Expression of the Molecular Chaperone Hsp70 in Detergent-resistant Microdomains Correlates with Its Membrane Delivery and Release*

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Accumulating evidence suggests that some heat shock proteins (Hsps), in particular the 72-kDa inducible Hsp70, associate to the cell membrane and might be secreted through an unknown mechanism to exert important functions in the immune response and signal transduction. We speculated that specialized structures named lipid rafts, known as important platforms for the delivery of proteins to the cell membrane, might be involved in the unknown mechanism ensuring membrane association and secretion of Hsp70. Lipid rafts are sphingolipid-cholesterol-rich structures that have been mainly characterized in polarized epithelial cells and can be isolated as detergent-resistant microdomains (DRMs). Analysis of soluble and DRM fractions prepared from unstressed Caco-2 epithelial cells revealed that Hsp70, and to a lesser extent calnexin, were present in DRM fractions. Increased expression of Hsps, through heat shock or by using drugs acting on protein trafficking or intracellular calcium level, induced an efficient translocation to DRM. We also found that Hsp70 was released by epithelial Caco-2 cells, and this release dramatically increased after heat shock. Drugs known to block the classical secretory pathway were unable to reduce Hsp70 release. By contrast, release of the protein was affected by the raft-disrupting drug methyl-β-cyclo-dextrin. Our data suggest that lipid rafts are part of a mechanism ensuring the correct functions of Hsps and provide a rational explanation for the observed membrane association and release of Hsp70.

Different Hsps are found in different cellular compartments and organelles. Among the major Hsps currently identified, the stress-inducible 72-kDa Hsp70 or the constitutively expressed 73-kDa Hsc70 are mainly found in the cytosol, but following stress they migrate to the nucleus and associate to nuclear proteins (2, 3). Hsps also include a group of analogous proteins confined to the endoplasmic reticulum (ER). Among these are the 78-kDa glucose-regulated protein (Grp78) located in the ER lumen and the calcium-binding proteins calreticulin and calnexin, the luminal and transmembrane-associated ER Hsps, respectively. There is recurrent evidence suggesting that several Hsps (ER-resident and cytosolic) are also expressed at the cell membrane (4–7). The origin of membrane-associated Hsps still remains elusive, and it has recently been suggested that they may also be secreted by cells (8–11) before being associated with the external leaflet of cell membranes. Membrane-associated or secreted Hsps (Hsp70 in particular) have been suggested to exert a necessary, but still poorly understood, role in the immune response and signal transduction (7–9, 12, 13). However, there is still no available data on the mechanisms by which Hsp70 might be released from cells. Since Hsp70 and some other Hsps are intracellular proteins and lack any secretory signal sequence, one should hypothesize that these proteins are not released through the classical exocytic pathway.

Our initial studies, aimed at defining the nature of the membranes to which Hsp70 may attach, have suggested that specialized membrane microdomains may be involved. These microdomains, also termed lipid rafts, are formed within the exoplasmic leaflet of the Golgi membrane (14, 15). Lipid rafts are characterized by their resistance to non-ionic detergent extraction at low temperature combined with their ability to float during density gradient centrifugation (16). These detergent-resistant microdomains (DRMs) are enriched in cholesterol, glycosphingolipids, glycosylphosphatidylinositol-anchored proteins, and some other acylated or transmembrane proteins (16).

In the present study, a model of cultured human intestinal cells, namely Caco-2, was used to study the expression and membrane association of Hsps. Different Hsps were found to associate with DRMs to a variable extent depending on the particular Hsp considered, and association of Hsps with these microdomains could be modulated by stress. DRM fractions obtained from unstressed cells contained consistent levels of Hsp70, while Hsc70 and ER chaperone levels were very low or undetectable. The stress response (heat shock, depletion of ER calcium, and inhibition of protein trafficking) resulted in an increased accumulation of some Hsps into DRM fractions as compared with unstressed cells. Notably, in stressed Caco-2 cells, the amount of Hsp70 was selectively increased in DRMs, and this was correlated with a robust stimulation of Hsp70

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† The abbreviations used are: Hsp, heat shock protein; DRM, detergent-resistant microdomain; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; LDH, lactate dehydrogenase.
release. Manipulating the lipid composition of DRMs resulted in a concomitant modulation of Hsp70 release, thus suggesting that lipid rafts may represent a cellular mechanism for membrane delivery and release of Hsps.

EXPERIMENTAL PROCEDURES

Reagents, Media, and Antibodies

If not otherwise stated all reagents were purchased from Sigma. The protease inhibitor mixture (Complete mini tablets) was purchased from Roche Applied Science. The lactate dehydrogenase (LDH) viability determination kit was from Roche Applied Science. Dulbecco’s modified Eagle’s medium, fetal calf serum, penicillin-streptomycin, non-essential amino acids, phosphate-buffered saline (PBS), trypsin-EDTA (0.05% trypsin, 0.02% EDTA), SDS, and polyacrylamide were from (Invitrogen). Radiolabeled cholesterol, [1,2-3H]-cholesterol (1 μCi/ml) and enhanced chemiluminescence (ECL) detection reagents were from Amersham Biosciences. Bicinchoninic acid (BCA) protein assay reagent was from Pierce.

The antibodies used were as follows: mouse anti-Hsp70 monoclonal antibody (catalog no. SPA-810), rat anti-Hsc70 monoclonal antibody (catalog no. SPA-815), mouse anti-calreticulin monoclonal antibody (catalog no. SPA-601), mouse anti-KDEL monoclonal antibody that recognizes Grp78 (catalog no. SPA-827), and rabbit anti-calnexin polyclonal antibody (catalog no. SPA-880) (StressGen, Victoria, Canada). Peroxidase-conjugated secondary antibodies, fluorescein isothiocyanate-conjugated F(ab’)2 fragments of rabbit anti-mouse IgG, and goat anti-rabbit IgG were from DAKO (Glostrup, Denmark). The Hsp70 enzyme-linked immunosorbent assay (ELISA) kit (catalog no. EKS-700) was from StressGen.

Cell Culture

Human colon adenocarcinoma (Caco-2) cells (passage 50–80) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum, 1% penicillin-streptomycin, and 1% non-essential amino acids. Cells were plated 21 days before experiments and maintained at 37 °C in 10% CO2. These conditions allow differentiation of epithelial membrane into apical (brush border) and basolateral domains. Some experiments were performed with a non-tumoral epithelial cell line, Madin-Darby canine kidney, cultured as confluent monolayers. Some experiments were performed with a non-tumoral epithelial cell line, Madin-Darby canine kidney, cultured as confluent monolayers. Some experiments were performed with a non-tumoral epithelial cell line, Madin-Darby canine kidney, cultured as confluent monolayers.

Heat Shock

Cells were exposed to thermal stress for 30 min in culture flasks partially immersed in a thermostatically regulated water bath. Cells were allowed to recover at 37 °C in 10% CO2 to allow the synthesis of Hsps.

Pretreatments with Agents Perturbing Golgi or ER Functions and Protein Traffic

Tunicamycin (25 μg/ml), monensin (25 μM), brefeldin A (15 μM), and thapsigargin (0.5 μM) were added to Dulbecco’s modified Eagle’s medium 18 h before experiments in supplemented culture medium.

DRM Preparation

DRM preparation was adapted from a procedure described previously. Adherent cells (1–2 × 10^5/cm² culture flask) were washed twice in PBS, lysed with 2 ml of extraction buffer (20 mM Tris-HCl, pH 7.4, NaCl 150 mM, EDTA 1 mM) supplemented with 1% Triton X-100 and a protease inhibitor mixture. Lysates were scraped from the flasks, sheared by 20 passages through a 22-gauge needle, left for 20 min before mixing with OptiPrep® density gradient medium (final concentration, 40% (v/v)), and placed at the bottom of a 12-ml ultracentrifuge tube. A discontinuous Optiprep gradient was formed by overlaying 4 ml of 30% Optiprep and 4 ml of 5% Optiprep (prepared by dilution of OptiPrep in extraction buffer). Gradients were ultracentrifuged at 100,000 × g for 4 h in a SW41 rotor (Beckman). A distinct Triton X-100-insoluble whitish band that floated to the 5–30% interface was designated as the DRM fraction. The last 4 ml of the gradient tube were mixed and designated as the soluble fraction. The whole procedure was performed at 0-4 °C. DRM and soluble fractions were collected and subjected to electrophoresis, Western blot, and ELISA analysis.

Electrophoresis and Western Blot

Cell proteins (5 μg/lane) were separated electrophoretically on onedimensional SDS, 7.5% polyacrylamide gels under reducing conditions.

FIG. 1. Expression of Hsp70 in Caco-2 cells. Effect of increasing temperature and recovery time after heat shock. A, Caco-2 cells were exposed to increasing temperatures (40, 42, 43, 44, and 45 °C) for 30 min and allowed to recover for 6 h at 37 °C. B, Caco-2 cells were exposed to heat shock (44 °C for 30 min) and allowed to recover for increasing times at 37 °C in supplemented culture medium. Hsp70 expression was determined by electrophoresis and Western blot.

Proteins of the gels were transferred to nitrocellulose membranes (Hybond, 0.45 μm, Amersham Biosciences) and probed with antibodies against Hsps (anti-Hsp70 diluted to 1:5,000, anti-Hsc70 diluted to 1:2,500, anti-KDEL diluted to 1:2,000 followed by anti-mouse or anti-rabbit peroxidase-conjugated antibodies diluted to 1:20,000). Blots were developed using ECL detection reagents according to the protocol supplied by the manufacturer. Immunoblots were scanned with a densitometric scanner (Arcus II, Agfa, Mortsel, Belgium) and quantified using Scion Image software.

Flow Cytometry

Analysis by flow cytometry was performed in cells detached with trypsin-EDTA, fixed with 3% paraformaldehyde, and permeabilized by 0.3% saponin. Primary antibodies were added for 1 h at 4 °C diluted at 1:100 (anti-Hsp70, anti-Hsc70, anti-calreticulin, and anti-calnexin) or diluted to 1:50 (anti-KDEL) in PBS containing 1% bovine serum albumin. Secondary antibodies were added for 1 h at room temperature in the dark: fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG or fluorescein isothiocyanate-conjugated goat anti-rabbit IgG were diluted to 1:50 in PBS-bovine serum albumin. In nontpecific controls, cells were incubated with the secondary antibody alone. Fluorescence light scatters were analyzed in a BD Biosciences fluorescence-activated cell sorter for green fluorescence detected through a 525-nm filter. Data are expressed as the percentage of positive cells above nonspecific controls.

Additional Methods

ELISA—Quantitative comparisons between the expression of Hsp70 in DRM and soluble fractions of different preparations from normal and stressed cells were performed using a commercial ELISA kit as recommended by the manufacturer. This assay was also used to quantify Hsp70 expression in cell supernatants.

Proteins—Protein concentrations were determined using BCA protein assay reagent and bovine serum albumin as standard according to the manufacturer’s instructions.

Cholesterol Depletion—To deplete cholesterol cells (1–2 × 10^5) were treated with methyl-β-cyclodextrin (10 mM) and rocked for 6 h at 37 °C. To estimate cholesterol depletion cells were pretreated with 3 μCi/ml [3H]cholesterol for 18 h in complete culture medium. At the end of the incubation, aliquots of the supernatants were collected, and their radioactivity was counted by liquid scintillation (LS 6000 SC, Beckman). Cells were then washed twice in PBS and lysed for DRM preparation. The ability of methyl-β-cyclodextrin to deplete cholesterol from different fractions obtained was estimated according to their radioactivity measured by liquid scintillation.

Alkaline Phosphatase and Dipeptidyl-peptidase IV Activities—Alkaline phosphatase and dipeptidyl-peptidase IV activities were determined spectrophotometrically at 410 nm using p-nitrophenyl phosphate and glycyl-l-proline p-nitroanilide as substrate, respectively. LDH—LDH was determined spectrophotometrically at 340 nm using a commercial kit as recommended by the manufacturer.

Statistics—Data are presented as mean ± S.E. Comparisons between groups were performed using the Wilcoxon’s signed ranked test. A p value ≤0.05 was considered significant.

RESULTS

Expression of Hsps in Control and Stressed Epithelial Cells—Preliminary experiments were conducted to analyze the heat shock response in Caco-2 epithelial cells by measuring Hsp70 expression as a function of temperature and recovery time after heat shock. Heat shock response increased as a function of
temperatures (40, 42, 43, 44, and 45 °C) to reach maximal expression, without loss of viability, at 44 °C (Fig. 1A). The expression of Hsp70 was maximal by 6 h postheat shock and declined thereafter (Fig. 1B). Kinetic and dose-response experiments were also performed using flow cytometry to determine optimal effects of drugs that interfere with protein folding and trafficking by perturbing Golgi or ER functions. Maximal induction of Hsps occurred after an incubation period of 18–24 h with 25 μM monensin (that blocks the transport out of the Golgi apparatus), 15 μM brefeldin A (the fungal metabolite that blocks the anterograde transport from ER to Golgi), 25 μg/ml tunicamycin, the inhibitor of N-glycosylation, or 0.5 μM thapsigargin (the ATPase inhibitor that depletes calcium from the ER). At the end of each experiment, LDH activity was measured to test cell viability and indicated that, at the concentrations used, none of the drugs had significant cytotoxic effects (Table I). As shown in Table II, Caco-2 cells expressed significant levels of all the Hsps tested even in their resting state. Although usually restricted to cells experiencing stress, the presence of Hsp70 in unstimulated cells is not unique to Caco-2 because in humans and primates inducible Hsp70 has also been detected in normally growing cells (3). Hsp70 was the major protein induced by heat shock (44 °C for 30 min), and low induction was observed for other Hsps. Agents perturbing Golgi or ER functions such as monensin or brefeldin A elicited preferentially classical ER-resident Hsps, while the calcium-binding proteins calnexin and calreticulin were particularly sensitive to thapsigargin that depletes calcium from the ER. Exposure of epithelial cells to the glycosylation inhibitor tunicamycin also led to a specific induction of the expression of ER Hsps. Results qualitatively similar to those described by flow cytometry were observed using Western blot (data not shown).

Expression of Hsps in Membrane Microdomains of Epithelial Cells: Modulation by Heat Shock and Drugs That Interfere with Protein Traffic—As a first step toward the demonstration that Hsps may associate with epithelial cell membranes, flow cytometry experiments were performed on non-permeabilized cells. As shown in Table III, a significant amount of Hsp70 was detected in unpermeabilized Caco-2 cells that likely corresponded to a membrane staining. To further characterize this membrane association and since it has been suggested that, in monocytes, Hsp70 may associate with membrane microdomains (18), the distribution of Hsp70 in Caco-2 cells was studied on an Optiprep gradient after Triton X-100 extraction. As shown on Fig. 2A, this protocol allowed isolation of a DRM-enriched fraction (fraction 4) in which alkaline phosphatase, a glycosylphosphatidylinositol-anchored brush border protein known as a good raft marker, was strongly enriched (70.0 ± 0.9%, n = 3) and soluble fractions (fractions 8–12) in which dipeptidyl-peptidase IV, a brush border hydrolase known to be mostly excluded from rafts (19), was almost exclusively recovered (94.0 ± 0.2%, n = 3). The expression of Hsp70 was determined in this gradient by Western blot (Fig. 2B), and a significant proportion of Hsp70 (14.6 ± 5.3%, n = 3) was detected in DRM fractions of untreated Caco-2 cells. This pattern of expression was also recovered from another epithelial cell line, Madin-Darby canine kidney, indicating that Hsp70 expression in rafts is not restricted to Caco-2 cells. The effect of heat shock on the level of expression and the distribution between the DRM and soluble fractions was dramatic. Besides the expected increase in total expression of Hsp70, a clear redistribution of Hsp70 from the soluble fractions to DRMs was observed as a function of temperatures from 37 to 45 °C (Fig. 2C), indicating that Hsp70 association to membrane microdomains is modulated by stress. Note that in this and the following experiments only two fractions were routinely considered: the DRM fraction 4 located at the interface (5–30%) and the soluble fraction that corresponded to a pool of fractions 8–12.

To further characterize this phenomenon, the level of expression and membrane distribution of different Hsps were studied in resting or stressed cells (Fig. 2D). Strikingly only two Hsps, namely Hsp70 and calnexin, displayed a redistribution to DRMs upon stress conditions, whatever the nature of the stress. The other Hsps displayed an expected overexpression without any redistribution. It is important to note that no change was observed in the distribution of alkaline phosphatase and dipeptidyl-peptidase IV between DRM and soluble fractions when cells were switched from a resting to a stressed situation (data not shown). The association of Hsp70 to DRMs was further confirmed using a quantitative ELISA protocol. Data from ELISA, described in Table IV, were consistent with those obtained by Western blot: Hsp70 is an abundant protein in Caco-2 cells (∼500 and 1,600 ng/mg of protein in control and heat-shocked Caco-2 cells, respectively). In unstimulated cells, the amount of Hsp70 present on DRMs is only 17% of the total cellular content, most of which resides in the soluble fraction. Following heat shock, expression of Hsp70 was enriched in DRMs to reach 35% of the total cellular content (Table IV). We next examined whether association of Hsp70 to DRM might result in an extracellular localization of the protein.

**Table I**

| Heat shock | Memotensin | Brefeldin A | Thapsigargin |
|------------|------------|-------------|--------------|
| Control n = 6 | 44 °C, 30 min | 25 μM | 15 μM | 0.5 μM |
| LDH (n = 6) | 4.2 ± 0.7 | 4.9 ± 1.4 | 4.5 ± 0.4 | 4.7 ± 0.9 | 5.1 ± 1.5 |

**Release of Hsp70 through Lipid Rafts**

Release of Hsp70 during a 6-h incubation period was 1.7 ± 0.4% of the total Hsp70 content in resting Caco-2 cells. Heat shock induced a high increase of Hsp70 release that increased up to 14.2 ± 2.2% of the total Hsp70 content (p < 0.01, n = 10) (Fig. 3). It should be pointed out that this result cannot be ascribed to an increased cell mortality since the amount of Hsp70 was corrected for LDH release (results are expressed as percentage of Hsp70 released — percentage of LDH released). Addition of the three drugs used to perturb protein traffic and secretion (namely monensin, brefeldin A, or tunicamycin) did not significantly affect Hsp70 secretion from resting or heat-shocked Caco-2 cells, a result expected from the above data indicating that these drugs did not elicit any significant up-regulation of Hsp70 (Fig. 3). However, it is important to note that, although these drugs strongly interfere with the classical exocytic pathway, they did not reduce Hsp70 release, demonstrating that Hsp70 is released through a pathway independent of the classical secretory route.

Whether Hsp70 release may be related to its association with lipid raft was finally studied. Lipid rafts result from a preferential packing of cholesterol and sphingolipids onto which specific proteins attach. Cholesterol has generally been considered as the major lipid molecule for maintaining raft stability, and together with sphingolipids its concentration is highly enriched...
in DRMs. Methyl-β-cyclodextrin was used to disturb raft organization by specifically removing cholesterol (20). We then compared both the association of Hsp70 to DRMs and the release of the protein in the absence and in the presence of methyl-β-cyclodextrin. To confirm the efficiency of methyl-β-cyclodextrin in cholesterol withdrawal, Caco-2 cells were incubated with [3H]cholesterol, and radioactivity was measured before and after methyl-β-cyclodextrin treatment in different fractions. After 24 h of incubation, 73.6 ± 2.5% (n = 6) of [3H]cholesterol was incorporated in Caco-2 cells, and 6 h of incubation with 10 mM methyl-β-cyclodextrin resulted in 58.03 ± 7.8% cholesterol removal from cell lysates. DRMs were then isolated from normal and cholesterol-deficient cell lysates and submitted to immunoblot analysis. Our data revealed that after partial cholesterol removal by methyl-β-cyclodextrin, Hsp70 expression was significantly decreased in supernatants from cholesterol-depleted cells (Fig. 4A) suggesting that released Hsp70 localizes preferentially in lipid rafts whose integrity is required for efficient release of the protein. Accordingly we observed a redistribution of Hsp70 expression from the DRM to the soluble fraction (Fig. 4B).

**DISCUSSION**

A primary finding of this report is that Hsps (Hsp70 in particular) exist in two forms within epithelial cells: a major Triton X-100-soluble form and a minor detergent-insoluble form associated to DRMs. The association of Hsps to DRMs appears to be physiologically modulated. Thus, following heat shock, increased amounts of Hsp70 become incorporated into DRMs as compared with unstimulated cells. Drugs that perturb ER-Golgi protein traffic such as monensin and brefeldin A do not prevent incorporation of Hsps into DRMs. Indeed preferential partitioning of Hsps into DRMs is observed with drugs that interfere with calcium homeostasis and protein traffic. Furthermore we report here that Hsp70 is released from Caco-2 cells via a pathway that presumably does not follow the classical route involving vesicular trafficking through the ER-Golgi compartments. Instead Hsp70 release was affected by the raft-disrupting drug methyl-β-cyclodextrin suggesting that protein release occurs through an atypical pathway involving lipid rafts. Treatment with methyl-β-cyclodextrin affected both the release and the recruitment of Hsp70.

**Expression of Hsps in DRM and soluble fractions of Caco-2 cells: modulation by heat shock and drugs interfering with traffic**

| Treatment          | Control | Heat shock | Monensin | Brefeldin A | Tunicamycin | Thapsigargin |
|--------------------|---------|------------|----------|-------------|-------------|--------------|
| Hsp70              | 3.3 ± 0.8 | 52.5 ± 4.1  | 6.8 ± 2.8  | 6.9 ± 1.9  | 5.4 ± 1.0  | 2.8 ± 1.1 |
| Hsc70              | 2.0 ± 0.1 | 2.5 ± 0.6   | 3.8 ± 0.8  | 4.0 ± 1.0  | 5.0 ± 1.8  | 1.5 ± 0.3  |
| Grp78              | 1.3 ± 0.5 | 3.7 ± 1.8   | 5.3 ± 0.9  | 7.4 ± 0.8b | 11.6 ± 3.8b| 10.6 ± 2.5b|
| Calnexin           | 1.7 ± 0.4 | 4.7 ± 2.9   | 6.7 ± 1.4b | 6.8 ± 1.6b | 7.7 ± 1.8b | 10.6 ± 2.6b|
| Calreticulin       | 3.2 ± 0.7 | 4.3 ± 2.1   | 4.9 ± 0.4  | 6.8 ± 1.6  | 5.8 ± 1.6  | 12.9 ± 3.4 |

*p < 0.01 versus control.

**Expression of Hsp70 in permeabilized and non-permeabilized Caco-2 cells**

| Treatment          | Permeabilized | Non-permeabilized |
|--------------------|---------------|-------------------|
| Hsp70              | 3.3 ± 0.8     | 1.0 ± 0.87        |
| Heat shock         | 52.5 ± 4.1    | 15.6 ± 1.8        |

**TABLE II**

**TABLE III**

**Fig. 2.** Expression of Hsps in DRM and soluble fractions of Caco-2 cells: modulation by heat shock and drugs interfering with protein traffic. A, different fractions obtained from the Optiprep gradient were characterized using two enzyme markers, alkaline phosphatase (AP), a raft marker (open bars), and dipeptidyl-peptidase IV (DPP IV), a protein known to be excluded from rafts (dark bars). The curve illustrates the protein content corresponding to each fraction. B, Hsp70 expression was measured in 12 different fractions recovered from the top to the bottom of the Optiprep gradient. Experiments were performed in Caco-2 and Madin-Darby canine kidney (MDCK) cell lines. C, Hsp70 expression was determined in DRM (D) and soluble (S) fractions prepared from Caco-2 cells exposed to increasing temperatures (37, 40, 42, 43, 44, and 45 °C). Data are representative from three different experiments. D, Caco-2 cells were left untreated (control) or exposed to heat shock (HS) (44 °C for 30 min followed by 6 h of recovery) or treated for 18 h in supplemented culture medium with 25 μM monensin (Mon), 25 μg/ml tunicamycin (Tun), or 0.5 μM thapsigargin (Thaps). DRM (D) and soluble (S) fractions were prepared as detailed under “Experimental Procedures” and subjected to electrophoresis and Western blot. Data are representative from four independent experiments.
**Fig. 3. Release of Hsp70 in epithelial Caco-2 cells.** Hsp70 accumulated during 6 h of incubation was measured by ELISA in cells, and supernatants from untreated or heat-shocked cells were cultured alone (open bars) or in the presence of monensin (25 μM) (black bars), brefeldin A (15 μM) (hatched bars), or tunicamycin (25 μg/ml) (dotted bars). Results are the mean ± S.E. from 3–10 separate experiments and are expressed as percentage of release from the total Hsp70 content. Values were corrected for LDH release.

**Fig. 4. Effect of methyl-β-cyclodextrin on DRMs and to release of Hsp70 in Caco-2 cells.** A, control or heat-shocked cells were left untreated (open bars) or exposed to methyl-β-cyclodextrin (10 mM, 6 h) (black bars). Hsp70 expression was determined in cells and supernatants by ELISA. Results are the mean ± S.E. from 5–10 separate experiments and are expressed as percentage of release from the total Hsp70 content. Values were corrected for LDH release. **, p < 0.01 versus untreated cells. B, control or heat-shocked (HS) cells were left untreated or pretreated with methyl-β-cyclodextrin (CD) (10 mM, 6 h) to deplete cholesterol. DRM (D) and soluble (S) fractions were then prepared as detailed under “Experimental Procedures” and subjected to electrophoresis and Western blot. Quantitative densitometry of the immunoblot illustrates the redistribution of Hsp70 expression from DRM to soluble fractions. Data are expressed as the DRM/total expression ratio in untreated cells (white bars) and in cells treated with methyl-β-cyclodextrin (black bars). One experiment representative from four is shown.

**TABLE IV**

| Expression of Hsp70 in DRM and soluble fractions of Caco-2 cells | Homogenate | DRM | Soluble |
|---------------------------------------------------------------|-----------|-----|---------|
| Expression of Hsp70 was determined by ELISA. Results are expressed as mean ± S.E. from four separate experiments. |           |     |         |
| Control | 459.4 ± 23.7 | 63.3 ± 14.3 | 320.5 ± 17.7 |
| Heat shock | 1578.9 ± 270.1 | 459.3 ± 65.6 | 869.4 ± 230.7 |

**An interesting aspect of the externalization of Hsp70 is the consequence for immune responses. Hsps are among the dominant antigens recognized by the immune system and play an important role in the immune response against cancer (7, 12, 13). Hsp70 released from cells may allow the transfer of antigenic peptides to antigen-presenting cells or may enhance the ability of intact cells to process and present endogenous tumor antigens to specific T cells (24). Consistent with this hypothesis, major histocompatibility complex class II molecules are found to be concentrated in lipid rafts whose integrity appeared to be important to allow efficient antigen presentation in antigen-presenting cells (25). Our data indicate that expression of Hsp70 in DRM is not restricted to tumor cells since both Caco-2 and the non-tumoral cell line Madin-Darby canine kidney exhibit a similar expression pattern. Indeed raft microdomains appear to be a ubiquitous feature of mammalian cells that likely allow small fractions of Hsps to be transported to DRM, suggesting that the drug altered protein-raft interactions. Consistent with our data on Hsp70 release, this protein was previously shown to be released by cultured rat embryo cells by a mechanism other than the classical secretory pathway (10). In addition, Hsp70 has been described in the circulation, and its levels were increased in pathological conditions related to inflammation (11). Our findings are also consistent with previous studies indicating that Hsp70 interacts with fatty acid chains of membrane lipids (21, 22). This interaction is likely to occur within lipid rafts and may be related to the unknown function of the previously described Hsp70-lipid association. Hsp70-lipid affinity represents an alternative explanation for the effect of methyl-β-cyclodextrin since this drug may interfere with the association of the protein to membrane lipids, thereby preventing Hsp70-lipid raft association. On the basis of our findings, lipid rafts can be considered as plausible platforms by which Hsps reach the cell membrane and are externalized by cells. This mechanism is likely not restricted to Hsps and indeed has already been suggested for other cytosolic proteins lacking signal sequence such as galectin 4, a member of the β-galactoside-binding mammalian lectins (23).**
evidence for extracellular functions of Hsp70 and provide new elements that may explain how Hsp70 escapes the intracellular compartment. The presence of Hsp70 in lipid rafts may also allow physical proximity between Hsp70 and CD14 (a glycosphosphatidylinositol-anchored protein known to be present in lipid rafts), thereby facilitating their interaction as recently suggested (18). Our data also suggest that cell membrane expression and related functions of Hsp70 may not be restricted to innate immune cells (monocytes and macrophages) and may be extended to epithelial cells and to other molecular chaperones such as ER-resident Hsps. In agreement, cell surface expression of molecular chaperones other than Hsp70 (ER-resident in particular) has already been described (5, 26).

In conclusion, here we have generated three lines of evidence suggesting a raft-based mechanism in the targeting of Hsps (Hsp70 in particular) to the extracellular space of epithelial cells: first, the presence of Hsps in DRMs; second, the lack of an inhibitory effect of drugs affecting the classical ER-Golgi secretory route; and third, observations revealing a methyl-inhibitory effect of drugs affecting the classical ER-Golgi secretory route. The presence of Hsp70 in lipid rafts may also allow physical proximity between Hsp70 and CD14 (a glycosphosphatidylinositol-anchored protein known to be present in lipid rafts), thereby facilitating their interaction as recently suggested (18). Our data also suggest that cell membrane expression and related functions of Hsp70 may not be restricted to innate immune cells (monocytes and macrophages) and may be extended to epithelial cells and to other molecular chaperones such as ER-resident Hsps. In agreement, cell surface expression of molecular chaperones other than Hsp70 (ER-resident in particular) has already been described (5, 26).

Acknowledgments—We are grateful to Michelyne Breton for valuable help and advice in DRM preparation. We also thank Serge Chwetzoff and Christelle Lenoir for helpful advice and Michel Kornprobst for assistance in flow cytometric analysis.

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J. Biol. Chem. 2003, 278:21601-21606.
doi: 10.1074/jbc.M302326200 originally published online April 7, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302326200

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