70 or was omitted (Fig. 6 B, panel 2). Likewise, incubation of No staining above background was detected when Hsp70 the incubation for binding at 0°C. CD40, apparently because binding was examined in the absence of nucleotide and peptide substrate. Our observations are fully consistent with those of Wang et al. (2001), as we find that human Hsp70 and DnaK both bind to independent sites on CD40. Nevertheless, both chaperones are able to elicit signaling via the CD40 receptor to a similar extent. This signaling is an established function of the interaction of CD40 with CD40 ligand, which induces a signal transduction cascade that activates NFκB, leads to secretion of TNFα and interferon-γ, and involves uptake of the receptor–ligand complex (Manning et al., 2002). The presence of a dileucine motif in the cytoplasmic domain of CD40 suggests a mechanism of uptake by endocytosis via the adaptor/clathrin system (Kirchhausen et al., 1997). Thus, in contrast to bacterial DnaK, binding of human Hsp70 has a dual role. In addition to stimulating activation of p38, human Hsp70 mediates the uptake of peptide bound to its substrate-binding domain. Evidently, this latter function cannot be served by DnaK, considering that its substrate-binding site is occupied by CD40. The less pronounced dependence on ADP of DnaK binding to CD40 may be explained by the fact that DnaK has a higher intrinsic ATPase activity than Hsp70, generating ADP-bound DnaK in the incubation.

Clearly, the most remarkable feature of Hsp70 binding to CD40 is the dramatic dependence of the interaction on substrate peptide bound to C70. Our results suggest that CD40 interacts exclusively with the ATPase domain of Hsp70, in the presence of ADP, and not by an allosteric conformational change, preventing recognition by CD40. This inhibitory effect is released when peptide is bound (Fig. 5, A and B). Conversely, it follows that CD40 stabilizes the ATPase domain in the ADP state that holds bound peptide stably. A similar effect in stabilizing Hsp70 in the ADP state has previously been described for the Hsp70 cochaperone Hip (Höhfeld et al., 1995). Indeed, binding of Hip to the ATPase domain of Hsp70 blocks the

Discussion

Our results establish the cell surface protein CD40 as a receptor for exogenously added human Hsp70–peptide complexes. The functional properties of the CD40–Hsp70 interaction ensure surface binding and uptake of Hsp70-associated peptide (Fig. 7). Binding to CD40 is mediated by the ATPase domain of Hsp70, and depends critically on the ADP-loaded state of the chaperone, which binds peptide tightly. Moreover, complex formation between CD40 and Hsp70 is strongly enhanced by the presence of Hsp70 peptide substrate. We suggest that the CD40–Hsp70 interaction shares important functional features with the interaction between Hsp70 and certain intracellular cochaperones, such as Hip.

Although a variety of cell surface proteins have been reported to stimulate the immune system upon binding of molecular chaperones in model cell systems (Introduction), only in the case of CD40 has such an interaction been established directly. Wang et al. (2001) have shown recently that the bacterial homologue of mammalian Hsp70 (DnaK) is able to bind to HEK293T cells transfected with CD40 cDNA, and can be coimmunoprecipitated with CD40 from monocytes/macrophages (THP1 cells. However, this interaction is mediated by the COOH-terminal substrate-binding domain of DnaK, as opposed to the ATPase domain. In fact, Wang et al. (2001) concluded that human Hsp70 does not bind to CD40, apparently because binding was examined in the absence of nucleotide and peptide substrate. Our observations are fully consistent with those of Wang et al. (2001), as we find that human Hsp70 and DnaK both bind to independent sites on CD40. Nevertheless, both chaperones are able to elicit signaling via the CD40 receptor to a similar extent. This signaling is an established function of the interaction of CD40 with CD40 ligand, which induces a signal transduction cascade that activates NFκB, leads to secretion of TNFα and interferon-γ, and involves uptake of the receptor–ligand complex (Manning et al., 2002). The presence of a dileucine motif in the cytoplasmic domain of CD40 suggests a mechanism of uptake by endocytosis via the adaptor/clathrin system (Kirchhausen et al., 1997). Thus, in contrast to bacterial DnaK, binding of human Hsp70 has a dual role. In addition to stimulating activation of p38, human Hsp70 mediates the uptake of peptide bound to its substrate-binding domain. Evidently, this latter function cannot be served by DnaK, considering that its substrate-binding site is occupied by CD40. The less pronounced dependence on ADP of DnaK binding to CD40 may be explained by the fact that DnaK has a higher intrinsic ATPase activity than Hsp70, generating ADP-bound DnaK in the incubation.
interaction of Hsp70 with CD40, suggesting that Hip and CD40 recognize overlapping regions on the ATPase domain.

The functional features of the CD40–Hsp70 interaction may be adapted to a role in the uptake of Hsp70–peptide complexes into APCs for cross priming. Fig. 7 describes a model for the binding of peptide antigen to Hsp70 in a tumor cell, followed by release after necrotic cell lysis and CD40-mediated uptake of the Hsp70–peptide complex by an APC. Peptide binding to Hsp70 would be facilitated by the high intracellular concentration of ATP and the activity of the Hsp70 cochaperone Hsp40 in catalyzing peptide loading (Minami and Minami, 1999). During cell necrosis, the internal concentration of ATP relative to ADP drops markedly (Bradbury et al., 2000). A further dilution of ATP (and of Hsp70 cochaperones) would occur upon lysis and release of cytosol content into the extracellular medium. As a result, peptide-bound Hsp70 remains in its ADP state, the stability of which determines the half-life of the Hsp70–peptide complex. Importantly, although peptide loading onto Hsp70 is possible in the absence of nucleotide with low efficiency (Minami et al., 1996), low nucleotide concentration would prohibit the reformation of an Hsp70–ADP–peptide complex in the extracellular space. Thus, the strong preference of CD40 for Hsp70–ADP–peptide ensures not only the binding of peptide-loaded Hsp70, but would also guarantee that intracellular peptide antigen is made available for cross priming. Thus, the uptake of circulating extracellular peptides, potentially triggering autoimmune reactions, would be avoided. Future work will be directed toward testing this model, with a focus on the fate of Hsp70-bound peptide after uptake into APCs, including possible peptide representation on the cell surface via MHC I.

Materials and methods

Plasmids and protein preparation

Human Hsp70, N70 and C70, Hip, and Bag-1 were expressed and purified as His-tagged proteins (Höhfeld et al., 1995; Scheufler et al., 2000; Sondermann et al., 2000, 2001). DnaK was expressed as described previously (Sabo et al., 1994). After ATP-sepharose affinity chromatography as described previously (Sondermann et al., 2000) Hsp70, N70, and DnaK were extensively dialyzed (twice against a 200-fold volume of PBS) in order to obtain essentially nucleotide-free proteins. Equine GST was purchased from Sigma-Aldrich. Biotinylated recombinant Hsp70 and equine GST were prepared by labeling with the FluoReporter Biotin-XX Protein Labeling Kit (Molecular Probes, Inc.).

For binding of endogenous Hsp70 and Hsc70 from HeLa cell lysate, HeLa cells were lysed as described above and centrifuged to SDS-PAGE, and were analyzed by immunoblotting with an anti-Hsc/Hsp70 antibody and an anti-Hsp90 antibody (SPA822 and SPA835; StressGen Biotechnologies). Human Hsp70, N70 and C70, Hip, and Bag-1 were expressed and purified after transfection in 50 ml of each protein was incubated with 200 mM ATP, 2 mM ADP, or 2 mM ADP and 28 mM peptide C in 50 ml binding buffer consisting of the following (mM): 10 MOPS-KOH, pH 7.2, 150 mM KC1, 2 ADP, and 3 MgCl2. Excess of unbound peptide C-biotin was removed by gel filtration on Sephadex G-50 (Amer- sham Biosciences). For competition experiments, 20 mM ADP was added to the eluates was subjected to SDS-PAGE and analyzed by immunoblotting for CD40 expression using an anti-CD40 antibody (CSA180; StressGen Biotechnologies).

Analysis of CD40 expression

For immunoblot analysis, ANA-1 cells were stimulated with LPS or mock treated as described under Cell culture and fluorescence microscopy, harvested, lysed by repeated passage through a needle (0.4–0.8 mm; Braun) in the presence of protease inhibitors (complete, EMD free; Roche), and fractionated by centrifugation at 100,000 g at 4°C for 1 h. The pellet was resuspended in 0.1% Triton X-100/PBS, and protein concentrations were determined in total lysate; supernatant and pellet fractions were determined by the Bradford assay (Bio-Rad Laboratories). Equal amounts of protein were subjected to SDS-PAGE, and were analyzed by immuno blotting with an anti-CD40 antibody (CSA180; StressGen Biotechnologies).

Binding assays with GST-CD40

For binding of endogenous Hsp70 and Hsc70 from HeLa cell lysate, HeLa cells were lysed as described above and centrifuged to SDS-PAGE, and were analyzed by immunoblotting with an anti-CD40 antibody and an anti-Hsp90 antibody (SPA822 and SPA835; StressGen Biotechnologies).

For binding assays with recombinant His-tagged Hsp70 and its His-tagged domains or DnaK, 2.8 M of each protein was incubated with 200 mM ATP, 2 mM ADP, or 2 mM ADP and 28 mM peptide C in 50 ml binding buffer consisting of the following (mM): 10 MOPS-KOH, pH 7.2, 150 mM KC1, and 3 MgCl2 for 30 min at 37°C. In competition experiments, binding Hsp70–peptide receptor complex | Becker et al. 1283
was probed with either a 10-fold excess of Hsp70, the two domains, DnaK, or with a fivefold excess of Hip or Bag-1 for 10 min at 0°C. To this end, Hsp70, N70, C70, and DnaK were preincubated with ADP, ATP, and peptide C as described under Plasmids and protein preparation. For comparison of CD40 binding of Hsp70 and its NH2-terminal domain, Hsp70 was preincubated in the presence or absence (mock treatment) of a 30-fold molar excess of peptide C. 3 μM GST or GST-CD40 was added to the samples and incubated for 20 min at 16°C. For titration of peptide C, 5–50-fold excess of peptide C (15–150 μM) was added to the preincubation of Hsp70. Thereafter, the samples were combined with 20 μl of 1% BSA-treated Glutathione Sepharose 4 Fast Flow and incubated in the presence of 2 mM DTT for another 20 min at 16°C. Unbound proteins were removed by washing the beads three times with 1 ml PBS, and immobilized proteins were eluted with 30 μl elution buffer for 20 min at 22°C. 10 μl of the eluates were subjected to SDS-PAGE and analyzed by immunoblotting using a Penta-His antibody (Qiagen). Blot signals were quantified by Quantity One software (Bio-Rad Laboratories).

Binding assay with His6-tagged Hsp70 and Ni-NTA agarose

ANA-1 cells were stimulated with LPS as described above and lysed in lysis buffer (150 mM Tris-HCl, pH 7.5, 1% CHAPS) for 45 min at 4°C. Cell lysate was centrifuged at 100,000 × g for 15 min at 4°C. 500 μl of the supernatant was incubated with 10 μg His6-tagged Hsp70, preincubated with ADP and a 30-fold excess of peptide C, or mock-treated, as described above for biotinylated proteins, for 30 min at 4°C. Thereafter, the samples were added to 20 μl of 1% BSA-treated Ni-NTA agarose (Qiagen) and incubated for 30 min at 4°C. Unbound protein was removed by washing the beads three times with 1 ml lysis buffer, and immobilized proteins were eluted with 10 μl 500 μM elution buffer by incubation for 5 min at 95°C. Eluates were analyzed by immunoblotting using an anti-CD40 antibody (CSA-180; StressGen Biotechnologies).

p38 kinase assay

24 h before p38 kinase assays, HEK293T-CD40 and HEK293T-MCAT cells were seeded into a 24-well plate coated with collagen and incubated with 100 nM CD40L (Alexis Biochemicals Corp.), HSP70, N70-domain, C70-domain, or DnaK for 20 min at 37°C. DnaK, Hsp70, N70, and C70 were preincubated with either 2 mM ADP and 30 μM peptide C or 40 μM AMPPNP (Sigma-Aldrich). On stimulation, cells were washed twice with ice-cold PBS and lysed in SDS–sample buffer. Equal amounts of protein were analyzed by immunoblotting with antibodies directed against phosphorylated p38 (Promega), and with a monoclonal antitubulin antibody (J. Wehland, German Research Centre for Biotechnology, Braunschweig, Germany).

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