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Role of Scavenger Receptors in the Binding and Internalization of Heat Shock Protein 70

Jimmy R. Thériault,* Hideki Adachi,† and Stuart K. Calderwood2*

Extracellular heat shock protein 70 (Hsp70) exerts profound effects both in mediating tumor rejection by Hsp70-based vaccines and in autoimmunity. Further progress in this area, however, awaits the identification of the cell surface receptors for extracellular Hsp70 that mediate its immune functions. We have examined a wide range of candidate Hsp70 receptors and find significant binding through two main families of cell surface proteins, including 1) the scavenger receptor (SR) family and 2) C-type lectins of the NK family. In addition, given that the anticancer effects of Hsp70 vaccines have been shown to involve uptake of Ags by APC exposed to Hsp70-tumor Ag complexes, we have examined the ability of the receptors identified here to internalize Hsp70-peptide complexes. Our findings indicate that the three SR family (lectin-like oxidized low density lipoprotein receptor 1; fasciclin, epidermal growth factor-like, laminin-type epidermal growth factor-like, and link domain-containing scavenger receptor-1; and SR expressed by endothelial cells) are able to bind Hsp70-peptide complexes and mediate its efficient internalization. Indeed, each of the SR was able to mediate efficient uptake of Hsp70 when transfected into Chinese hamster ovary cells previously null for uptake. Curiously, Hsp70 internalization occurs independently of the intracellular domains of the SR, and Hsp70 uptake could be detected when the entire intracellular domain of lectin-like oxidized low density lipoprotein receptor 1 or SR expressed by endothelial cells was truncated. The existence of a wide repertoire of cell surface Hsp70-binding structures may permit intracellular responses to extracellular Hsp70 that are cell specific and discriminate between Hsp70 family members. The Journal of Immunology, 2006, 177: 8604–8611.

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3 Abbreviations used in this paper: Hsp, heat shock protein; Hsp70, Hsp70-PC, Hsp70-peptide complex; LDL, low-density lipoprotein; oxLDL, oxidized LDL; acLDL, acetylated LDL; LRP1, LDL receptor-related protein-1; SR, scavenger receptor; LOX-1, lectin-like oxidized low-density lipoprotein receptor 1; PEEL-1, fasciclin, epidermal growth factor-like, laminin-type epidermal growth factor-like, and link domain-containing scavenger receptor-1; SREC-1, SR expressed by endothelial cells-1; PSOX, SR that binds phosphatidylserine and oxidized lipoprotein; CHO, Chinese hamster ovary; ID, intracellular domain. Grp94, glucose-regulated protein 94; NKG2D, killer cell lectin-like receptor subfamily K, member 1.
recently been expanded to other molecules including Gram-positive and Gram-negative bacteria, LPS, advanced glycation end products, fucoidan, and polyanionic ligands (16). The SR family can be subdivided into eight different subclasses (A–H), and most receptors belonging to this family are expressed on the surface of APC (16, 17). Class A and B SR such as SR-A, macrophage receptor with collagenous structure, human CD36 and LIMP-II analogous receptor (CLA-1), and CD36 are integral parts of the immune response increasing phagocytosis of bacteria and yeast (16). Class F SR expressed by endothelial cells-1 (SREC-1) interacts with a ligand of the chemokine receptor CXCR6 (CXCL16) (21, 22). The class G SR that binds phosphatidylserine and oxLDL has also been discovered as a ligand of the chemokine receptor CXCR6 (CXCL16) (21, 22). The class G SR that binds phosphatidylserine and oxLDL possesses angiogenetic and proliferative properties (23, 24). Finally, class H receptor FEEL-1 also named stabilin-1/CLEVER-1 has been initially characterized as a receptor interacting with advanced glycation end products and mediating trans-migration of lymphocyte and vascular cells on the endothelium (25–28). Intriguingly, members of the heat shock protein family other than Hsp70 have been shown to interact with some SRs. Gpr94/glycoprotein 96 and calreticulin show significant affinity to SR-A and are internalized by this receptor (29). Extra-cellular calreticulin uptake can also be mediated by SREC-1 but apparently not by LOX-1 (29, 30).

As mentioned earlier, a receptor-mediated Ag uptake mechanism appears to mediate Hsp70-induced cross-presentation. Receptor-ligand complex internalization is mainly governed through motifs within the IDs of endocytic receptors. Such motifs are sufficient to trigger ligand uptake through coated pits or lipid raft caveolin-related mechanisms (31, 32). Tyrosine-based and dileucine-based motifs account for the vast majority of intracellular sorting signals and usually direct ligands to specific intracellular compartments such as lysosome or endosome (32). After internalization of extracellular Hsp70.PC by APC, the complex is thought to traffic into the cytoplasm or endosomal vesicles before the release, processing and re-presentation of the immunogenic peptide cargo by class I and/or II MHC receptors at the surface of APC (2, 33).

In this study, we show that a subgroup of the SR family including LOX-1, SREC-1 and FEEL-1 can bind avidly both to Hsp70.PC purified from mammalian cells and to purified recombinant Hsp70. Interestingly, the nature of the adenosine phosphate moiety associated with Hsp70 affects its binding to all three SR. We show that each of the SR is subsequently able to mediate efficient internalization of Hsp70.PC after expression in CHO cells previously null for uptake. Surprisingly, deletion mutation studies indicate that sequences in the ID of both LOX-1 and SREC-1 are dispensable for Hsp.PC uptake, ruling out conventional pathways of internalization involving internalization motifs found in the ID of many receptors.

Materials and Methods

Cell culture

Wild-type CHO K1 cells and all stable CHO transfectant cells were grown in Ham’s F-12 medium supplemented with 10% FBS. In the case of stable transfectant CHO human SREC-1, CHO human FEEL-1, CHO human Dectin-1, CHO human CD94-NKG2A, and CHO human CD94-NKG2C, clonal selection was kept in 0.4 mg/ml G418, whereas LOX-1 expression (CHO-LOX-1) was maintained within the supernatant. Stable CHO-Dectin-1 transfectant was produced through transfection of pCDNA3 Flag human Dectin-1 and populations of G418 (0.4 mg/ml)-resistant cells were generated after 2 wk of cell culture.

Generation of LOX-1 deletion mutants

LOX-1 deletion mutants were constructed especially for this study using a PCR-based Site-directed Mutagenesis Kit (ExSite; Stratagene). Briefly, PCRs were performed on pCDNA3 myc human LOX-1 using as template two 21-bp oligonucleotides corresponding of the extremities of the deleting site of each mutant and the ExSite PCR-Base Site Directed Mutagenesis kit (Stratagene) according to the manufacturer’s suggestions. After amplification, the reaction products were digested with DpnI (Invitrogen Life Technologies), and the PCR products were ligated with T4 DNA ligase (Invitrogen Life Technologies).

Alexa 488-labeled purified Hsp70 preparation

Human melanoma A375-MEL cells or mouse MISA cells have been used as starting material for mammalian Hsp70.PC preparations. Recombinant Hsp70 was purified from competent BL-21 Escherichia coli (Invitrogen Life Technologies) transformed with a pET-5 plasmid encoding human HSP70.1 DNA (BarnHI-HindIII insertion). Hsp70 purification was conducted as previously reported (34). Briefly, a 10-ml cell pellet was homogenized in 40 ml of hypotonic buffer (10 mM NaHCO3, 0.5 mM PMSF, pH 7.1) by Dounce homogenization. Bacterial rHsp70 was produced by first growing the BL-21 bacteria (dilution 1:100) in 1 liter of LB with ampicillin (50 mg/ml) for 3 h at 37°C. Hsp70 expression was induced by adding 1 mM isopropyl β-D-thiogalactoside for 4 h. A 1-liter bacterial pellet was washed twice with 30 ml of buffer A (50 mM HEPES, 50 mM NaCl, 10 mM DTT, 0.5 mM EDTA, 0.5 mM PMSF, and 1× protease inhibitor mixture from Roche; pH 7.4) and then sonicated and homogenized (Dounce homogenization) in 12.5 ml in buffer A supplemented with 2 mM MgCl2, 1.25 mg/ml lysozyme and 11.2 μM Dnase I. The homogenate was first centrifuged at 10,000 × g for 30 min, and the supernatant was recentrifuged for 60 min at 100,000 × g. The sample buffer was changed to buffer D (20 mM Tris-acetate, 20 mM NaCl, 15 mM 2-ME, 3 mM MgCl2, and 0.5 mM PMSF, pH 7.5) using a PD-10 column (Amersham Biosciences). The sample was applied directly to a 5-ml ADP-agarose column (Sigma-Aldrich) which was equilibrated with buffer D. Hsp70 was eluted from ADP-agarose column with 3 mM ADP or 3 mM ATP in buffer D (ADP-bound Hsp70 vs ATP-bound Hsp70). The sample buffer was changed to FPLC buffer (20 mM sodium mono- and diphosphate, 20 mM NaF, 1 mM EDTA, 0.7 mM DTT) with a B-10 column. Hsp70 was eluted with the FPLC buffer containing 150 mM NaCl. All proteins were quantitated with Bradford assay. Alexa 488 labeling on Hsp70.PC was conducted according to the manufacturer’s instructions (Molecular Probes). BSA was used as negative control. Almost no degradation (<2%) of the Hsp70 purified preparation was observed by Coomassie staining, and the presence of Hsp70 in the preparation was confirmed by Western blotting using a mouse mAb specific against Hsp70 (SPA-810; Stressgen).

Hsp70 binding assay

Nontryptsinized cells (2 × 105) were washed twice in PBS containing 0.5% FBS, 0.05% NaN3, and 1 mM CaCl2 and incubated with 150 nM Alexa 488-labeled BSA, mammalian Hsp70.PC, ATP-bound recombinant Hsp70 and ADP-bound recombinant Hsp70 for 30 min on ice with gentle shaking. The cells were washed in PBS containing 0.5% FBS, 0.05% NaN3, and 1 mM CaCl2 twice. And Alexa 488-labeled Hsp70.PC binding was monitored by flow cytometry (BD Biosciences).

Transfection and Western blot

CHO-K1 cells (2.5 × 105) were transiently transfected with 5 μg of empty vector (pCDNA3) or pCDNA3 plasmids encoding various myc-tagged human LOX-1, human LOX-1 deletion mutants, full-length human SREC-1, or SREC-1 D370 (deletion of the last 370 aa of the C terminus) for 48 h.
using the Superfect transfection reagent (Qiagen) according to the manufacturer’s instructions. The cells were harvested in 200 ml of SDS-PAGE sample buffer once, and 20 mg of protein extracts were loaded on SDS-PAGE, transferred, and subjected to Western blot using the mouse monoclonal Myc Ab (clone 9E10; Stratagene).

**Hsp70 internalization and immunofluorescence experiments**

In an 8-well CultureSlide (Labtek), 2 \times 10^4 cells were rinsed twice in ice-cold medium (Ham’s F-12, 10% FBS) and incubated with 750 nM (50 mg/ml) Alexa 488-labeled BSA (negative control) or Hsp70 for 30 min with intermittent shaking at 4°C. After the incubation, the cells were washed four times with ice-cold medium and once with warm medium (37°C). Hsp70 internalization was induced by incubating the cells for 15 min at 37°C. Then, the cells were washed twice in Dulbecco’s PBS and fixed in 3.7% paraformaldehyde for 15 min and processed for microscope visualization. LOX-1 and SREC-1 surface expression was illustrated using 9E10 and SREC-1 (SR-4) (dilution, 1/100) Ab. Images were obtained using a fluorescent microscope NIKON Eclipse E600, at \( \times 40 \) magnification; with a SPOT RT color camera and SPOT advanced software (Diagnostics Instruments).

**Results**

**Binding of Hsp70 to cell surface SRs**

We first examined the ability of Hsp70.PC from human tumor cells to bind to CHO cells transfected with expression plasmids encoding a number of the known SRs. Binding of Hsp70 to wild-type CHO was minimal, whereas significant binding was seen with cells stably transfected with the SR family members LOX-1, SREC-1, and FEEL-1 (Fig. 1). Cell surface expression of the overexpressed receptors was confirmed by immunofluorescence as shown below. Each of the three SRs was able to bind mammalian Hsp70.PC with a different relative avidity compared with Alexa-labeled BSA (negative control) which did not show significant binding to cells expressing these receptors or to wild type CHO (Fig. 1A). Binding to these SR family members is relatively selective as previous studies show that, whereas LOX-1 binds Hsp70, a number of other SR family members including SR-A1, CD36, macrophage receptor with collagenous structure, and CLA-1 do not (5). These experiments therefore show selective association of Hsp70 with a subset of the SR. We next conducted similar experiments using recombinant human Hsp70 preparation, purified from *E. coli* and observed binding to the LOX-1-, SREC-1-, and FEEL-1-expressing cell lines (Fig. 1B). However, in the case of LOX-1 and SREC-1, such binding was significantly reduced when compared with the degree of association with the preparation of Hsp70 purified from mammalian cells (Fig. 1B). Interestingly, the association of the recombinant Hsp70 with CHO-SREC-1 was greatly reduced and almost at the minimal level of Hsp70 binding observed in wild-type CHO cells (Fig. 1A). By contrast, in the case of FEEL-1-expressing cells, recombinant Hsp70 binds at similar levels compared with mammalian purified Hsp70 (Fig. 1). The nature of the adenosine phosphate moiety (ATP vs ADP) bound to Hsp70 also affected the degree of Hsp70 interaction with LOX-1, SREC-1, and FEEL-1 expressed in CHO cells (Fig. 1A). ADP-bound Hsp70 slightly surpassed the binding level of ATP-bound Hsp70 to all 3 SR (Fig. 1B). Differences in Alexa 488 fluorochrome incorporation in the ADP-bound or ATP-bound Hsp70 preparations used here were unlikely to be responsible for the differences in binding to SR because fluorochrome incorporation into each of the Hsp70 preparations was similar (5.25 Alexa 488 molecules per ATP-bound Hsp70 vs 5.75 Alexa 488 molecules per ADP-bound Hsp70). Overall, these experiments demonstrate that the source of the Hsp70 preparation used (recombinant Hsp70 compared with Hsp70 from mammalian cells) and the adenosine phosphate moiety (ATP vs ADP) bound to Hsp70 are important factors affecting Hsp70 binding to LOX-1, SREC-1, and FEEL-1.

Some members of the SR family, including SR-A and CLA-1, have been shown to participate in bacterial LPS uptake raising concerns about the potential involvement of such endotoxins in SR-mediated Hsp70 internalization (35, 36). Indeed, the presence of endotoxins in Hsp70 preparations has been recently proposed to participate in Hsp70-mediated proinflammatory responses, suggesting that LPS could also take part in Hsp70 internalization (37, 38). However, SR-mediated LPS internalization is observed only when LPS is preassociated to LPS binding protein, soluble CD14, or even high-density lipoprotein signifying that LPS cannot be internalized by itself and requires prior binding to a putative SR binding ligand (35, 39). Although there is no indication that SR-A and CLA-1 directly interact with Hsp70, it is still possible that Hsp70 can act as a SR-binding ligand facilitating LPS internalization (5). Nevertheless, we conducted Hsp70 binding experiments comixing LPS-FITC and Alexa 488-labeled Hsp70 and saw no additive effect of LPS on Hsp70 binding to RAW 264.7 cells (data not shown).
FIGURE 2. Mammalian Hsp70.PC binds to specific C-type lectin receptors. CHO K1 cells or CHO cells stably overexpressing human C-type lectin receptors Dectin-1, NGK2D-Ly49 chimera, CD94-NKG2A, or CD94-NKG2C were incubated with Alexa 488-labeled BSA or mammalian Hsp70.PC at a concentration of 10 ng/ml (150 nM) on ice for 30 min with gentle shaking. Mammalian Hsp70.PC binding was monitored by flow cytometry. Shaded gray and thick black line histograms corresponded, respectively, to BSA and mammalian Hsp70.PC binding. Experiments were conducted three times with similar results.

HSP70 binding to C-type lectin receptors

Besides its functional relationship to other SR family members, LOX-1 shares structural homology to type V C-type lectin family members such as Dectin-1, killer cell lectin-like receptor subfamily K, member 1 (NKG2D), and CD94 (40). These C-type lectin receptor family members usually exist as homo- or heterodimers (40). Previous studies have demonstrated that another type V C-type lectin CD94 binds to Hsp70 but not to type II C-type lectin DC-SIGN (12, 41). From these experiments, it has been suggested that the C-type lectin domain, a conserved ligand interaction domain in these receptors, is responsible for the association with Hsp70. To determine whether the C-type lectin domain can function as an Hsp70 binding site, we examined Hsp70 binding to CHO cells overexpressing a number of members of the V-type lectin receptor family. Although Dectin-1 has been shown to possess the greatest structural homology to LOX-1 of the C-type lectins, no Hsp70 binding was not detected in CHO overexpressing Dectin-1 (Fig. 2). However, a significant affinity was seen between Hsp70 and NKG2D expressing cells and to a lesser extent in cells expressing the heterodimer CD94/NKG2A but not the CD94/NKG2C heterodimer (Fig. 2). Binding to these members of the C-type lectins showed selectivity in that a number of other family members overexpressed in CHO cells, including DC-SIGN, CLEC-1 and CLEC-2 showed minimal Hsp70 association (J. Theriault and S. K. Calderwood, unpublished observations). Thus, these findings suggest that a number of C-type lectins can serve as receptors for Hsp70 although the C-type lectin domain was not the sole determinant required for Hsp70 interaction.

SR-induced Hsp70 endocytosis

Because Hsp70-induced Ag cross-presentation requires Hsp70.PC uptake after binding to receptor(s) on APC, we next examined whether the SR mediated such Hsp70.PC internalization (1). We chose not to examine CD94/NKG2A and NKG2D internalization because these C-type lectin receptors function mainly in NK cell signaling. Moreover, NK cells are not known to efficiently participate in extracellular Ag endocytosis. We have instead concentrated our effort on LOX-1, SREC-1, and FEEL-1 which are most likely to be involved in Hsp70 uptake. These receptors have been shown previously to mediate ligand internalization and LOX-1, SREC-1, and FEEL-1 can mediate oxLDL and/or acLDL uptake (42–44). Hsp70 internalization was initiated by permitting Alexa 488-labeled Hsp70 to bind to the cell surface at 4°C and then incubating for an additional 15 min at 37°C to trigger energy-dependent Hsp70 uptake before fixation. Hsp70 internalization was assessed by visualization of intracellular punctuate staining (vesicle staining) using fluorescence microscopy. Fluorescence images are in each case accompanied by matched phase-contrast images of the same field. As seen in Fig. 3, CHO cell lines overexpressing, respectively, each of the SR, LOX-1, SREC-1, or FEEL-1 were able to internalize mammalian Hsp70.PC, whereas uptake was not seen in wild-type CHO control cells or cells transfected with empty expression plasmid. Control experiments indicate minimal uptake of Alexa-labeled BSA by the SR transfectants (Fig. 3).

Recombinant Hsp70 uptake was also mediated by each of the three SR, although with contrasting efficiency (Fig. 4). Indeed, SREC-1-mediated HSP70 uptake was relatively ineffective, resulting in a fairly low percentage of CHO-SREC-1-expressing cells internalizing Hsp70 (<5% of cells scored) as well as a lower fluorescence intensity (Fig. 4B). The greatest degree of internalization was observed in CHO-FEEL-1 cells and magnitude of hsp70 internalization was positively correlated with Hsp70 binding (Fig. 1A). No obvious differences were observed between ATP- and ADP-bound Hsp70 internalization (Fig. 4). In conclusion therefore, the expression of LOX-1, SREC-1, or FEEL-1 at the cell surface was sufficient to mediate the endocytosis of extracellular Hsp70.PC.

The cytoplasmic portion of LOX-1 and SREC-1 is dispensable for Hsp70 internalization

Receptor-mediated uptake of many ligands is initiated by the association of adaptor molecules with consensus endocytic motifs such YXXQ and NPXY on the ID of the receptor (31, 32). However, even though LOX-1 and SREC-1 are able to internalize extracellular Hsp70, no such consensus internalization motif is present in the ID of either receptor. Therefore, to evaluate the role of the ID of LOX-1 and SREC-1 in Hsp70 uptake, constructs of LOX-1 and SREC-1 with the ID truncated were generated by deletion mutagenesis, stably transfected into CHO cells and subjected to Hsp70 internalization assays (Figs. 5 and 6). The relative levels of expression of the deletion mutants in CHO transfectant cells were examined by Western blot and/or immunofluorescence using an anti-myc Ab to detect expression of a LOX-1-myc tag fusion protein and an anti-SREC-1 Ab (SR-4) for SREC-1 (Figs. 5B and 6). LOX-1 glycosylation (presence suggested by a band doublet) was observed with the full length LOX-1 and other
LOX-1 deletion mutants except for LOX-1 D2–35 (Fig. 5B). A LOX-1 cytoplasmic arginine-rich motif (KKAK) could potentially serve as a positive motif required for the expression of LOX-1 at the cell surface. However, the full ID deletion mutant LOX-1 D2–35 was expressed at the cell surface indicating that this motif may be nonfunctional (Fig. 5B). To rule out the possibility of a deleterious effect of this large truncation on LOX-1 receptor structure, function or stability, we also prepared another, smaller deletion mutant removing only the arginine-rich region in the ID of LOX-1 (LOX-1 D21–27) and evaluated its cell surface receptor expression. LOX-1 D21–27 was still expressed at the cell surface confirming that the KKAK motif does not play a significant role in LOX-1 surface sorting (Fig. 5C). Each LOX-1 deletion mutant, as well as full-length LOX-1 was able to mediate the internalization of mammalian Hsp70-PC (Fig. 5C). Because there was no indication that the ID of LOX-1 is involved in Hsp70 uptake, this led us to investigate the albeit unlikely possibility that adjacent extracellular regions could be involved in Hsp70 internalization. Intriguingly, a putative extracellular dileucine motif (DXXXLL) was present in the extracellular portion of LOX-1 closely adjacent to the transmembrane domain (Fig. 5A). We tested the possibility that this motif was required for Hsp70 internalization. However deletion of this 62–67 region (sequence containing the dileucine motif) likewise did not affect Hsp70 uptake (Fig. 5C). A similar series of experiments was executed with deletion of the ID of SREC-1, generating deletion mutant (SREC-1 CD370, which truncates the

**FIGURE 3.** Mammalian Hsp70-PC was internalized in CHO cells overexpressing LOX-1, SREC-1, and FEEL-1. Fluorescence microscopic analysis of wild-type CHO K1 (a–d), CHO LOX-1 (e–h), SREC-1 (i–l), and FEEL-1 (m–p) incubated with Alexa 488-labeled mammalian Hsp70 or Alexa 488-labeled BSA. Hsp70 uptake was induced by preincubating the cells with 750 nM Alexa 488-labeled Hsp70-PC or BSA for 30 min at 4°C followed by an additional 15 min of incubation at 37°C. Phase contrast images of the cells were taken at the same time. Experiments were conducted three times with similar results. wt, Wild type.

**FIGURE 4.** Effect of nucleotide (ATP or ADP) bound to Hsp70 internalization. Fluorescence microscopic analysis of wild-type CHO K1 (a–f), CHO LOX-1 (g–l), SREC-1 (m–r), and FEEL-1 (s–x) after exposure to Alexa 488-labeled rATP-bound Hsp70 (H70-ATP), ADP-bound Hsp70 (H70-ADP), or Alexa 488-labeled BSA. Hsp70 uptake was induced by preincubating the cells with 750 nM Alexa 488-labeled Hsp70-PC or BSA for 30 min at 4°C followed by an additional incubation of 15 min at 37°C. Phase contrast images of the cells were taken at the same time. Experiments were performed multiple times with reproducible results.
370 intracellular amino acids from human SREC-1). As with LOX-1, we observed that the ID of SREC-1 is not essential for the internalization of mammalian Hsp70.PC (Fig. 6). Interestingly, the full ablation of the ID of the SREC-1 is similarly ineffective in blocking SREC-1-mediated acLDL internalization, showing convergence between requirements for uptake of modified LDL molecules and Hsp70 (19). These experiments demonstrate that the ID regions of LOX-1 and SREC-1 are not required for Hsp70 uptake and suggest alternative mechanisms for the endocytic functions mediated by the SR.

Discussion

One of the keys to understanding the role of extracellular molecular chaperones in cell regulation is to discern the receptors/acceptor proteins on the cell surface that mediate extracellular activity. This has proved an exceedingly protracted process, and many candidate receptors with more or less validity have been proposed. Here we have begun to screen for high avidity Hsp70 receptors and show that three members of the SR when expressed on the surface of CHO-K1 cells (previously null for binding) have ability to bind Hsp70 (Fig. 1). Three receptors from the SR family and two members of the NK family of C-type lectins have been uncovered as Hsp70 receptors in this study, although the significance of these findings of multiple Hsp70 receptors is not yet entirely clear. However, the different receptors may mediate cell-specific effects through expression on different cell types or may recognize different members of the Hsp70 family. This is likely to be true of the NK family receptors NKG2A and NKG2D which are expressed on NK cells, and in the case of NKG2D, NK cells as well as some classes of T lymphocytes (45). These receptors may play more significant roles in the lysis of tumor cells expressing Hsp70 bound to the cell surface than in tumor Ag cross-presentation (45–47). Our experiments also suggest some selectivity for the SR in recognizing different Hsp70 isoforms. There are at least 12 members of the human Hsp70 family many of which are expressed in tumor cells constitutively (48). Our experiments show that human Hsp70 affinity purified from mammalian cells as well as recombinant Hsp70 which is the product of the Hsp70.1 (HSPA1A) gene binds to LOX-1 and FEEL-1 (Fig. 1). However, SREC-1 only showed significant avidity to the mammalian Hsp70 preparation and bound poorly to the HSPA1A product (Fig. 1).

The Hsp70 preparation purified from mammalian cells is in fact a mixture of the intracellular Hsp70 family gene products, including the HSPA1A product, but also heat shock 70 KDa protein 8, to a lesser extent, Grp78 and likely other Hsp70 family proteins (data not shown). We concentrated on this Hsp70 preparation as it forms the basic building block of Hsp70-based vaccines and likely contains peptides loaded under physiological conditions and the correct form of physiological modification. The absence of interaction between SREC-1 and the recombinant HSPA1A preparation suggests selectivity in Hsp70 family member binding to the same receptor (Fig. 1). Many of the previous studies investigating the intracellular transport and exit from the endosomal compartment have focused on the role of the ID regions.
properties of extracellular Hsp70 receptors used exclusively commercial preparations of the recombinant HSPA1A purified from E. coli, and use of this single protein may thus not identify effects mediated by other Hsp70 family members and their receptors (5, 10, 14). In addition, posttranslational modifications such as phosphorylation or glycosylation of Hsp70 purified from mammalian cells could also be modulating factors affecting binding to SR. Future studies of recombinant Hsp70 will use baculovirus expression, a condition that more closely reproduces the milieu of the mammalian cell. ADP-bound Hsp70 interacted with slightly more avidity with LOX-1, SREC-1, and FEEL-1 than ATP-bound Hsp70 although the differences were of marginal significance. The affinities for Hsp70-SR binding are currently not known, although binding with Hsp70 concentrations as low as 15 nM can be readily detected. Attempts to study affinity using surface plasmon resonance with soluble (extracellular domains) of LOX-1 and SREC-1 were unsuccessful perhaps due to incorrect folding of these forms of the SR.

Because the receptors that bind Hsp70 are divergent in sequence, it is also currently unclear which motifs are involved in Hsp70 recognition by such receptors. It has been shown that oxLDL and Hsp70 can compete for binding to cell surface LOX-1; it has thus been assumed that both molecules possess overlapping binding sites on LOX-1 (49). The oxLDL binding site is located in the LOX-1 C-type lectin domain, and negative charges on the surface of the oxLDL particle appear to interact specifically with basic residues disposed at the surface of LOX-1 homodimer (50, 51). The elucidation of the crystal structure of LOX-1 has revealed that these basic residues are in fact positioned diagonally on the surface of a heart-shaped dimer (52, 53). In addition, the analysis of the NKG2D crystal structure demonstrates that a fairly similar pattern of basic residues on the surface of the protein are also responsible for its interaction with one of its own ligand MHC class I polypeptide-related sequence A (53). Moreover, the shared ability of LOX-1, SREC-1 and FEEL-1 to bind Hsp70 preparations suggests that LOX-1 shares functional homology with these SR. Indeed, FEEL-1 contains a LINK domain that was originally discovered as a hyaluronan proteoglycan repeat binding site and has been shown to be a simpler quaternary structural version of the C-type lectin extracellular domain, missing only one unstructured big loop (40, 54). Therefore, these considerations suggest that negatively charged amino acids, located in the surface residues of extracellular Hsp70, may interact with a special disposition of basic residues present at the surface of C-type lectin receptors and possibly SR.

The observation that SR-mediated Hsp70 internalization can occur even after deleting the ID of LOX-1 or SREC-1 suggests that the functions of these SRs in endocytosis may be confined to ligand binding and that other cell surface structures are required to trigger Hsp70/PC uptake (Figs. 5C and 6). The internalizing receptor CD91/LRP1 has been proposed to mediate Hsp-mediated cross-presentation, although our previous experiments suggest that these properties are not mediated through direct binding (11, 12). However, CD91/LRP could function in an alternative role, as a coreceptor necessary for LOX-1- and SREC-1-mediated Hsp70 uptake. Indeed, CD91/LRP can modulate platelet-derived growth factor endocytosis by acting as a platelet-derived growth factor receptor coreceptor (55). We have found that LOX-1-mediated Hsp70 internalization is markedly reduced in CD91/LPR1-deficient CHO cells (CHO 13-5-1) although traces of uptake were still seen (J. R. Theriault and S. K. Calderwood, unpublished observations). Thus, CD91/LRP or molecules with a homologous cellular function could play a role in Hsp70/PC complex uptake. By analogy with LOX-1 and SREC-1, CD94-NKG2A heterodimer receptor internalization occurs without the requirement of tyrosine-based motifs located in the ID of NKG2A (56). In addition, NKG2D does not possess a cytoplasmic tail but can be efficiently internalized (57). Thus, mechanisms for endocytosis that function independently of internalization motifs in the ID have been shown previously in functionally related receptors, suggesting a precedent for our findings in the present study. However, there is still much to learn regarding the mechanisms of Hsp70/PC uptake by the SR.

Our experiments therefore indicate that Hsp70 can be recognized by a number of cell surface molecules of the interrelated SR and C-type lectin families. Indeed, it is not clear whether further Hsp70 receptors may remain to be uncovered. This plethora of receptors may, however, play a functional role in permitting selectivity in responding to different Hsp70 family members in different cellular contexts. Future studies will determine the significance of these interactions in mediating the extracellular functions of Hsp70.

Disclosures
The authors have no financial conflict of interest.

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