Characteristics of the Interaction between Hsc70 and the Transferrin Receptor in Exosomes Released during Reticulocyte Maturation*

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The transferrin receptor (TfR) of reticulocytes is released in vesicular form (exosomes) during their maturation to erythrocytes. The heat shock cognate 70-kDa protein (Hsc70) has been demonstrated to interact with the cytosolic domain of the TfR and could thus trigger the receptor toward this secretion pathway. We investigated the characteristics of the interaction between Hsc70 and the TfR in exosomes with an in vitro binding assay using TfR immobilized on Sepharose beads and purified Hsc70. The results show that Hsc70 binds to exosomal TfR with characteristics expected of a chaperone-peptide interaction. We demonstrated that heat-denatured luciferase competed for in vitro binding, dependent on the nucleotide bound to Hsc70, and that this interaction activates the ATPase activity of Hsc70. Moreover, we used immunosuppressive agents that interact with Hsc70, thus decreasing Hsc70 binding to TfR in our in vitro binding assay and enabling us to assess the role of this interaction in vivo during reticulocyte maturation.

Exosomes are small membrane-bound vesicles secreted in the extracellular medium by hematopoietic cells, such as reticulocytes (1), B lymphocytes (2), or dendritic cells (3). These vesicles are formed by membrane budding into the lumen of an endocytic compartment, leading to the formation of multivesicular intracellular structures. Fusion of these multivesicular structures with the plasma membrane leads to the release of internal vesicles (exosomes) into the medium (4). The protein composition of exosomes depends on the cell type. Some membrane proteins become enriched in exosomes, whereas others are excluded. For example, the transferrin receptor (TfR) is a major protein of exosomes secreted by reticulocytes, leading to the complete loss of TfR from maturing reticulocytes, whereas no anion transporter is lost during maturation into erythrocytes. By contrast, TfR is absent from the surface of exosomes secreted by B lymphocytes, which instead are enriched with major histocompatibility complex class II molecules (2).

The molecular basis of the process of protein sorting into exosomes is yet not known. However, it seems specifically related to the particular cell involved because TfR is a major actor in reticulocyte and prereticulocyte function and major histocompatibility complex class II is essential in B lymphocyte physiology. In reticulocyte exosomes, another protein is enriched in a stoichiometric ratio with TfR. This protein has been identified as clathrin uncoating ATPase, also known as the heat shock cognate 70-kDa protein (Hsc70) (5). This protein has been shown to interact with the cytosolic domain of the TfR (6), and this interaction was suggested to induce sorting of the receptors into exosomes. This conclusion is strengthened by the fact that Hsc70 is also present in exosomes secreted by dendritic cells (3) and thus may be a general marker of exosome formation. Interestingly, however, clustering of TfRs on the reticulocyte cell surface by using antibodies or lectins was shown to induce receptor sorting into exosomes (7). These observations demonstrated that protein sorting into exosomes is not necessarily induced by cytosolic sorting machinery. The fact that glycosylphosphatidylinositol-anchored proteins such as acetylcholinesterase, CD55, CD58, and CD59 are enriched in reticulocyte exosomes (1, 8), whereas they do not cross the plasma membrane, supports the idea that protein sorting into exosomes may occur in the absence of a cytoplasmic domain.

The proposal that protein aggregation may be the signal triggering molecules toward the exosome pathway could account for these observations. For example, Hsc70 could interact with proteins following partial unfolding of the cytoplasmic domain. If TfR were to unfold, Hsc70 would become associated with the TfR without participating further in the sorting process. It is known that, besides its role in clathrin uncoating of coated vesicles through interaction with auxilin (9), Hsc70 has general chaperone properties and is involved in protein folding and unfolding (10).

Previous studies have described the general characteristics of the Hsp70 class of molecular chaperones in binding to peptides (11). Hsc70 binds preferentially to hydrophobic sequences containing basic amino acids. The ADP-bound form of Hsp70 has a high affinity for peptides, whereas the ATP form has a lower affinity, thus resulting in dissociation (12). In the present study, we carried out an in vitro binding assay to study characteristics of the interaction between Hsc70 and TfR. Our results demonstrate that Hsc70 binds to exosomal TfR with the characteristics expected of a chaperone/peptide interaction. We used deoxyaspergualin (DSG) and LF15-0195, two immunosuppressive agents that interact with Hsc70 (13, 14) (Fig. 1). Both compounds diminished the interaction between Hsc70 and TfR in our in vitro binding assay. Both induced an increase in TfR release in exosomes when added during in vitro maturation of reticulocytes. These data demonstrate that TfR aggregation...
instead of Hsc70 binding may be the signal targeting TfR molecules toward the exosome pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rat monoclonal antibody raised against Hsc70 (SPA-815) and mouse monoclonal antibody raised against rat TfR (CL-071AP) were from StressGen (Victoria, Canada) and Cedarlane (Hornby, Canada), respectively. A mouse anti-NeuK/ 

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**Aggregation Assay by Centrifugation**—Recombinant firefly luciferase (58 pmol) was incubated in buffer E (25 mM Tris-HCL, pH 7.8, 50 mM KCl, 1 mM dithiothreitol) containing the indicated combinations of nucleotides (10 μM Hsc70 and 2.6 μM transferrin) for 10 min at 4 or 42 °C. Reaction mixtures were centrifuged in an air-driven ultracentrifuge (Beckman Airfuge with 18° A-100 rotor) at 5 p.s.i. for 10 min. Supernatants were analyzed by SDS-PAGE and Coomassie staining.

**ATPase Assay**—ATPase assay was performed as described (18, 19) with slight modifications. The assay was carried out in buffer F (20 mM Hepes, pH 7.2, 250 mM KCl, 10 mM NH₄SO₄) at 30 °C for 1 h. [α-32P]ATP (10 μCi) was diluted in 25 μl of a 50 μM solution of ATP (8 μCi/μmol ATP) was aliquoted to achieve 3.8 μCi/μg of purified Hsc70. Incubation was conducted for 40 min at 30 °C. The sample was applied to a 1-ml Sephadex G-25 spin column. 32P-Labeled full-length Hsc70 complex was recovered by centrifugation and added to immobilized TfR for 2 h at 30 °C. As control, 0.4 μCi of [α-32P]ATP was added along with 100 μg of adenine 5′-triphosphatase (Sigma), 9 μl of buffer F, and protein A-Sepharose beads incubated with 400 μg of exosomal proteins and anti-TfR antibody. Aliquots were spotted on thin layer chromatography plates (polyethylenimine-cellulose) and developed using 0.4 M NH₄HCO₃ buffer. [α-32P]ADP and [α-32P]ATP were visualized by autoradiography.

**Western Blot Analysis**—Proteins were separated by 10% SDS-PAGE according to Laemmli (17) and electrophoretically transferred to PVDF membrane (Immobilon-P, Millipore), unless otherwise specified, as described below.
Purified Hsc70 binds to immobilized exosomal TfR. A, Hsc70 was purified from rat brain. Lane 1, Coomassie Blue staining of SDS-PAGE from brain extract; lane 2, purified Hsc70; lane 3, Western blot of purified Hsc70. For immobilization of exosomal TfR, protein A-Sepharose beads were incubated with solubilized exosomal proteins, in the absence (lanes 4 and 6) or presence (lanes 5 and 7) of anti-TfR antibody. Purified Hsc70 was then added for 1 h at 30 °C before washing the Sepharose beads as described under “Experimental Procedures.” Bead-associated proteins were separated by SDS-PAGE and transferred on PVDF membrane. Western blot of TfR (lanes 4 and 5) and Hsc70 (lanes 6 and 7) was then carried out on the membrane. Molecular mass standards are indicated to the left. B, increasing amounts of TfR were immunoprecipitated using the same quantities of anti-TfR antibody and protein A-Sepharose beads and increasing amounts of solubilized exosomes (1, 4, 8, and 16 μg of protein, respectively, in lanes 1, 2, 3, and 4). Purified Hsc70 was then added, and the beads were treated as described in A. Membrane was blotted for TfR (upper part) and Hsc70 (lower part).

RESULTS

Hsc70 Binding to Immobilized Exosomal TfR—Association of Hsc70 with exosomal TfR has been demonstrated by chemical cross-linking, coimmunoprecipitation, and immobilization experiments (6). To further characterize this association, we developed a binding assay using Hsc70 purified from rat brain and TfR immunoprecipitated from rat exosomes. As shown in Fig. 2A, a single band of ~70 kDa was obtained by Coomassie staining (lane 2), and recognized by an anti-Hsc70 antibody (lane 3). TfR was immunoprecipitated from rat reticulocyte exosomes using a monoclonal antibody against the extracellular domain of the receptor. As shown in Fig. 2A (lane 5), a band with a molecular mass (about 94 kDa) corresponding to the TfR monomer, was detected by Western blot after immunoprecipitation. Note that a band with a lower molecular mass (approximately 85 kDa) was also detected on the membrane, corresponding to the soluble fragment of a cleaved receptor already described (21, 22). TfR immobilized on protein A-Sepharose beads was then used to characterize binding of purified Hsc70. As highlighted by the Western blot in Fig. 2A (lane 7), after incubation of the purified chaperone with immobilized TfR and several washes, Hsc70 was found to be associated with the TfR-Sepharose beads. Protein A-Sepharose incubated with Hsc70 without immobilized TfR did not result in any chaperone copelleting (Fig. 2A, lanes 4 and 6). To confirm the specificity of the association between TfR and Hsc70, we examined whether increasing amounts of TfR would bring down an increasing amount of Hsc70. As shown in Fig. 2B, there was an increase in the amount of Hsc70 brought down by the TfR immobilized on Sepharose beads. Quantification of TfR and Hsc70 Western blots was done for each lane, and the ratio (Hsc70/TfR) gave similar values.

Purified Hsc70 Binds to Exosomal TfR through Its Chaperone Activity—Contrary to other members of the Hsp70 protein family, Hsc70 is constitutively expressed in almost all living cells. One of its best known roles in cell physiology is to remove clathrin triskelions from coated vesicles and to provide recycled components for the formation of nascent coated pits (23). Hsc70 is also involved in other cellular functions that require binding to partially unfolded proteins during synthesis or en route to degradation. The fate of the proteins can then vary. For example, the chaperone can prevent aggregation of a misfolded protein as well as assist its refolding with cochaperones (24), or it may target some cytosolic proteins to lysosomes (25). The chaperone activity of purified Hsc70 was assessed by measuring its binding to heat-denatured firefly luciferase (26). Luciferase unfolding was obtained by 10 min of incubation at 42 °C. This led to protein aggregation and was followed by pelleting during centrifugation (Fig. 3A, lanes 1 versus 2). However, when Hsc70 was present during incubation at 42 °C, luciferase aggregation and pelleting was prevented. The addition of transferrin instead of Hsc70 had no effect (Fig. 3A, lanes 3 and 4), and luciferase was removed from the supernatant. If Hsc70 is limiting and binds to exosomal TfR via its chaperone capacity, the addition of denatured luciferase could compete with the Hsc70-bound TfR and lower the amount of Hsc70 associated with Sepharose-immobilized TfR. As shown in Fig. 3B, incubation in the presence of heat-denatured luciferase significantly decreased Hsc70 binding to TfR, whereas the addition of native luciferase did not significantly affect Hsc70/TfR binding. The minor effect of native luciferase could be the result of a partial unfolding of luciferase during the 1-h incubation at 30 °C, as suggested by the lower luciferase activity (not shown). Note that although aggregation and pellet formation of heat-denatured luciferase were prevented by the addition of Hsc70, the latter could not bring about protein refolding, as measured by restoration of its enzymatic activity (not shown).

Effect of Nucleotides on Hsc70 Binding to Exosomal TfR—The Hsp70 family binds peptides under regulation by ATP binding and hydrolysis. The ADP-bound form of Hsc70 binds peptides with high affinity and has a slow rate of substrate release, whereas the ATP-bound form is more prone to peptide release. Moreover, the complex between Hsc70 and the nucleotide (ATP or ADP) is very stable, and when purified from a cell homogenate, special methods are required to obtain nucleotide-free Hsc70 (27, 28). To study the effects of added nucleotides, we thus generated nucleotide-free chaperone, loaded it with ADP or ATPγS, and assessed binding of the two forms to exosomal TfR. As shown in Fig. 4A, more ADP-bound Hsc70 than ATPγS-bound Hsc70 was retained by TfR immobilized on Sepharose beads. Western blot quantification indicated that about three times more of the ADP form was associated with
the TfR-bead pellet than the ATPγS bound form. These data are all consistent with chaperone behavior.

Ssa1p, the predominant yeast cytosolic Hsp70, was demonstrated to be inactivated by NEM when in the nucleotide-free form but not when bound to nucleotides (28). It was suggested that NEM modification may disrupt the conformation of Ssa1p or interfere with nucleotide binding. To assess the effect of NEM in this system, nucleotide-free or ATP-bound Hsc70 were treated with NEM before NEM treatment as described under “Experimental Procedures.” Hsc70 was set to 100%. B, nucleotide-bound Hsc70 is protected from NEM treatment. Purified nucleotide-free Hsc70 was loaded or not with ATP before NEM treatment as described under “Experimental Procedures.” Hsc70 binding to exosomal TfR was carried out as described above. TfR and Hsc70 Western blots are presented in the upper part of the panel. Data of Hsc70 immunoblot quantification are given in the lower part. (Image quantification: NEM-untreated ATP-Hsc70 was set to 100%).

siae (30) stimulate ATPase activity of chaperone proteins. As shown in Fig. 5, two radioactive spots appeared (lane 8) when a purified [α-32P]ATP-Hsc70 complex was added to TfR immobilized on Sepharose beads followed by 2 h of incubation at 30 °C and polyethyleneimine-cellulose chromatography. The reaction mixture at t0 gave only one radioactive spot (lane 7). Migration comparison with unlabeled nucleotides identified the spots as ADP and ATP, as indicated. Spots corresponding to AMP, ADP, and ATP were also obtained by incubation of [α-32P]ATP with excess porcine brain ATPase (lanes 3 and 4). Neither spontaneous (lanes 1 and 2) nor immobilized-TfR-induced (lanes 5 and 6) [α-32P]ATP hydrolysis was found during the incubation period. The nucleotide hydrolysis induced by binding of ATP-Hsc70 to exosomal TfR thus confirmed that the interaction between the two proteins was that expected of an interaction with a chaperone.

FIG. 3. Purified Hsc70 binds to immobilized exosomal TfR through its chaperone activity. A, purified Hsc70 has chaperone activity. Recombinant luciferase (Lucif.) was treated at 4 °C (lane 1) or 42 °C (lanes 2–4) for 10 min in the presence (lane 2) or absence (lanes 1, 2, and 4) of purified Hsc70, plus transferrin (Tf; lane 4). Mixtures were ultracentrifuged, and supernatants containing nonaggregated proteins were analyzed by SDS-PAGE and Coomassie Blue staining. B, heat-denatured luciferase competes with Hsc70 binding to exosomal TfR. Binding of Hsc70 to immobilized exosomal TfR was carried out as described in Fig. 2, except that purified Hsc70 was first loaded with ADP and preincubated (lanes 2 and 3) or not (lane 1), with recombinant luciferase for 10 min at 4 °C (lanes 1 and 2) or 42 °C (lane 3). Washed beads were resuspended with Laemmli buffer and loaded on SDS-PAGE. Proteins were transferred to PVDF membrane and immunoblotted using an anti-Hsc70 antibody.

FIG. 4. Effects of nucleotides on Hsc70 binding to exosomal TfR. A, ADP-loaded Hsc70 binds TfR with a higher affinity than ATPγS-loaded chaperone. Hsc70 was freed from nucleotide and loaded with ADP or ATP-γS by incubation with 5 mM nucleotides, as described under “Experimental Procedures.” Hsc70 binding to Sepharose-immobilized exosomal TfR was carried out as in Fig. 2. Hsc70 immunoblots (upper part) were scanned and quantified using ImageQuanNT software (lower part). (Image quantification: ADP-Hsc70 was set to 100%). B, nucleotide-bound Hsc70 is protected from NEM treatment. Purified nucleotide-free Hsc70 was loaded or not with ATP before NEM treatment as described under “Experimental Procedures.” Hsc70 binding to exosomal TfR was carried out as described above. TfR and Hsc70 Western blots are presented in the upper part of the panel. Data of Hsc70 immunoblot quantification are given in the lower part. (Image quantification: NEM-untreated ATP-Hsc70 was set to 100%).
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FIG. 5. Stimulation of the Hsc70 ATPase by immunoprecipitated TfR. [32P]ATP-Hsc70 complex was obtained as described under “Experimental Procedures.” [32P]ATP (lanes 5 and 6) or [32P]ATP-Hsc70 (lanes 7–10) were added to immobilized TfR (lanes 7 and 8) or to protein A-Sepharose beads (lanes 9 and 10) for 2 h at 30 °C. Aliquots of the reaction mixtures were spotted on polyethylenimine-cellulose plate at the beginning of incubation (lanes 5, 7, and 9) or 2 h later (lanes 6, 8, and 10). Nucleotides were visualized by autoradiography after migration, as described under “Experimental Procedures.” Spontaneous hydrolysis of [32P]ATP during the incubation period at 30 °C was visualized by incubation of the nucleotide alone and spotting at t₀ (lane 1) and after 2 h (lane 2). Partial and total [32P]ATP hydrolysis was obtained by [32P]ATP incubation in the presence of adenosine 5’-triphosphatase and spotting at t₀ (lane 3) and after 2 h (lane 4). Nucleotide species are indicated to the left.

DSG binds Hsc70 with a Kₐ in the micromolar range (13), suggesting that Hsc70 may be a target for the immunosuppressive action of DSG in vivo.

First we assessed the effect of the compounds on in vitro interaction between Hsc70 and exosomal TfR. Purified Hsc70 was preincubated with or without DSG or LF15-0195 before using it for in vitro binding to TfR. As shown in Fig. 6, preincubation of both immunosuppressive agents with the chaperone decreased its association with immobilized TfR. Radiolabeled Hsc70 was used in the binding assay to improve the quantification. As shown in Fig. 6, the data in both assays gave similar values.

To improve our understanding of the nature and role of the Hsc70/TfR interaction during reticulocyte maturation, we took advantage of the inhibition of Hsc70 binding to TfR by DSG. DSG is transported across the plasma membrane and accumulates in the cell cytoplasm (32). We were thus able to assess the consequences of decreasing Hsc70/TfR interaction during exosome formation. The immunosuppressive agents were added during in vitro maturation of reticulocytes, and the time course of exosome release was monitored after the addition of the immunosuppressive compounds.

Reticulocytes from phlebotomized sheep that were cultured in vitro for 3–4 h or for up to 2 days, in the presence of DSG or methyl-DSG, showed an increase in the amount of TfR released via exosomes (Table I and Fig. 7). Although the extent of increase was variable, the direction of the response was consistent. Immunoblotting for Hsc70 from exosomes did not show a comparable quantitative increase in the presence of DSG. Similar results with DSG and LF15-0195 were obtained using rat reticulocytes from animals treated with phenylhydrazine (not shown). This increase of TfR release in exosomes in response to the immunosuppressive agents in two different species argues against a possible fortuitous event.

Other membrane proteins have been demonstrated to be selectively sorted into exosomes, together with TfR (1). If TfR release were part of a general sorting mechanism, the presence of DSG during reticulocyte maturation would also increase Na⁺/K⁺-ATPase and nucleoside transporter release. The presence of DSG during reticulocyte maturation increased the amount of [3H]nitrobenzylthioinosine binding in the released exosomes (Table II). [3H]Nitrobenzylthioinosine is a nucleotide analog frequently used to assess the level of equilibrating nucleoside transporter (1). In addition, in a single experiment, there was also evidence of enhanced externalization of the α-subunit of the Na⁺/K⁺-ATPase.

DISCUSSION

Release of exosomes during reticulocyte maturation was first described about 20 years ago (33) and is now a well known process (34). More recently, this process has been demonstrated to occur in other hematopoietic cells (2, 3, 35). Depending on the cell type, the occurrence of the phenomenon of vesicle release may have different but important implications. In the case of reticulocytes, it likely represents a mechanism to remove obsolete proteins from the cell surface. It is known that the plasma membrane of reticulocytes has a greater variety and a higher amount of proteins than that of the mature erythrocyte. In the case of antigen presenting cells, exosome release could be a way to increase the cellular immunologic response (36). However, the molecular basis of protein sorting into exosomes is still not known. It was suggested that Hsc70, the only known cytoplasmic protein enriched in reticulocyte exosomes, could be involved in TfR sorting. In fact, Hsc70 was demonstrated to interact better with the TfR present in exosomes than with those on the plasma membrane (6). Moreover, the interaction in exosomes appeared stoichiometric.

In this study, we set up an in vitro binding assay using purified Hsc70 and immobilized exosomal TfR to characterize the nature of the interaction between these two molecules. We found that binding of Hsc70 to exosomal TfR followed the pattern expected of an interaction with a chaperone. Thus we found that (i) heat-denatured luciferase was able to compete with TfR for Hsc70 binding, (ii) binding of Hsc70 to TfR was dependent upon the nucleotide bound to the chaperone, (iii) binding to exosomal TfR activated Hsc70 ATPase activity, and (iv) compounds known to interact and modify the chaperone characteristics of Hsc70 decreased binding of Hsc70 to TfR. Thus, during reticulocyte maturation, it is possible that the cytoplasmic domain of the TfR becomes partially unfolded, which would then trigger Hsc70 binding. It is noteworthy that a peptide sequence: 23FSLARQV29 in the TfR cytoplasmic domain has the characteristic motif recognized by Hsc70. Hsc70 binds to peptides enriched in large hydrophobic and aromatic residues and containing basic amino acids. For example, it has been shown that the peptide sequences FSGLWKL and LSRTLTSV were responsible for high affinity binding (~200 μM) of peptides P17G and P10K, respectively, to Hsc70 (11). Moreover, the peptide sequence 23FSLARQV29 of TfR is also part of the protein kinase C phosphorylation motif 22RFSLAR27. Thus, binding of the chaperone with its target domain should impair further phosphorylation of the serine residue by PKC. If Hsc70
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**TABLE I**

**DSG increases release of exosomal TfR**

Sheep reticulocytes enriched to contain at least 80% reticulocytes were incubated as described for the times indicated. The exosomes were separated from the medium by ultracentrifugation of the cell-free supernatant. An aliquot of exosomes corresponding to the number of packed cells from which the exosomes were obtained were then subjected to SDS gel electrophoresis, transferred to nitrocellulose membranes, and blotted with a monoclonal mouse antibody to the cytoplasmic domain of the human TfR (obtained from Trowbridge). A sheep anti-mouse horseradish peroxidase-antibody was used as secondary antibody. The blots were visualized with chemiluminescence reagents according to the manufacturer's instructions and analyzed with a Bioimage system. The arbitrary OD units (AOD) are given. Three separate experiments are shown, two of which were done in triplicate. An immunoblot corresponding to experiment 1 is shown in Fig. 7.

| Conditions | Time of incubation | AOD units |
|------------|--------------------|-----------|
| Control    | 4 h                | 4.0 (± 0.2, n = 3) |
| +40 μg/ml DSG | 4 h                | 4.7 (± 0.2, n = 3) |
| +40 μg/ml MeDSG | 4 h                | 7.5 (± 0.2, n = 3) |
| Control    | 16 h               | 2.2 (± 0.2, n = 3) |
| +100 μg/ml DSG | 16 h               | 3.2 (± 0.1, n = 3) |
| +100 μg/ml MeDSG | 16 h               | 3.3 (± 0.3, n = 3) |
| Control    | 4 h                | 0.05 (n = 1) |
| +20 μg/ml DSG | 4 h                | 1.0 (n = 1) |
| +20 μg MeDSG | 4 h                | 0.2 (n = 1) |

**FIG. 6.** DSG and LF15 decrease Hsc70 binding to exosomal TfR. Binding of Hsc70 to immobilized exosomal TfR was carried out as described in Fig. 2, except that purified Hsc70 was first preincubated for 1 h at 30 °C with buffer alone or in the presence of DSG (10 μM) or LF15-0195 (5 μM). Washed beads were then resuspended with Laemmli buffer and loaded on SDS-PAGE. Proteins were transferred to PVDF membrane and immunoblotted using an anti-Hsc70 antibody. Hsc70 immunoblot was scanned and quantified using ImageQuaNT software. (Image quantification: untreated Hsc70 was set to 100%). [3H]Hsc70 was added to unlabeled chaperone to obtain a specific activity of ~20 cpm/ng. Radiolabeled Hsc70 was then used in the binding assay as described for unlabeled chaperone. Hsc70 association with Sepharose bead pellet was determined by radioactivity counting. (The 100% binding obtained with CTRL corresponded to ~10000 cpm associated with Sepharose beads). CTRL, control.

**TABLE II**

**DSG increases release of exosomes bearing the nucleoside transporter**

Exosomes from sheep reticulocytes were collected after overnight incubation in the presence or absence of the drugs as indicated. [3H]Nitrobenzylthioinosine at a concentration of 1.0 μM was incubated with the recovered exosomes as described using a sample of exosomes obtained from 150 μl of sheep reticulocytes. To obtain specific binding, a control containing 1000× excess of unlabeled [3H]nitrobenzylthioinosine was carried out with each sample. The nonspecific uptake, which represented no more than 5% of the total uptake, has been subtracted from the values given.

| Conditions       | Transporter released pmol/100 μg protein |
|-----------------|----------------------------------------|
| Control         | 4.0                                    |
| +100 μg/ml DSG  | 4.9                                    |
| +100 μg/ml MeDSG| 4.3                                    |

**FIG. 7.** Immunoblot corresponding to experiment 1.

remained associated with the TfR upon immunoprecipitation of the latter, this could explain why immunoprecipitates of the TfR from the plasma membrane were phosphorylated by PKC in vitro (37), whereas the immunoprecipitates of exosomal TfR, which are more likely associated with Hsc70, could not be phosphorylated under identical conditions.

A common explanation of the role of Hsc70 in TfR externalization is that after many rounds of endocytic cycles, along with changes in the cytoplasm during cell maturation, partial unfolding of the TfR cytoplasmic domain would expose the hydrophobic sequence that would bind Hsc70. This would be analogous to the appearance of a ‘new’ signal during the lifespan of the receptor in red cells, leading to a change in its intracellular routing. This type of rerouting signal could also occur in other cell types where lysosomal degradation of proteins takes place at the end of their lifespan.

Although this model has attractive features, data obtained using DSG are inconsistent with this model and suggest that Hsc70 binding is not the real signal for a change in TfR sorting. On the contrary, DSG data suggest that TfR segregation into exosomes is inhibited by Hsc70 binding.

DSG is a synthetic analog of spergualin, a natural product isolated from *Bacillus laterosporus* that has been shown to interact specifically with Hsc70 and Hsp90, with *K*<sub>M</sub> values of 4 and 5 μM, respectively (13). Although its exact mechanism of action is still poorly understood, DSG was demonstrated to be a potent immunosuppressive agent (38). The ability of DSG to bind Hsc70 and modify the chaperone characteristics of Hsc70 in other nonimmunologic areas has recently been shown. It was demonstrated that DSG can rescue the ΔF508-CFTR trafficking defect (the most common mutant of the transmembrane conductance regulator encountered in cystic fibrosis). ΔF508-CFTR is purportedly retained and degraded in the ER through interaction with Hsp70. However, it was targeted to the plasma membrane when cells expressing the mutant protein were ex-
posed to DSG (39). In the present case, the addition of DSG or analogs during in vitro reticulocyte maturation increased the amount of TfR secreted via exosomes. If Hsc70 binding were required for externalization along with the prevention of binding by DSG, a decrease in exosome formation would be expected. This is in apparent contradiction with the data on in vitro binding, which demonstrated a decrease in Hsc70 binding to exosomal TfR in the presence of DSG (Fig. 6). The data suggest that Hsc70 binding may prevent or diminish sorting into exosomes. Thus, it is unlikely that binding of Hsc70 itself leads to TfR externalization. In this context, it is important to remember that one of the chaperone functions is to fight against protein unfolding and aggregation. Aggregation is thus probably the real signal triggering protein sorting into exosomes. Hsc70 would bind to TfR as a consequence of receptor unfolding but would not be the major factor of its segregation. Moreover, Hsc70 could delay TfR aggregation by acting as a chaperone, and this reaction would be impaired by DSG. The result would also be consistent with a greater effect of DSG at short maturation times. In this perspective, it is noteworthy that TfR has a marked tendency to interact strongly upon reconstitution into lipid bilayers (40). This characteristic probably favors the aggregation of TfR in the exosomal membrane and could account for the finding that TfR is often detected as a dimer on SDS-PAGE under reducing conditions. This is also consistent with our previous work demonstrating that external experimental aggregation of TfR induces its secretion through exosomes (7) and that aggregation of furin in post-Golgi compartments triggers its targeting to lysosomes (41).

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J. Biol. Chem. 2001, 276:9910-9916.
doi: 10.1074/jbc.M009641200 originally published online December 22, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M009641200

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