C-Mannosylated peptides derived from the thrombospondin type 1 repeat interact with Hsc70 to modulate its signaling in RAW264.7 cells

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The thrombospondin type 1 repeat (TSR) is a functional module of proteins called TSR superfamily proteins (e.g., thrombospondin, F-spondin, mindin, etc.) and includes a conserved Trp–x–x–Trp (W–x–x–W) motif, in which the first Trp residue is preferably modified by C-mannosylation. We previously reported that synthesized TSR-derived peptides (e.g., C-Man-WSPW) specifically enhanced lipopolysaccharide-induced signaling in macrophage-like RAW264.7 cells. In this study, we searched for the proteins that bind to C-mannosylated TSR-derived peptides in RAW264.7 cells and identified heat shock cognate protein 70 (Hsc70). The binding affinity of Hsc70 for C-mannosylated peptides in solution was higher than that for the peptides without C-mannose. The binding was influenced by a nucleotide-induced conformational change of Hsc70, and C-mannosylated peptides preferred the substrate-binding domain of Hsc70. Furthermore, in RAW264.7 cells, addition of Hsc70 stimulated cellular signaling to produce tumor necrosis factor-α, via transforming growth factor-β-activated kinase 1, and the Hsc70-induced signaling was enhanced more in the presence of the peptides with C-mannose than that without C-mannose, suggesting functional interaction between Hsc70 and the C-mannosylated peptides in the cells. Together, these results demonstrate a novel function of the C-mannosylation of TSR-derived peptides in terms of interaction with Hsc70 to regulate cellular signaling.

Keywords: C-mannosylation/Hsc70/macrophase/thrombospondin

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The W–x–x–W motif is found in the thrombospondin type 1 repeat (TSR), which is predicted to be a functional peptide module in various integral proteins called TSR superfamily proteins such as thrombospondin-1 (TSP-1) (Tucker 2004). The motif is known to bind with heparin, heparan sulfate proteoglycans, collagen and transforming growth factor-β (TGF-β), suggesting functional significance in cell–cell interaction and/or cellular signaling. In this respect, to investigate whether C-mannosylation exerts some influence on the W–x–x–W motif in the TSR, we examined the function of C-mannosylated TSR-derived peptides (e.g., C-Man-WSPW) in RAW264.7 cells and found that the peptides modulate lipopolysaccharide (LPS)-induced signaling including pathways involving mitogen-activating protein kinases such as c-Jun N-terminal kinase (JNK) and enhance the cytotoxicity of proteins such as thrombospondin-1 (TSP-1) (Tucker 2004).

In this study, to clarify how C-mannosylated TSR-derived peptides influence cell signaling in macrophages, we searched for specific proteins bound to chemically synthesized C-mannosylated TSR-derived peptides (e.g., C-Man-WSPW) in RAW264.7 cells. Here we report that the peptides interacted with heat shock cognate protein 70 (Hsc70) to modulate its signaling to produce tumor necrosis factor-α (TNF-α) in the cells.

**Results**

**C-Mannosylated TSR-derived peptides interact with Hsc70 in RAW264.7 cells**

To identify proteins interacting with C-mannosylated TSR-derived peptides in RAW264.7 cell, C-Man-WSPWC-biotin was chemically synthesized as described in Materials and methods and used as a probe. A non-mannosylated peptide derivative (WSPWC-biotin) was also prepared for use as a negative control probe. The cells were incubated with C-Man-WSPWC-biotin, WSPWC-biotin or biotin (10 μM) then treated with dithiobis[succinimidylpropioniate] (DSP), a membrane-permeable cross linker, as described in the methods. The cells were lysed, and cellular proteins bound to the biotin conjugates were separated by non-reducing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) then detected by blot analysis using peroxidase-conjugated avidin. As shown in [Figure 1A](https://academic.oup.com/glycob/article-abstract/20/10/1298/1988249) (right), various proteins were bound to C-Man-WSPWC-biotin to a greater extent than to WSPWC-biotin, under the conditions with DSP (arrows). The binding was not apparent under the conditions without DSP (Figure 1A, left), although some non-specific binding of peroxidase-conjugated avidin to proteins was detected (arrowheads). These results suggest specific but weak interaction between C-Man-WSPWC-biotin and the proteins in the cells. To identify the proteins interacting with C-Man-WSPWC-biotin, the cells were incubated with biotinylated peptides with or without C-mannose, then treated with DSP as described above, and the proteins bound to biotinylated peptides were isolated from cell lysate by using NeutrAvidin-conjugated agarose beads as described in the methods. Next, the samples were separated by reducing SDS-PAGE, and the proteins were visualized with silver staining. As shown in [Figure 1B](https://academic.oup.com/glycob/article-abstract/20/10/1298/1988249) (right), some specific protein bands (arrowheads) were detected in the sample bound to C-Man-WSPWC-biotin but not in that bound to non-mannosylated peptides. Among the proteins isolated, attention was focused on the protein band B4 (arrowhead B4), which was excised and digested in-gel with trypsin and then subjected to mass
In vitro binding of Hsc70 with C-mannosylated TSR-derived peptides in solution

To examine whether Hsc70 can bind C-mannosylated TSR-derived peptides in solution, fluorescence-labeled probes conjugated with C-Man-WSPW or WSPW were prepared, and their interaction with Hsc70 was examined in solution with the potassium-based buffer system by measuring fluorescence polarization as described in the methods. As shown in Figure 3A, the probes containing C-Man-WSPW and WSPW specifically interacted with Hsc70, compared with biotin-conjugated controls. The dissociation constant (Kd) for the probes with C-Man-WSPW and WSPW was 25 ± 6 and 75 ± 22 nM, respectively. This indicates that Hsc70 has greater affinity for C-Man-WSPW than WSPW. However, when the measurements were performed with the sodium-based buffer system (i.e., 20 mM Tris-HCl [pH 7.2], 150 mM NaCl), the Kd values for C-Man-WSPW and WSPW were similar, approximately 75 nM (data not shown). These results indicate that Hsc70 binds C-Man-WSPW with higher affinity than it binds WSPW, especially in solution with the potassium-based buffer system.

Next, to know what portion of C-Man-WSPW contributes to the binding with Hsc70, the binding of Hsc70 (100 nM) with a fluorescence-labeled C-Man-WSPW probe (2 nM) was examined by fluorescence polarization assay in the presence of various related compounds, such as C-Man-WSPW, WSPW, C-Man-W, mannose and Trp. In Figure 3B, the results show that the polarization due to the binding of Hsc70 to the probe was apparently suppressed by C-Man-WSPW in a dose-dependent manner compared with slight suppression by WSPW. In addition, the polarization was little affected by C-Man-W, mannose and Trp. These results suggest that the entire structure of WSPW peptides containing C-mannose is required in the binding between Hsc70 and the fluorescent C-Man-WSPW probe.

C-Mannosylated TSR-derived peptides preferably interact with the substrate-binding domain of Hsc70

The structure of Hsc70 is altered in the ATP- and ADP-bound states to regulate chaperone functions (Mayer and Bukau 2005). To investigate whether the binding of Hsc70 with C-Man-WSPW is influenced by the conformational change of Hsc70 due to adenosine nucleotides, the effect of ATP or ADP (1 mM) on the binding of Hsc70 with C-Man-WSPW was examined by fluorescence polarization assay. As shown in Figure 4, the Kd value for the probes with C-Man-WSPW in the presence of 1 mM ATP and ADP was 10 ± 5 and 30 ± 8 nM, respectively. This indicates that Hsc70 has greater affinity for C-Man-WSPW in the presence of ATP, suggesting that the ATP-induced conformational change of Hsc70 influences the binding of Hsc70 to C-mannosylated TSR-derived peptides.

To further investigate the structural basis for the binding between Hsc70 and C-Man-WSPW peptide, functional do-
mains of Hsc70 (i.e., the nucleotide-binding domain [NBD] and substrate-binding domain [SBD]) (Mayer and Bukau 2005) were prepared to assess interaction with the TSR-derived peptides with or without C-mannose. As shown in Figure 5A, NBD and SBD were expressed in bacteria then purified as described in the methods. The interaction of these domains with C-Man-WSPW or WSPW was examined using fluorescence polarization measurements. In Figure 5B (upper), the results for the NBD of Hsc70 showed no difference between C-Man-WSPW and WSPW, and the Kd values were similar, approximately 180 nM. In contrast, in Figure 5B (lower), the results for the SBD of Hsc70 showed that the Kd values for the probes with C-Man-WSPW and WSPW were 40 ± 9 and 80 ± 23 nM, respectively.

Collectively, these results indicate that the SBD of Hsc70 has higher affinity for C-Man-WSPW than for WSPW. In contrast, the NBD shows less affinity for both C-Man-WSPW and WSPW, which suggests that the SBD plays the main role in the interaction with C-mannosylated TSR-derived peptides.

Effect of C-mannosylated TSR-derived peptides on Hsc70-induced production of TNF-α in RAW264.7 cells

To investigate whether the C-mannosylated TSR-derived peptides influence the function of exogenous Hsc70 added to cultured RAW264.7 cells, Hsc70-induced production of TNF-α was measured using a cytotoxicity assay. As shown in Figure 3, the addition of C-Man-WSPW or WSPW significantly increased the production of TNF-α compared to the control group. The production of TNF-α was dose-dependent, with the highest production observed at 1 mM concentrations of C-Man-WSPW and WSPW. These results suggest that C-mannosylated TSR-derived peptides can enhance the protective activity of Hsc70 against TNF-α-induced cytotoxicity.

Fig. 3. Evaluation of the interaction between C-mannosylated TSR-derived peptides and Hsc70 in solution using fluorescence polarization. (A) Fluorescent ligands conjugated with biotin, WSPWC-biotin or C-Man-WSPWC-biotin (2 nM) were incubated with different concentrations of Hsc70 at room temperature for 3 h in the dark. Fluorescence polarization was measured as described in the methods. Data were expressed as values relative to that for saturated Hsc70 binding with each ligand and were fit to a four-parameter logistic curve to deduce the Kd of Hsc70 for the ligands. Each value represents the mean ± SE for three to four independent experiments. (B) A fluorescent ligand conjugated with C-Man-WSPWC-biotin (2 nM) was incubated with Hsc70 (100 nM) in the presence or absence of various compounds as shown in the figure. Fluorescence polarization was measured as described in the methods. Each value represents the mean ± SE for at least five experiments.

Fig. 4. Effects of ATP/ADP on the interaction between C-mannosylated TSR-derived peptides and Hsc70 in solution. A fluorescent ligand conjugated with C-Man-WSPWC-biotin (2 nM) was incubated with different concentrations of Hsc70 in the presence or absence of ATP or ADP (1 mM) at room temperature for 3 h in the dark. Fluorescence polarization was measured as described in the methods. Data are expressed as values relative to that for saturated Hsc70 binding with each ligand and were fit to a four-parameter logistic curve to deduce the Kd of Hsc70 for the ligands. Each value represents the mean ± SE for three to four independent experiments.
α was examined in the presence or absence of factors such as mannose, C-Man-W, C-Man-WSPW and WSPW. The cells were treated with Hsc70 (0.01 μM) in the presence or absence of various compounds (10 μM), then the amount of TNF-α secreted from the cells was estimated by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 6A, the addition of purified Hsc70 apparently induced the production and secretion of TNF-α. The production was not influenced in the presence of mannose, C-Man-W (Figure 6A) or Trp (data not shown). In contrast, the secretion of TNF-α was enhanced more with Hsc70 plus C-Man-WSPW than with Hsc70 plus WSPW or with Hsc70 alone. The transcriptional expression of TNF-α was also examined by reverse transcription-polymerase chain reaction (RT-PCR) in the cells. The cells were treated for 1, 4 and 8 h with Hsc70 (0.01 μM) in the presence or absence of C-Man-WSPW or WSPW (10 μM), then total RNA was extracted and subjected to RT-PCR. As shown in Figure 6B, the transcriptional level of TNF-α was enhanced more with Hsc70 plus C-Man-WSPW than with Hsc70 plus WSPW or Hsc70 alone.

To estimate how is the binding or internalization of C-mannosylated TSR-derived peptides with Hsc70 in the cells, the cells were incubated with biotin, WSPWC-biotin or C-Man-WSPWC-biotin (10 μM) in the presence or absence of Hsc70 (0.01 μM) at 4°C for 30 min. After the incubation, internalization of the molecules was induced by incubating the cells at 37°C for 15 min. Then the biotin conjugates were visualized by fluorescence microscopy after incubation with DyLight 488-conjugated NeutrAvidin (Figure 6C). Under conditions in which cellular membranes were permeabilized by Triton X-100, strong fluorescence signals for all biotinylated conjugates showed a vesicular pattern in the cells, although the signal was weak under non-permeabilized conditions without Triton X-100. These results indicated that most biotinylated conjugates were internalized in the cells under the conditions. In our previous study using flow cytometry, binding capacity to the surface of RAW264.7 cells was higher with C-Man-WSPWC-biotin than with WSPWC-biotin or with biotin alone (Muroi et al. 2007). But the biotinylated conjugates were likely to be similarly internalized in the cells (Figure 6C, left). On the other hand, in the presence of exogenous Hsc70, the intensity of internalized signals was diminished in WSPWC-biotin and biotin, compared with those in the absence of Hsc70. In consequence, the intensity was slightly higher with C-Man-WSPWC-biotin than with WSPWC-biotin or with biotin alone (Figure 6C, right).

Taken together, these results indicate that C-Man-WSPW peptides enhance the Hsc70-induced production of TNF-α.
with a concomitant increase in their internalization in the cells.

Previously, we found that C-Man-WSPW peptides enhanced LPS-induced production of TNF-α through upregulation of the JNK pathway followed by phosphorylation of TGF-β-activated kinase 1 (TAK1) (Muroi et al. 2007). In the present study, we investigated whether C-Man-WSPW peptides influence TAK1 signaling in the Hsc70-induced production of TNF-α in RAW264.7 cells. The activation of TAK1 occurs with a concomitant autophosphorylation of serine or threonine (Kishimoto et al. 2000). The cells were treated with Hsc70 (0.01 μM) in the presence or absence of C-Man-WSPW or WSPW (10 μM), then the phosphorylation status of TAK1 and JNK was examined by immunoblotting using a specific antibody for each phosphorylated kinase. As shown in Figures 7A and B, the level of phosphorylated TAK1 was increased by Hsc70 in the presence or absence of WSPW or C-Man-WSPW. But it showed a moderate enhancement after 10 min, by the treatment with Hsc70 plus C-Man-WSPW, compared with Hsc70 plus WSPW or Hsc70 alone. In addition, JNK was slightly phosphorylated by Hsc70.
Fig. 7. Hsc70-induced TAK1 signaling is up-regulated in RAW264.7 cells treated with C-Man-WSPW. (A) Cells were treated with Hsc70, Hsc70 plus WSPW or Hsc70 plus C-Man-WSPW as described in Figure 6B. The activation status of TAK1 and JNK was examined in the cell lysate by immunoblot analysis using the anti-phospho-TAK1 (Thr-187) and anti-phospho-JNK (Thr-183/Tyr-185) antibodies as described in the methods. The expression of GAPDH is also shown as a control using specific antibodies. NS, non-specific band. (B) Quantitative data for the phosphorylation status of TAK1 (left) and JNK (54 kDa) (right) shown in A. The band intensity was estimated densitometrically, and the phosphorylation rate is expressed as the relative intensity of the phosphorylated protein/total protein. Each value represents the mean ± SE for three independent experiments. (C) Cells were cultured for 10 min with Hsc70 (0.01 μM) in the presence of various concentrations of C-Man-WSPW (0–10 μM). The activation status of TAK1 and JNK was examined in the cell lysate by immunoblotting using the anti-phospho-TAK1 and anti-phospho-JNK antibodies as described above. The band intensity was estimated densitometrically, and the phosphorylation rate is expressed as the relative intensity of the phosphorylated protein/total protein. Each value represents the mean ± SE for three independent experiments. (D) Cells were pretreated at 37°C for 30 min with a TAK1 inhibitor (5Z-7-oxozeaenol, 1 μM) then treated for 10 min with Hsc70 (0.01 μM). The activation status of TAK1 was examined in the cell lysate by immunoblotting using the anti-phospho-TAK1 antibody as described above. (E) Cells were pretreated at 37°C for 30 min with the TAK1 inhibitor and treated for 4 h with Hsc70 (0.01 μM) in the presence or absence of C-Man-WSPW (10 μM). Then, the amount of TNF-α expressed was estimated by RT-PCR as described in the methods. Data represent three to four independent experiments.
The level of phosphorylated JNK was increased 10–30 min after the treatment with Hsc70 plus C-Man-WSPW, although it was similarly increased after 60 min, by the treatment with Hsc70 plus WSPW or Hsc70 alone. Together, these results indicate that the signaling related to TAK1 and JNK is upregulated more rapidly by Hsc70 plus C-Man-WSPW than by Hsc70 plus WSPW or Hsc70 alone. This also suggests that C-Man-WSPW peptides enhance the TAK1 signaling especially at the early phase of signaling induced by Hsc70. To see if there is a dose-responsive effect of C-Man-WSPW on the Hsc70-induced phosphorylation of TAK1, cells were treated for 10 min with Hsc70 (0.01 μM) in the presence of various concentrations of C-Man-WSPW (0–10 μM). The phosphorylation status of TAK1 and JNK was examined as described above. The results showed that C-Man-WSPW enhanced the Hsc70-induced phosphorylation of TAK1 and JNK in a dose-dependent manner (Figure 7C).

Next, to investigate whether the signaling pathways of TAK1 are involved in the Hsc70-induced production of TNF-α, levels of TNF-α were examined in cells treated with Hsc70, Hsc70 plus WSPW, and Hsc70 plus C-Man-WSPW in the presence or absence of a signaling inhibitor for TAK1 (i.e., 5Z-7-oxozeaenol) (Ninomiya-Tsuji et al. 2003). The cells were pretreated for 30 min with the TAK1 inhibitor (1 μM) then treated for 4 h with Hsc70 (0.01 μM) in the presence or absence of C-Man-WSPW or WSPW (10 μM). In Figure 7D, the phosphorylation status of TAK1 was examined as described above. The results showed that the Hsc70-induced phosphorylation of TAK1 was apparently suppressed by the pretreatment with the TAK1 inhibitor. Next in Figure 7E, the expression of TNF-α was examined by RT-PCR as described. The results showed that the expression was significantly suppressed by the TAK1 inhibitor in all cases, indicating that TAK1 plays an important role in the Hsc70-induced expression of TNF-α.

Collectively, these results demonstrate that the Hsc70-induced production and secretion of TNF-α via TAK1 signaling is enhanced more in the presence of the peptide with C-mannose than without C-mannose. They also suggest functional interaction between Hsc70 and C-mannosylated TSR-derived peptides in the Hsc70-induced signaling in cells.

**Discussion**

The TSR is a functional module found in various extracellular proteins called TSR superfamily proteins including TSPs, ADAMTSs, F-spondin and properdin (Tucker 2004). Especially in the case of TSPs, the module has a variety of cellular functions, such as cell attachment, inhibition of cell proliferation and angiogenesis, induction of apoptosis and neurite outgrowth (Lawler 2004; Carlson et al. 2008). TSR-derived peptides containing W–x–x–W have also been shown to possess anti-angiogenic effects (Iruela-Arispe et al. 1999) and to induce promyelocytic leukemia cell differentiation (Brueil et al. 2005). In the W–x–x–W–x–x–W motif of TSP1, the first or second Trp is preferably C-mannosylated (Hofsteenge et al. 2001), which suggests the functions of the W–x–x–W motif to be influenced by C-mannosylation.

Previously, by using chemically synthesized C-mannosylated peptides, we found that C-mannosylated TSR-derived peptides such as C-Man-WSPW specifically enhanced LPS-induced cell signaling and damage in macrophage cells (Muroi et al. 2007). The results showed that the enhanced LPS-induced signaling with C-Man-WSPW was not simply due to enhanced binding of LPS to the cells treated with LPS plus C-Man-WSPW. On the other hand, the binding of C-Man-WSPW to the cells was greater than that of biotin or WSPWC-biotin on flow cytometry, suggesting some specific binding of C-Man-WSPW peptides to the cell surface targets. However, it was not clear how the C-mannosylated TSR-derived peptides could influence the cell signaling.

In the present study, to investigate the molecular mechanism of action of C-mannosylated TSR-derived peptides on cells, we searched for specific targets, which could bind the peptides (i.e., C-Man-WSPW) in RAW264.7 cells. Using chemical cross linkers, various proteins were found to bind C-mannosylated but not non-mannosylated peptides, and Hsc70 was identified as a target of the C-mannosylated peptides in RAW264.7 cells. Hsc70 is a member of the heat shock protein (Hsp) 70 family and constitutively expressed in a variety of tissues and cells (Hightower and Leung 1997). Hsp70 is a stress-induced chaperone exerting a variety of cellular functions, such as protein quality control, protein translocation, cellular signaling and so forth (Mayer and Bukau 2005; Schmitt et al. 2007). Hsc70 is likely to share similar cellular functions with Hsp70 but also possesses specific functions related with membrane trafficking in the cell (Eisenberg and Greene 2007). In this study, the binding of purified Hsc70 with C-Man-WSPW or WSPW peptides was examined by fluorescence polarization-based solution-phase binding assays. Hsc70 showed higher affinity for C-Man-WSPW (Kd = 25 ± 6 nM) than WSPW (Kd = 75 ± 22 nM), especially in solution with the potassium-based buffer system. The value with WSPW was similar to that of DnA, a bacterial homologue of Hsp70, with hydrophobic substrate peptides (Davis et al. 1999). Hutchison et al. (1992) reported that ATP-dependent association of glucocorticoid receptor with Hsp70 and Hsp90 was enhanced by K+, NH4+ and Rb+ but not by Na+ and Li+. This work suggests monovalent cation selectivity for the molecular recognition of Hsp70 family, and it seems consistent with the finding that Hsc70 has greater affinity for C-mannosylated peptides with the potassium-based than sodium-based system. This suggests the interaction between Hsc70 and C-mannosylated peptides to be enhanced more in the cytoplasmic environment, although further investigation is needed to clarify how the interaction is regulated under physiological conditions in the cell. On the other hand, we also examined the binding by ELISA-based solid-phase binding assay using 96-well plastic plates coated with C-Man-WSPW or WSPW peptides and found that Hsc70 bound similarly to both peptides with and without C-mannose (data not shown). Collectively, these results indicate that C-mannosylation on the WSPW peptide enhances the affinity of the peptide for Hsc70 under specific conditions such as in solution.

Hsc70 is composed of two functional domains, a NBD located at the amino-terminal and a SBD at the carboxyl-terminal. The conformation of Hsc70 changes in a cycle between ATP-bound and ADP-bound states (Mayer and Bukau 2005). Hsc70 in the ADP-bound form binds unfolded substrates then releases them when it is in the ATP-bound form. In this study, we found that the binding of Hsc70 with C-Man-WSPW was apparently en-
hanced in the presence of ATP but not ADP, suggesting that it is controlled by the nucleotide-induced conformational change of Hsc70. Furthermore, in the assay using two functional domains of Hsc70, C-Man-WSPW showed similar affinity for the SBD of Hsc70 and full-length Hsc70 but showed less affinity for the NBD of Hsc70. Collectively, these results strongly suggest that Hsc70 interacts with C-mannosylated TSR-derived peptides via chaperone-like functions through its SBD, although further investigation is required to clarify the precise binding mechanism.

Hsp70 and Hsc70 interact as chaperones with the hydrophobic peptide parts of misfolded proteins in the cell. In addition, they are known to bind other non-peptidic molecules, such as phosphorylserine (Arispe et al. 2002; Arispe et al. 2004; Harada et al. 2007), and acidic glycolipids (e.g., sulfatide, GM3 and GD3 [Mamelak and Lingwood 2001; Mamelak et al. 2001; Harada et al. 2007]). In the case of phosphorylserine, its interaction with Hsp70 on the plasma membrane causes damage in various cells (Arispe et al. 2002; Schilling et al. 2009). It was also revealed that Hsp70/Hsc70 binds with the AU-rich region of mRNA to control the mRNA’s stability in cells (Henics et al. 1999; Laroia et al. 1999). Furthermore, it was reported that Hsp70 binds an O-linked β-N-acetylgalactosamine residue (O-GlcNAc) in proteins (Guinez et al. 2004). Although the precise binding mechanism is not clear, Hsp70 may bind the O-GlcNAc portion of modified proteins to protect them from degradation under certain stressful conditions (Guinez et al. 2008). As described above, there are a variety of candidates interacting with Hsp70/Hsc70 in the cell, and these results suggest some biological functions through their interactions. In this respect, it would be interesting to clarify the relation of the C-mannosylated TSR module in proteins with each Hsp70/Hsc70-binding molecule in the cell.

A variety of C-mannosylated proteins have been identified, some of which, such as the TSR superfamily, mainly function in the extracellular spaces (Furmanek and Hofsteenge 2000). It was reported that TSP-1 and 2 are cleaved by ADAMTS1, some of which, such as the TSR superfamily, mainly function in the extracellular spaces (Furmanek and Hofsteenge 2000). It was reported that TSP-1 and 2 are cleaved by ADAMTS1, resulting in the production of functional anti-angiogenic peptides (Arispe et al. 2002; Mamelak et al. 2001; Harada et al. 2007). In the case of phosphorylserine, its interaction with Hsp70 on the plasma membrane causes damage in various cells (Arispe et al. 2002; Schilling et al. 2009). It was also revealed that Hsp70/Hsc70 binds with the AU-rich region of mRNA to control the mRNA’s stability in cells (Henics et al. 1999; Laroia et al. 1999). Furthermore, it was reported that Hsp70 binds an O-linked β-N-acetylgalactosamine residue (O-GlcNAc) in proteins (Guinez et al. 2004). Although the precise binding mechanism is not clear, Hsp70 may bind the O-GlcNAc portion of modified proteins to protect them from degradation under certain stressful conditions (Guinez et al. 2008). As described above, there are a variety of candidates interacting with Hsp70/Hsc70 in the cell, and these results suggest some biological functions through their interactions. In this respect, it would be interesting to clarify the relation of the C-mannosylated TSR module in proteins with each Hsp70/Hsc70-binding molecule in the cell.

The receptor functions seem to overlap with the peptide-carrier function and cytokine-inducing function (Asea et al. 2000), but the precise relation is not fully understood. In addition, it is still controversial whether the activation of macrophages and release of inflammatory cytokines induced by Hsp70 in vitro are caused by contamination from pathogen-associated molecules such as LPS in the recombinant Hsp70 prepared from bacteria (Quintana and Cohen 2005). In this respect, pathogen-associated molecules must be a concern in studies using recombinant proteins, although there appear to be in vivo and in vitro data to support that extracellular chaperones have immunomodulatory effects (Lehnardt et al. 2008; Chen et al. 2009; Tsan and Gao 2009).

In the present study, we also observed that TNF-α expression was induced in RAW264.7 cells by adding Hsc70 to the culture medium. The Hsc70-induced expression of TNF-α was regulated in part through the TAK1 signaling pathway, and initiation of the signaling was accelerated more by Hsc70 plus C-Man-WSPW than by Hsc70 plus WSPW or Hsc70 alone. These results indicate that the interaction between Hsc70 and C-mannosylated TSR-derived peptides is highly relevant to the biological functions of Hsc70 in the cell. It was demonstrated that exogenous Hsc70 associated with RAW264.7 cells through multiple Hsp70 receptors in a flow cytometric analysis (Thériault et al. 2005), but the precise mechanism for the binding is not known. It would be interesting to know whether the Hsc70-induced signaling is influenced by C-mannosylated peptides through Hsc70’s peptide-carrier function, cytokine-inducing function or both. In addition, it was reported that Hsp70 and Hsp90 associate with Toll-like receptor 4 upon stimulation with LPS, also suggesting a LPS-induced clustering of Hsp70, Hsp90 and the LPS-receptor in the cell (Triantafilou and Triantafilou 2004). If so, is the enhancing effect of C-mannosylated TSR-derived peptides on Hsc70-induced signaling linked with the LPS-induced signaling, which is also enhanced by the C-mannosylated peptides (Muroi et al. 2007)? However, the binding of C-Man-WSPW with endogenous Hsc70 seems not a direct cause of cell signaling because TNF-α-producing signaling was not induced in RAW264.7 cells solely treated by C-Man-WSPW or WSPW (Muroi et al. 2007). Further investigation is required to clarify precisely how the binding with C-mannosylated TSR-derived peptides modulates the Hsc70-induced signaling via TAK1 in the cell.

In conclusion, C-mannosylated TSR-derived peptides have an enhancing effect on Hsc70-induced signaling to produce TNF-α through binding to Hsc70. This study demonstrates a novel regulatory role of C-mannosylation on the TSR-derived peptide motif in the immune response of macrophages and also suggests unrevealed functions of C-mannosylated TSR family proteins or peptides in macrophages.
C-Mannosylated TSR and Hsc70

Materials and methods

Materials

Antibodies against JNK, phospho-JNK (Thr-183/Tyr-185), TAK1 and phospho-TAK1 (Thr-187) were purchased from Cell Signaling Technology (Beverly, MA). The mouse antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Millipore (Chemicon, Temecula, CA). Peroxidase-conjugated secondary antibodies against IgG of rabbit and mouse were from Dako (Denmark). DSP and DSS were from Thermo Scientific (Pierce Biotech., Rockland, IL). The TAK1 inhibitor, 5Z-7-oxozeaenol, was obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). The other reagents used in the study were all of high grade, from Sigma or Wako pure chemicals (Osaka, Japan).

Chemical synthesis of C-mannosylated peptides and derivatives

Cα-α-d-C-mannosylpyranosyl-l-tryptophan (C-Man-W) and C-mannosylated TSR-derived peptides (C-Man-WSPW and C-Man-WSPWC) were synthesized essentially as described previously (Manabe and Ito 1999; Manabe et al. 2003). WSPW peptides, in which the first W has the potential to be C-mannosylated, are derived from TSR2 of human TSP-1, and the first W corresponds to Trp423 in the amino acid sequence (Lawler and Hynes 1986; NCBI Accession number NP_003237). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry spectra were measured with a Shimadzu AXIMA-CFR using 2,5-dihydrobenzoic acid or α-cyano-4-hydroxycinnamic acid as a matrix. 1H-NMR (nuclear magnetic resonance) spectra were recorded at ambient temperature (23~24°C) in CDCl3, CD3OD or DMOS using a JEOL EX 400 MHz spectrometer. The biotin derivative was purchased from Thermo Scientific. Chemical synthesis of WSPWC-biotin and C-Man-WSPWC-biotin was performed as described before (Muroi et al. 2007). The purity of C-Man-W-containing peptides was determined by 1H-NMR spectroscopy at 400 MHz. The purity of other peptides (i.e., not containing C-Man-W) was determined by both 1H-NMR spectroscopy and high-performance liquid chromatography (Iertsil ODS column, water:CH3CN containing 0.1% trifluoroacetic acid). The purity of all peptides was more than 95%.

Cell lines and culture

RAW264.7 cells, a clonal line of mouse macrophage-like cells, were obtained from American Type Culture Collection (TIB-71). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum under a humidified atmosphere of 95% air and 5% CO2 at 37°C.

Estimation of C-Man-WSPWC-biotin binding to the cells

Cells were incubated at 37°C for 20 min in phosphate-buffered saline (PBS; 10 mM sodium phosphate [pH 7.4] and 150 mM NaCl) with biotin, WSPWC-biotin or C-Man-WSPWC-biotin (10 μM). After incubation, the cells were treated for 10 min with or without 1 mM DSP or DSS, membrane-permeable cross linkers. After quenching with 1 M Tris-HCl (pH 7.2), the cells were washed with PBS and harvested by using a scraper. The cells were lysed in lysis buffer (20 mM Tris-HCl [pH 7.2], 130 mM NaCl and 1% NP-40 including protease inhibitors [20 μM p-(aminophenyl) methanesulfonyl fluoride hydrochloride, 50 μM pepstatin and 50 μM leupeptin]). Protein samples were electrophoresed on 7.5% SDS-polyacrylamide gels under non-reducing conditions and then transferred to a nitrocellulose membrane as described before (Ihara et al. 2005). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.5] and 150 mM NaCl) and then incubated at 4°C for 10 min with the peroxidase-conjugated NutriAvidin (Thermo Scientific) in TBS containing 0.1% Tween 20. After a wash with TBS containing 0.1% Tween 20, the blots were developed using an ECL chemiluminescence detection kit (GE Healthcare Biosciences) according to the manufacturer’s instructions. Proteins bound to C-Man-WSPW peptides were also pulled down from cell lysate by using C-Man-WSPWC-biotin and NutriAvidin-conjugated agarose beads (Thermo Scientific) then separated by 7.5% SDS-PAGE and visualized by silver staining (Silver stain MS kit, Wako, Osaka, Japan).

Immunoblot analysis

Cultured cells were harvested and lysed in lysis buffer as described above. Protein samples were electrophoresed on 7.5~10% SDS-polyacrylamide gels under reducing conditions and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBS and then incubated at 4°C overnight with the primary antibody in TBS containing 0.1% Tween 20. The blots were coupled with the peroxidase-conjugated secondary antibodies, washed and then developed using an ECL chemiluminescence detection kit (GE Healthcare Biosciences).

Vector construction

Various deletion constructs of the Hsc70 gene were generated by PCR with Advantage HF-2 DNA polymerase (Clontech Lab. Inc.) using the human full-length TrueClone cDNA for Hsc70 in pCMV6-XL5 (OriGene Tech. Inc., Rockville) as a template. Human cDNA for Hsc70 was used for this study because human and mouse Hsc70 differ in only one amino acid (aa 579) unrelated to the substrate-binding function (NCBI accession number; NP_006588.1 and NP_112442.2), suggesting quite similar biological functions of Hsc70 between human and mouse. Primers used were as follows: for full domains of Hsc70 (amino acids 1~412), forward: 5′-CAC CTG GCC TCC ACA GAC AAG GGA CCT GCA GTT GG-3′; reverse: 5′-TTC ATC AAC CTC TTC AAT GGT GGG CCC-3′; for the nucleotide-binding domain of Hsc70 (amino acids 1~383), forward: 5′-GGC CAT GGG GAT GTC CAA GGG ACC TG-3′; reverse: 5′-CGG GTA CCT TAC TCA GAC TTG TCT CCA GAC-3′; and for the substrate-binding domain of Hsc70 (amino acids 384~650), forward: 5′-GGC CAT GGG GAA TGT TCA AGA TTT GCT GC-3′; reverse: 5′-CCG GTA CCT TAA TCA ACC TCT TCA ATG G-3′. To produce a His-tagged Hsc70 protein in the bacterial expression system, a fragment of the Hsc70-coding region was subcloned into the bacterial expression vector pDEST-17 (Invitrogen) to produce pDEST17-Hsc70 by a LR recombination reaction according to the manufacturer’s directions. To produce His-tagged nucleotide-binding and substrate-binding domains of Hsc70 in the bacterial ex-
pression system, fragments of the coding region were sub-
cloned into the NcoI-KpnI site of the bacterial expression
vector pPROEX HTa (Invitrogen).

**Purification of Hsc70 expressed in bacteria**

Human Hsc70 proteins were expressed with a His-tag at their N-terminus in bacterial BL21 cells by using expression vectors as described above. His-tagged Hsc70 proteins were purified by using Ni²⁺-agarose and ATP-agarose columns according to the methods of **Blond-Elguindi et al. (1993)** with a slight modification. Endotoxin was removed from purified proteins using Detoxi-Gel Endotoxin Removing Gel composed of im-
mobilized polymixin B (Thermo Scientific), and then the samples were desalted by gel filtration using NAP-10 (GE Healthcare Biosciences) for the subsequent assay.

**Fluorescence polarization measurements**

Fluorescent ligands were prepared by coupling DyLight488-
conjugated NeutrAvidin (Thermo Scientific) with biotin, WSPWC-biotin or C-Man-WSPWC-biotin. Fluorescent ligands (final concentration, 2 nM) were incubated with different concentrations of Hsc70 protein in 100 μL of potassium-based binding buffer (20 mM Tris-HCl [pH 7.2] and 150 mM KCl) in 6 × 50 mm borosilicate glass tubes, at room temperature for 3 h in the dark. For the experiment to estimate the effect of adenylnucleotides, 1 mM ATP or ADP (with 5 mM MgCl₂) was contained in the binding buffer. Fluorescence polarization was measured as described (Davis et al. 1999) using a Panvera BEA-
CON 2000 Fluorescence Polarization System (Invitrogen) at 25°C with excitation at 490 nm and emission at 535 nm. Data were fit to a four-parameter logistic curve to deduce dissociation constant (Kd) values with GraphPad PRISM ver.4 software.

**TNF-α assay**

Cells were plated in 96-well plates (10,000 cells/well) and cul-
tured overnight. The medium was renewed. Then, cells were stimulated with Hsc70 (0.01 μM) in the presence or absence of mannose, C-Man-W, C-Man-WSPW or WSPW (10 μM). After the incubation, the culture medium was collected, and the concentration of TNF-α in the sample was evaluated using a mouse TNF-α ELISA kit according to the manufacturer’s instructions (BioSource International, Camarillo, CA). All ex-
periments were done in quadruplicate.

**Reverse transcription-polymerase chain reaction**

Total RNA was prepared from cultured cells using standard methods and was reverse transcribed using a One Step RNA PCR Kit (AMV) (TaKaRa Biomedicals, Shiga, Japan) with avian myoblastosis virus-derived reverse transcriptase XL ac-
cording to the manufacturer’s instructions. PCR was run for 19 cycles of 95°C for 0.5 min/65°C for 0.5 min/72°C for 1.5 min. Primer sequences were as follows: for mouse TNF-α (NCBI accession number NM_013839), forward: 5’-TCT CAG CCT CTT CTC ATT CC-3’; reverse: 5’-GTC CCA GCA TCT TGT GTT TC-3’; and for mouse GAPDH (GenBank accession num-
ber M32599), forward: 5’-CAG TAG AGG CAG GGA TGA TG-3’; reverse: 5’-GGA TTT GGC CGT ATT GGG CG3’.

**Fluorescence microscopy**

Cells were grown on a cover glass for 24 h and incu-
bated with biotin, WSPWC-biotin or C-Man-WSPWC-biotin (10 μM) in the presence or absence of Hsc70 (0.01 μM) at 4°C for 30 min. After the incubation, internalization of mo-
lecules was induced by incubating the cells at 37°C for 15 min. The cells were washed with PBS and fixed with 4% paraformaldehyde in PBS and permeabilized for 10 min with 1% Triton X-100 in PBS. The cells were then blocked with PBS containing 1% bovine serum albumin, and coupled with DyLight 488-conjugated NeutrAvidin (Thermo Scientific) in so-
lution. The biotinylated conjugates were visualized by laser scanning confocal microscopy (LSM5Pascal, Carl Zeiss Co., Jena, Germany) and analyzed using PASCAL analytic software.

**Supplementary data**

Supplementary data mentioned in the text is available to sub-
scribers in Glycobiology online.

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**Abbreviations**

DSP, dithiobis[succinimidylpropionate]; DSS, disuccinimidyl suberate; ELISA, enzyme-linked immunoabsorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; NBD, nucleotide-binding domain; PAGE, polyacrylamide gel elec-
trophoresis; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; SBD, sub-
strate-binding domain; SDS, sodium dodecyl sulfate; TAK1, TGF-β-activated kinase 1; TBS, Tris-buffered saline; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor-
α; TSP, thrombospondin; TSP, thrombospondin type 1 repeat.

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