# Hsp-70 is closely associated with the transferrin receptor in exosomes from maturing reticulocytes

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The presence of the heat shock protein (hsp-70) has been detected in exocytosed vesicles (which are named exosomes) from mammalian and avian immature red cells (i.e. reticulocytes) as well as from a differentiating avian erythroleukaemic cell line. The close, but non-covalent, association of hsp-70 with the transferrin receptor (TFR) in exosomes is demonstrated by: (1) the ability to cross-link hsp-70 to TFR; (2) the co-immunoprecipitation of hsp-70 and TFR with an antibody against TFR, and the co-immunoprecipitation of TFR and hsp-70 with antibody against hsp-70; and (3) the retention of TFR by hsp-70 bound to ATP–agarose and the simultaneous elution of both proteins by excess ATP. Semi-quantitative analysis of the relative efficiency of cross-linking of these proteins in exosomes versus plasma membranes shows that TFR in exosomes is preferentially bound to hsp-70. From an analysis of the relative amounts of hsp-70 and TFR regenerated from the cross-linked complex, the ratio of TFR monomer bound to hsp-70 is approximately 1.5 to 1. Given the presence of hsp-70 in exosomes from several species and its close association with TFR (the major protein lost during reticulocyte maturation) it is proposed that hsp-70 plays a role in exosome formation and/or release in immature red cells.

## INTRODUCTION

The postulated role of the highly conserved and ubiquitously found hsp-70 'heat shock' or 'stress proteins' [1,2] has now extended beyond the original proposal that this class of proteins offers protection against, and recovery from, stress-induced denaturation of proteins. It is now evident that this class of proteins plays many roles, from (a) targeting (damaged or abnormal) proteins for degradation [3,4], (b) directing protein-folding [5], and (c) maintaining an unfolded protein structure, competent for translocation across cellular membranes [6,7] to (d) preventing premature association of the monomers of a multimeric protein [8,9].

One of the first identified actions of the constitutive hsp-70 proteins was associated with removal of clathrin from coated vesicles [10,11]. However, the amount of hsp-70 found in some cells (i.e. in red cells) is far greater than the amount of clathrin, suggesting that other functions for this protein are likely. Recent work has borne out this supposition.

In studies on formation of exosomes (particles of approx. 50 nm containing phospholipids and membrane proteins) during sheep reticulocyte maturation, we were struck by the characteristic and selective protein content of the exosomes [12] compared with that of the plasma membrane. In contrast, the phospholipid composition of sheep exosomes was very similar to that of the sheep red cell plasma membrane [13] with relatively high sphingomyelin content compared with phosphatidylcholine in both preparations.

Of the myriad peptides in, or associated with, maturing red cell membranes, two peptides form the majority ($\geqslant 50\%$) of the exosomal protein content. One of these proteins, the transferrin receptor (TFR), is evident in sheep reticulocyte membranes, but is absent from mature cells [12]. The other protein, the hsp-70 'heat shock' cognate protein, although not a membrane protein, is the second major protein in exosomes and is identical to the red cell clathrin-uncoating ATPase [14]. The dual presence of TFR and hsp-70 in the exosomes is unlikely to be a fortuitous event since exosomes from three other species of reticulocytes (rat, pig and chicken) also contain both TFR and hsp-70.

It is known that hsp-70 binding to its targets is associated with stretches of amino acids in the ligated protein which are in non-native conformations [15,16]. Therefore we considered the possibility that during red cell maturation, membrane proteins targeted for externalization may become altered, exposing normally hidden domains and thereby forming a close association with hsp-70. Such a complex could then act as a signal to direct the protein in question for externalization. If this proposal is valid, the targeted protein and hsp-70 in the released exosomes might be expected to be in close proximity, permitting cross-linkage of the two proteins. Moreover, if closely associated, and both specifically and tightly bound, the two proteins might undergo co-immunoprecipitation, even without cross-linkage, with antibodies directed against either component. The experiments that follow provide evidence consistent with our predictions using the TFR as the protein targeted to exosomes.

## MATERIALS AND METHODS

### Reticulocyte and exosome preparation

Reticulocytes were obtained from phlebotomized sheep [17,18] and plasma membranes were prepared as described [18]. To obtain exosomes, the reticulocytes were cultured *in vitro* overnight and the exosomes recovered from the cell-free culture medium by high-speed centrifugation [13]. Triton X-100 (TX-100) extracts of membranes and exosomes were prepared [13] and the clear supernatants used for most of the experiments described below.

Unless otherwise indicated the experiments outlined below were executed a minimum of two times, the majority three times and yielded similar results. Representative experiments are shown.

Abbreviations used: hsp, heat shock protein; TFR, transferrin receptor; $TFR_{CD}$, cytoplasmic domain of transferrin receptor; DMSO, dimethyl sulphoxide; TX-100, Triton X-100; PVDF, polyvinylidene difluoride; ME, 2-mercaptoethanol; DSP, dithiobis(succinimidylpropionate).

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### Cross-linking experiments

The cleavable cross-linking agent dithiobis(succinimidyl-propionate) (DSP), which reacts with the epsilon amino group on lysine residues, was used. DSP, dissolved in 100% dimethyl sulphoxide (DMSO), was added to give a final concentration of 1.11 mM DSP in 2% (v/v) DMSO. Cross-linking was carried out at room temperature for up to 1 h. L-Lysine (50 mM) was added to stop the reaction. Control samples had lysine present prior to addition of DSP [19] to prevent cross-linking of the proteins. In some experiments, albumin in excess was included in the cross-linking reaction mixture to detect any non-specific linking and to assess whether the specific interaction could be abolished. When used, the ponderal excess of albumin added (in micrograms) was six times more than the total protein content of the exosomes, of which TFR and hsp-70 account for approx. 50% of the total protein. After cross-linking, all samples were prepared for SDS/PAGE by addition of SDS-sample buffer (2% SDS, 10% glycerol, 0.0675 M Tris/HCl, pH 6.8, without reducing agents), and incubation at 37 °C for 30 min. To cleave the cross-linked derivative and regenerate the original proteins, SDS-sample buffer containing 10% 2-mercaptoethanol (SDS-ME) was used. The samples were heated at 95 °C for 5 min prior to electrophoresis. SDS/PAGE was carried out according to the procedure of Laemmli [20]. The gels were either stained (with Coomassie Blue or silver) or the proteins were transferred to nitrocellulose for immunodetection of hsp-70 or TFR. Hsp-70, once cross-linked, was not detectable immunologically with anti-hsp-70 antibodies. Therefore, loss of this immunoreactivity can serve as a guide to the fraction of hsp-70 in the sample undergoing cross-linkage. TFR can be detected in the cross-linked complex with an antibody to a stretch of the cytoplasmic domain peptide of TFR. However, the cross-linked TFR, unlike native TFR, can no longer be immunoprecipitated with a monoclonal anti-TFR antibody to the extracellular domain of TFR.

To obtain the cross-linked complexes for further study and regeneration of the original peptides, duplicate gels of the cross-linked samples were run. One set was immunoblotted with an antibody against the cytoplasmic domain of the TFR (anti-$TFR_{CD}$) to locate the cross-linked TFR complex. The duplicate gel was stained with Coomassie Blue. The cross-linked complex was excised from the stained gel using the blot as an overlay to detect the boundaries of the peptide in question. The proteins were electroeluted using a Bio-Rad electroelution unit according to the manufacturer's instructions. The electroeluted complex was then reduced using SDS-ME, the constituent proteins were separated by a second electrophoresis step and visualized by silver staining or sequential immunoblotting with both anti-TFR and anti-hsp-70 antibodies.

### Calculation of the ratio of hsp-70:TFR in the exosomes

The ratio of hsp-70 to TFR in exosomes was measured in three ways, as described below.

(1) The cross-linked complex electroeluted from the gel was rerun on SDS/PAGE under reducing conditions to regenerate free hsp-70 and TFR. After silver staining the gels, the individual bands were quantified by laser densitometry and the ratio of the arbitrary absorbance ($A$) units was obtained. It was assumed that the absorbance was proportional to the amount of protein since the molecular masses of the proteins are roughly of the same order.

(2) The reduced peptides regenerated from the complex (as above) were electroblotted on to polyvinylidene difluoride (PVDF) membranes and the relative amounts of each peptide estimated (see below) by quantitative amino acid analysis after making appropriate allowance for the carbohydrate content of TFR.

The PVDF membrane was stained with Coomassie Blue to locate the two bands. The bands of TFR and hsp-70 were excised from the PVDF membrane and washed thoroughly in distilled water. Each band, after cutting into 1 mm × 2 mm pieces, was transferred to clean (previously pyrolysed) acid hydrolysis tubes, and dried under reduced pressure in preparation for acid hydrolysis. Hydrolysis was at 150 °C for approx. 2 h employing a Pico-Tag Work Station, as recommended by the manufacturer. Aliquots of 6 M HCl plus phenol (50 $\mu$l) were added to each hydrolysis tube. After hydrolysis and cooling to room temperature, the tubes were placed in a Savant speed vacuum centrifuge and the contents were taken to dryness. The contents of each hydrolysis tube were suspended in sample loading buffer (Beckman: pH 2.2, sodium citrate) containing norleucine, allowed to wet thoroughly, vortexed and finally centrifuged prior to removing an aliquot for analysis. Amino acid analysis was performed on a Beckman Model 6300 amino acid analyser (ion-exchange chromatography/ninhydrin detection), according to the manufacturer's recommendations. Sets of amino acid standards were also analysed. The picomolar quantities, and hence picograms, of each amino acid were estimated, for TFR and hsp-70 individually, by comparison with standards. The sum of the masses of individual amino acids gave an estimated total mass of unglycosylated protein. From this, and the known molecular mass of each protein, the pmol quantity of each protein could be derived.

(3) Coomassie Blue-stained gels of native exosomes were scanned and the arbitrary absorbance units of TFR and hsp-70 compared.

### Association of hsp-70 with immobilized TFR

In addition to the standard immunoprecipitation procedure used routinely for the TFR [21], the protocol as described by Smith et al. [22] was used to assess whether TFR association with hsp-70 showed temperature dependence as described for the progesterone receptor. To immobilize TFR, Protein A–Sepharose was incubated in 100 mM potassium phosphate, pH 8.0, with anti-TFR (1 mg of IgG per ml of packed gel) for 30–60 min at room temperature. The gel was washed twice in the potassium phosphate buffer and finally resuspended 1:1 (v:v) in TG buffer [10 mM Tris/HCl, pH 7.6, 50 mM NaCl and 10% (w/v) glycerol]. Of this slurry 30 $\mu$l was used per sample. Extracts of reticulocyte membranes or exosomes, solubilized in 20 mM sodium phosphate buffer with 0.5% TX-100, were added to the Protein A–Sepharose. EDTA-TG buffer (TG buffer with 4 mM EDTA) equivalent to one-third of sample volume was added, followed by incubation on ice for 1 h, with gentle resuspension at 5 min intervals. The Sepharose pellet, containing the bound TFR, was then washed three times with EDTA-TG buffer to remove any non-specifically adhering proteins (including endogenous hsp-70), followed by two washes with 20 mM potassium Hepes buffer, pH 7.4. Centrifugations of 15 s duration in a microfuge were used to wash the Sepharose. To determine whether exogenous hsp-70 would associate with immobilized TFR, a membrane-free lysate formed from a 1:5 dilution of red cells was applied to the gel. The lysate was prepared in 5 mM phosphate buffer, pH 7.4, containing 0.1 mM PMSF. Prior to use, $MgCl_2$ and $CaCl_2$ at 2 mM and KCl and urea at 67 mM were added. Urea was included to decrease the level of non-specific hsp-70 association with the Sepharose. The samples were then incubated at 30 °C or 4 °C for 30 min with gentle vortexing every

5 min, followed by four quick washes with TG buffer. The retained proteins were eluted off the beads with SDS-ME and used for gel electrophoresis.

### Co-immunoprecipitation of TFR with anti-hsp-70

A monoclonal antibody SPA-820 (StressGen Biotechnologies Corp.), to human hsp-73 (constitutive) and hsp-72 (inducible), was utilized to immunoprecipitate hsp-72/73 from exosome extracts. Antibody SPA-820 bound to Protein A–Sepharose was incubated overnight at 4 °C with exosome extracts. For non-specific immunoprecipitation, non-immune mouse IgG was used as control. After overnight incubation, the Protein A–Sepharose pellet was washed with PBS and the bound proteins eluted with SDS-ME for gel electrophoresis. The electrophoresed proteins were transferred to nitrocellulose for immunoblotting using SPA-820, or anti-TFR antibody.

### Association of TFR with hsp-70 immobilized on ATP–agarose

ATP–agarose gel was swollen and washed in 20 mM PBS containing 2 mM $MgCl_2$ (PBS/$MgCl_2$) and resuspended in the same buffer to provide a 2 % (w/v) suspension of beads. Aliquots of 50 $\mu$l of the agarose were used per sample. Exosome extracts containing 2 mM $MgCl_2$ were added to the ATP–agarose and stirred gently on ice for 15 min. In duplicate samples, ATP (20 mM) was added to compete with the immobilized ATP for binding to hsp-70. To prepare the samples for SDS/PAGE the agarose was washed three times in PBS/$MgCl_2$ followed by eluting the residual bound proteins with SDS-ME and heating at 95 °C for 5 min.

Millipore Bioimage System scanning of immunoblots and protein-stained gels was carried out according to the manufacturer's instructions.

### Materials

Eagle's minimum essential medium, glutamine, penicillin/streptomycin and non-essential amino acids, all used for culturing reticulocytes, were purchased from Gibco, Canada. The cross-linking agent DSP was from Pierce Chemicals, Rockford, IL, U.S.A. Protein A–Sepharose CL-4B was from Pharmacia LKB, Uppsala, Sweden. DMSO was from Anachemia, Montreal, Quebec. TX-100 was from Fisher Scientific. ATP–agarose, inosine, adenosine, BSA, aprotinin and PMSF were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Hepes (free acid) was obtained from Calbiochem, La Jolla, CA, U.S.A. EDTA and Tris base were from Boehringer Mannheim Corp., Indianapolis, IN, U.S.A.

#### Antibodies

Polyclonal antiserum to sheep red cell hsp-70 was raised in a rabbit using hsp-70 protein purified from sheep red cell lysates according to the procedure described in [23] except that the Sepharose in the procedure was replaced by Sephacryl S-400 from Pharmacia LKB, Uppsala, Sweden. Monoclonal mouse anti-(human hsp 72/73) (SPA-820) was purchased from StressGen Biotechnologies Corp., Victoria, B.C. Monoclonal mouse anti-(human TFR), which recognizes the extracellular domain of the TFR, was used for immunoprecipitation [24]. The antisera to detect the cytoplasmic domain of TFR were kindly provided by Dr. I. Trowbridge (Salk Institute, La Jolla, CA, U.S.A.) [25] and by Dr. T. Yoshimori, Osaka University, Osaka, Japan [26]. Non-immune mouse IgG was purchased from Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.

## RESULTS

### Cross-linking of TFR to hsp-70 in TX-100 extracts of exosomes

An examination of the protein content of sheep exosomes shows the presence of two major proteins, the TFR and the hsp-70 [14,27]. These data are duplicated in Figure 1(a) (lane 2). Immunoprecipitation of TFR at 4 °C with a monoclonal antibody against the extracellular domain of the TFR, shows that TFR is not covalently linked to hsp-70 (Figure 1a, lane 1) since no hsp-70 is found in this immunoprecipitate [13]. The same result is obtained whether reducing or non-reducing gels are used. Figure 1(a) shows a reducing gel where TFR runs as an approx. 94 kDa peptide (monomer).

That hsp-70 is closely associated with the TFR in exosomes is shown by the fact that in TX-100-treated extracts of exosomes, the two proteins may be cross-linked using DSP as a cross-linking agent. To detect the cross-linked product, non-reducing SDS/PAGE was carried out. As seen in the Coomassie Blue-stained gel [Figure 1b, left lane (without lysine)], treatment with DSP results in the formation of new peptide and the disappearance of the ~ 190 kDa (TFR dimer) and 70 kDa peptides (hsp-70). If the cross-linking is carried out in the presence of excess lysine, which scavenges the DSP and hence prevents the cross-linking, peptides at ~ 190 kDa and 70 kDa remain clearly evident [Figure 1b, right lane (with lysine)]. Note that unlike TFR, the mobility of hsp-70 is the same on reducing and non-reducing gels. Although only the data on TX-100-solubilized exosomes are shown here, identical results were obtained when intact exosomes were cross-linked.

That the ~ 240 kDa peptide formed upon cross-linking contains the TFR is shown by the immunoblot in Figure 1(c) [left lane (without $\beta$ME)] using anti-$TFR_{CD}$ antibody. Moreover, addition of ME after cross-linking results in the reappearance of free TFR, both dimer (~ 190 kDa) and monomer (~ 94 kDa), [Figure 1c, right lane (with ME)]. We have frequently observed some TFR dimer even when processed by SDS/PAGE under reducing conditions [28].

Although we attempted to blot the cross-linked product with anti-hsp-70 antibody, no reactivity was detected, suggesting that cross-linkage results in a loss of immunoreactivity. However, hsp-70 can be detected after reduction of the complex. The results in Figure 2(a) (lane 1) show a Coomassie Blue-stained non-reducing gel of the cross-linked product. In lane 3 in Figure 2(a), the same sample is shown after treatment with ME prior to electrophoresis (only the monomer of TFR is detected in this sample). If the high-molecular-mass complexes (~ 240 kDa) are electroeluted from the gel and the samples rerun on a reducing SDS gel and silver stained, again two peptides of the appropriate size (~ 94 and 70 kDa) reappear (Figure 2b, lanes 1 and 2). That the peptides regenerated are TFR and hsp-70 is shown in Figures 2(c) and 2(d), in which the immunoblots corresponding to the stained peptides in Figure 2(b) were probed with anti-$TFR_{CD}$ (Figure 2c) and anti-hsp-70 (Figure 2d). Figure 2(c) shows the monomer of TFR with a small amount of TFR dimer (top bands). Thus the data show that the cross-linked high-molecular-mass complex contains both TFR and hsp-70 and that the latter peptides are regenerated upon reduction of the cross-linked product.

The possibility was considered that cross-linking of these two proteins was fortuitous and without physiological significance since these two proteins are the major proteins in the exosomes. For this reason the cross-linking experiments were also carried out with excess albumin. The samples shown in Figure 2(a) (lanes 2 and 4; and lane 2 in Figures 2b, 2c and 2d) contained excess albumin. Except for the presence of additional contaminating

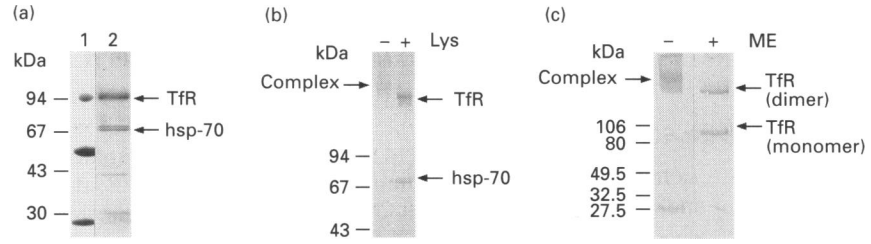


**Figure 1 Cross-linking of TFR to hsp-70 in sheep exosomes and regeneration of individual peptides with reduction**

(**a**) Coomassie Blue-stained gels showing: lane 1, immunoprecipitate of TFR from exosome extracts; lane 2, total exosome protein content. (**b**) Coomassie Blue-stained gel of cross-linked exosome extract: lane 1, non-reducing SDS gel; lane 2, non-reducing SDS gel with 50 mM lysine (control, non-cross-linked sample). (**c**) Anti-TFR immunoblot of cross-linked exosome extract (lane 1) and of a duplicate sample (lane 2) which has been treated with 2-mercaptoethanol (ME) to recover free TFR.

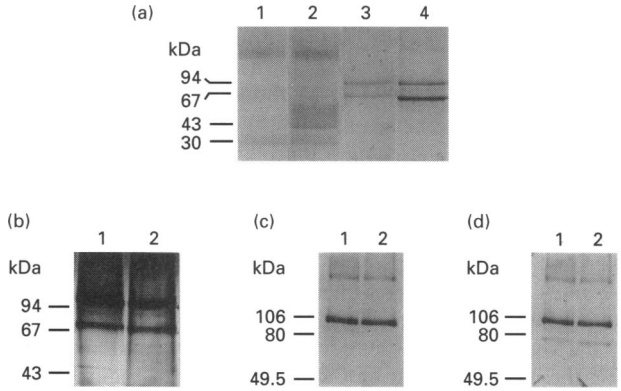


**Figure 2 Specificity of cross-linkage of TFR and hsp-70**

(**a**) Coomassie Blue-stained gel showing cross-linkage of exosome extracts carried out in the absence (lane 1) or presence (lane 2) of excess albumin. Effect of reduction with ME (lanes 3 and 4, minus and plus excess albumin respectively). (**b**) Silver-stained gel of peptides recovered after electroelution and reduction of ~ 240 kDa cross-linked complex formed in the absence (lane 1) or presence (lane 2) of excess albumin. (**c**) Same as (**b**) except immunoblotted for TFR with anti-$TFR_{CD}$–albumin. (**d**) Immunoblot from (**c**) reprobed with anti-hsp-70.

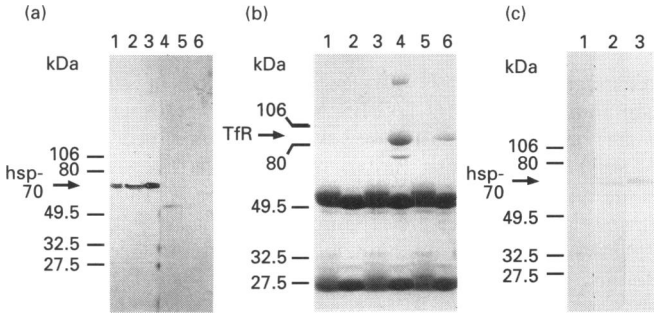


**Figure 3 Presence of hsp-70 in mature red cell membranes**

(**a**) Membranes from mature sheep red cells, from reticulocytes and exosomes were subjected to reducing SDS/PAGE, transferred to nylon membranes and immunoblotted with anti-hsp-70 antibody (lanes 1 to 3) or preimmune serum (lanes 4 to 6). Membranes from the same volume of packed cells were used for the mature cells and reticulocytes. Lanes 1 and 4, mature cells; lanes 2 and 5, reticulocytes; lanes 3 and 6, exosomes. (**b**) Absence of TFR in mature red cells. Coomassie Blue stains of immune and non-immune precipitates of the TFR are shown using a mouse monoclonal antibody against the external domain of TFR. The non-immune IgG came from mouse serum. Lanes 1 and 2, non-immune and immune precipitates respectively from mature cell membranes; lanes 3 and 4, as lanes 1 and 2 with reticulocyte membranes; lanes 5 and 6, as lanes 1 and 2 with exosomes. (**c**) TFR is required to form a high-molecular-mass complex with hsp-70. Membranes from mature cells, reticulocytes and exosomes were cross-linked as detailed in the Materials and methods section and the samples electrophoresed on non-reducing SDS gels. The band at 240 kDa (detected by Coomassie Blue staining and with anti-$TFR_{CD}$) was excised, eluted and rerun on a reducing SDS gel. The proteins were transferred to nylon membranes and blotted with anti-hsp-70. In contrast to reticulocytes and exosomes, with mature cell membranes no band was visible at 240 kDa, but an area corresponding to that molecular mass was excised and processed. Lane 1, mature membranes; lane 2, reticulocyte membranes; lane 3, exosome membranes.

peptides from albumin in lane 2 of Figure 2(a), the presence of albumin in excess does not alter the cross-linked product nor the peptides that reappear after reduction. Albumin migrates anomalously when cross-linked. Whether the cross-linkage is done with albumin alone (not shown) or in the presence of TX-100-treated extracts of exosomes (as in Figure 2a, lane 2) albumin migrates as a broad band, more characteristic of a protein of 40–50 kDa. Upon reduction of the cross-linked product, the presence of a peptide migrating at ~ 68 kDa characteristic of albumin is regenerated (Figure 2a, compare lanes 3 and 4). Moreover, the data in Figure 2(c) demonstrate that there was no albumin in the high-molecular-mass cross-linked complex. The anti-$TFR_{CD}$ used in Figure 2(c) was made against an albumin derivative of the cytoplasmic domain peptide of TFR [26] and it recognizes albumin (results not shown). Had albumin been present in the conjugate, a peptide of ~ 68 kDa would reappear. Clearly no such additional peptide is evident, both lanes 1 and 2 in Figure 2(c) showing identical patterns.

The possibility of homocross-linkage of TFR or hsp-70 was eliminated for the following reasons. A cross-linked dimer of TFR would give a complex of ~ 190 kDa, a cross-linked tetramer (a dimer of dimers) would be > 350 kDa. Complexes of these sizes were not detected (Figure 1b). A cross-linked dimer of hsp-70 would yield a complex of ~ 140 kDa. No peptides of this size were detected after cross-linking.

If the formation of homodimers of hsp-70 (~ 140 kDa), trimers (~ 210 kDa) or other higher-order homocomplexes of hsp-70 were to occur, their presence should become evident if the cross-linking were carried out in TFR-free exosome extracts. Immunoprecipitation of TX-100-solubilized exosome extracts with anti-TFR at 4 °C in absence of bivalent cations [under conditions where there is no concomitant co-precipitation of hsp-70 (see Figure 1a, lane 1)] followed by cross-linking of the residual extract failed to show the formation of complexes of 140 kDa or larger (results not shown). Moreover, mature erythrocyte plasma membranes contain hsp-70 (Figure 3a), but no TFR (Figure 3b). Reticulocyte membranes and exosomes contain both TFR and hsp-70. After cross-linking the membrane extracts from all three preparations followed by electroelution of

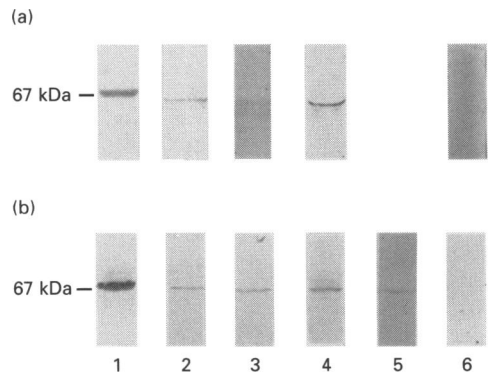


**Figure 4 Detection of hsp-70 in exosomes from different species**

Exosomes from maturing sheep reticulocytes (lane 1), rat reticulocytes (lane 2), embryonic chicken red cells (lane 3) and a chicken erythroleukaemic cell line (HD3) (lane 4) and exosomes isolated from the peripheral circulation of anaemic young piglets (lane 5), were probed with polyclonal rabbit anti-(sheep hsp-70) (**a**), or monoclonal mouse anti-(human hsp-72/73) (SPA-820) antibodies (**b**). Representative blots with pre-immune sera are shown in lane 6 in (**a**) and (**b**).

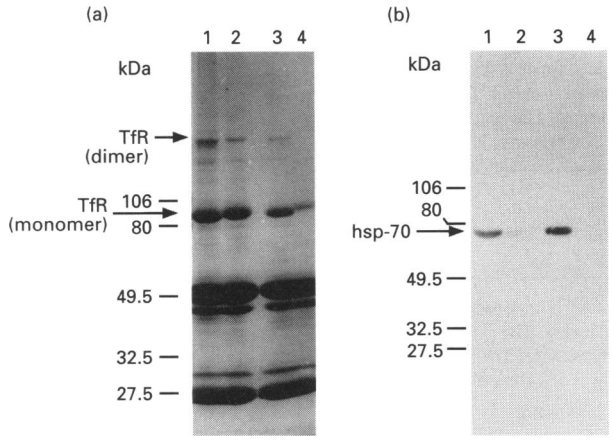


**Figure 5 Hsp-70 and TFR are preferentially cross-linked in exosome membranes**

(**a**) Exosomes and reticulocyte membranes were cross-linked with DSP in the presence and absence of excess lysine (the samples with excess lysine are not cross-linked and therefore give a measure of the total TFR or hsp-70 in the sample). After reacting with DSP, a monoclonal anti-TFR antibody against the external domain was used to immunoprecipitate the unreacted TFR to assess the fraction of free TFR remaining. In the cross-linked sample this antibody does not recognize TFR. The Coomassie Blue-stained gels were scanned with a Bioimager. Lane 1, reticulocyte membranes (not cross-linked, excess lysine); lane 2, reticulocyte membranes (cross-linked); lane 3, exosomes (not cross-linked, excess lysine); lane 4, exosomes (cross-linked). (**b**) As in (**a**) except that after cross-linking the samples were subjected to SDS/PAGE under non-reducing conditions, transferred to nylon membranes and immunoblotted with anti-hsp-70. Cross-linked hsp-70 no longer reacts with the antibody. Lane 1, reticulocyte membranes (not cross-linked, excess lysine); lane 2, reticulocyte membranes (cross-linked); lane 3, exosomes (not cross-linked, excess lysine); lane 4, exosomes (cross-linked). Note: Most of the exosomal TFR and hsp-70 disappear from the exosome sample upon cross-linkage.

the gel area at which a peptide of 220–240 kDa would migrate and SDS/PAGE of the eluate under reducing conditions, no hsp-70 or TFR was detected in the samples from mature red cells. In contrast, both were recovered from reticulocyte and exosome membranes. The hsp-70 recovered in this fashion is shown in Figure 3(c). Thus all the data are consistent with a cross-linked derivative of TFR and hsp-70 and the absence of complex formation in the absence of TFR. In all experiments cited above (whether shown or not), duplicate experiments were always carried out with excess lysine. In these controls there was no evidence for cross-linking.

### Presence of hsp-70 in exosomes from multiple species

If hsp-70 plays a role in recognizing obsolete membrane proteins (like TFR) for externalization during reticulocyte maturation and these two proteins are tightly associated in native exosomes, the simultaneous presence of hsp-70 and TFR would be expected in exosomes from other species. The results in Figure 4 show that hsp-70 can be detected in exosomes from sheep, rat and piglet reticulocytes and in exosomes derived from *in vitro* cultures of embryonic chicken red cells, which are all known to contain TFR [21,28]. In an avian erythroblast cell line (HD3), induction of differentiation results in release of TFR-containing exosomes [29] and hsp-70 can be detected in these exosomes as well (Figure 4, lane 4). Greater amounts of hsp-70 are detected in the exosomes with increased time of differentiation in conjunction with the appearance of more TFR (results not shown).

### Preferential association of hsp-70 with exosomal TFR

If hsp-70 is preferentially bound to exosomal TFR, it should be possible to obtain a greater yield of cross-linked complex from exosomes than from reticulocyte plasma membranes. The results in Figure 5 are consistent with this prediction. In this experiment the amounts of TFR and hsp-70 in reticulocyte membranes and exosomes were determined with and without cross-linkage. TFR was detected by Coomassie Blue staining of immunoprecipitates and hsp-70 by immunoblotting followed by scanning of the appropriate areas with a bioimage scanner.

The data in Figure 5(a) show that the membrane preparation had more total TFR than the sample of exosomes (compare lane 1 with lane 3 in Figure 5a). After cross-linkage, a substantial fraction of the TFR in the membrane sample remained free and was immunoprecipitated from the reaction mixture. (As noted earlier, the cross-linked complex cannot be immunoprecipitated with the anti-TFR monoclonal antibody). With the exosomes, after cross-linking, relatively little of the original TFR remained free.

A similar result is seen with the immunoblots of hsp-70. Anti-hsp-70 does not detect cross-linked hsp-70. Before cross-linking more hsp-70 is detected in the exosomes than in membranes (compare lane 1 with lane 3 in Figure 5b). After cross-linking, virtually 100% of the exosomal hsp-70 disappears, whereas a fraction of that in the membranes is still evident (compare lanes 2 and 4 in Figure 5b). In Table 1 are shown the numerical values obtained by bioimage scanning of these samples. Thus despite the presence of $\sim 17\%$ more hsp-70 in exosomes than membranes, $> 95\%$ of the exosomal hsp-70 became cross-linked. In membranes, about 80% of hsp-70 became linked despite the fact that there was nearly twice as much TFR available. Whereas $> 60\%$ of available TFR became cross-linked in exosomes, only $\sim 20\%$ of that from the plasma membranes was cross-linked. Normalized to a uniform amount of hsp-70, the proportion of TFR cross-linked in exosomes is approximately twice that in plasma membranes using the integrated absorbance units as a measure of the respective protein contents.

Additional evidence for a preferential association of hsp-70 with exosomal compared with plasma membrane TFR comes from studies on the immobilization of hsp-70 (Figure 6). In these experiments, TFR was first immunoadsorbed to the monoclonal anti-TFR antibody, the latter being immobilized by Protein A–Sepharose. Variable amounts of membranes and exosomes

**Table 1 Comparison of the association of hsp-70 with TFR in exosomes and plasma membrane extracts**

Data from Figure 5 after Bioimage scanning to obtain the integrated absorbances of the appropriate areas. With TFR, both the monomer and dimeric forms were scanned and the values given represent the sum of these values. The integrated absorbance units of dimer were 5–10% that of the monomer.

| | Integrated absorbances | |
|---|---|---|
| | Membranes | Exosomes |
| 1 Total TFR (monomer and dimer) | 21.5 | 12.2 |
| 2 Residual TFR | 17.5 | 4.0 |
| 3 Cross-linked TFR (1 − 2) | 4.0 | 8.2 |
| 4 % of TFR cross-linked | 19 | 67 |
| 5 Total hsp-70 | 6.9 | 8.4 |
| 6 Residual hsp-70 | 1.4 | 0.1 |
| 7 Cross-linked hsp-70 (5 − 6) | 5.5 | 8.3 |
| 8 % of hsp-70 cross-linked | 80 | 99 |
| TFR cross-linked per unit of hsp-70 | 4/6.9 = 0.58 | 8.2/8.4 = 0.98 |

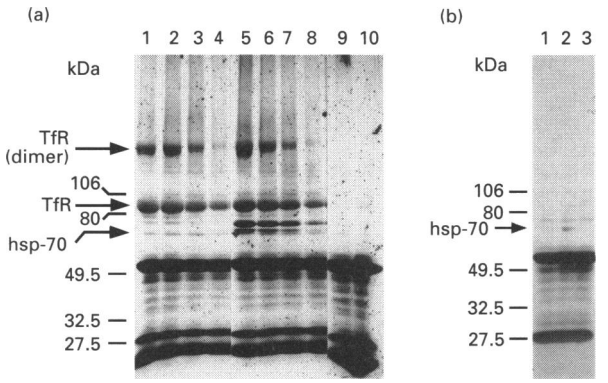


**Figure 6 (a) Immunoprecipitated TFR from exosomes binds hsp-70 preferentially to that from plasma membrane and (b) an anti-hsp-70 duplicate immunoblot of (a)**

(**a**) Variable quantities of reticulocyte membranes and exosomes were immunoprecipitated using a monoclonal anti-TFR antibody to the external domain of the receptor and immobilized with Protein A–Sepharose. To each Sepharose sample was added an identical quantity (0.2 ml) of a 20% membrane-free lysate and the sample was incubated for 30 min at 37 °C. The Sepharose pellet was washed thoroughly and the immunoprecipitate subjected to SDS/PAGE followed by silver staining or immunoblotting with anti-hsp-70. Lanes 1–4, immunoprecipitates from reticulocyte membranes; lanes 5–8, as in lanes 1–4 using immunoprecipitates from exosomes; lanes 9 and 10, lysate added to anti-TFR Protein A–Sepharose, but in absence of TFR. (**b**) Anti-hsp-70 immunoblot of duplicate of (**a**). Lane 1 in (**b**) corresponds to lane 1 in (**a**), lane 2 in (**b**) corresponds to lane 6 in (**a**); lane 3 in (**b**) corresponds to lanes 9 and 10 in (**a**). The prominent bands at 45 and 27 kDa are due to reaction of the secondary antibody with the IgG used to immunoabsorb TFR.

were immunoprecipitated to assure that near equivalent amounts of TFR from both samples would be available for comparison. To the immobilized TFR samples was added a uniform amount of membrane-free, red cell lysate, a known source of hsp-70 [23]. The results show that the hsp-70 bound to TFR is related to the amount of TFR present in either exosomes or membranes. However, in all cases the amount of hsp-70 retained by exosomal TFR is substantially greater than that retained by the plasma membrane TFR (compare lanes 5–8 with lanes 1–4 of Figure 6a). In the absence of TFR, no hsp-70 associates with the antibody on the gel support (Figure 6a, lanes 9 and 10; Figure 6b, lane 3).

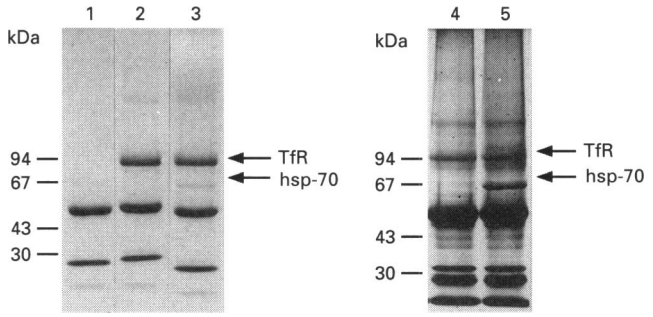


**Figure 7 Retention of hsp-70 by immobilized TFR: temperature and cation dependence**

Reticulocyte membranes solubilized in 0.5% Triton X-100 were applied to Protein A–Sepharose preincubated with anti-TFR. The TFR–anti-TFR–Protein A–Sepharose complex was then incubated under the conditions given below for 30 min at 4 °C or 30 °C. Then the bound proteins were released from the Protein A–Sepharose by treating with SDS/ME sample buffer and subjected to SDS/PAGE. Lanes 1–3 are stained with Coomassie Blue, lanes 4 and 5 stained with silver. Lane 1, Sepharose-bound anti-TFR + lysate + $Mg^{2+}$ + $Ca^{2+}$, 30 °C; lane 2, Sepharose-bound anti-TFR + TFR + lysate + $Mg^{2+}$ + $Ca^{2+}$, 4 °C; lane 3, Sepharose-bound anti-TFR + TFR + lysate + $Mg^{2+}$ + $Ca^{2+}$, 30 °C; lane 4, Sepharose-bound anti-TFR + TFR + lysate + $Mg^{2+}$ + $Ca^{2+}$ + EDTA, 30 °C; and lane 5, Sepharose-bound anti-TFR + TFR + lysate + $Mg^{2+}$ + $Ca^{2+}$, 30 °C. Membrane-free sheep red cell lysate was used as a source of hsp-70 (as in Figure 6).

That hsp-70 is bound in the presence of TFR is confirmed by the immunoblots shown in Figure 6(b).

The specific nature of the association of TFR with hsp-70 is illustrated by the cross-linking studies with plasma membranes. Unlike exosomes, TFR and hsp-70 are both relatively minor components of the total membrane proteins. The majority of hsp-70 is not membrane bound and can be purified from the cytosol [23]. Despite their relatively low concentrations, cross-linkage of the total plasma membrane fraction results in the formation of a complex which, after electroelution and SDS/PAGE under reducing conditions, shows the presence of both TFR (see legend to Figure 3c) and hsp-70 (Figure 3c). Thus, there appears to be a close association of some of the TFR with hsp-70 in the cell as well as in exosomes. The cross-linkage of plasma membrane TFR with hsp-70 is not surprising since a substantial fraction of the cellular TFR in sheep reticulocytes has been segregated to the multivesicular bodies which contain exosomes prior to externalization [30]. All the TFR in reticulocytes is membrane bound. However, we have not been able to obtain an intracellular compartment free from plasma membrane to assess the relative affinities for hsp-70 in the different cellular membrane fractions.

## Co-immunoprecipitation of TFR and hsp-70

As seen in Figure 1(a), immunoprecipitation of the TFR at 4 °C in the absence of bivalent cations does not result in co-precipitation of hsp-70. However, if the immunoprecipitation is carried out at 30 °C with bivalent cations, co-immunoprecipitation can be obtained (Figure 7). A similar result showing temperature- and bivalent cation-dependent immunoprecipitation has been previously reported for the co-immunoprecipitation of the progesterone receptor with hsp-90 and hsp-70 [22].

Using Protein-A–Sepharose–anti-TFR complex to immobilize TFR, the results in Figure 7 show that at 4 °C (lane 2), or in the presence of excess EDTA at 30 °C (lane 4), little hsp-70 is brought down by immobilized TFR. In contrast, at 30 °C and in

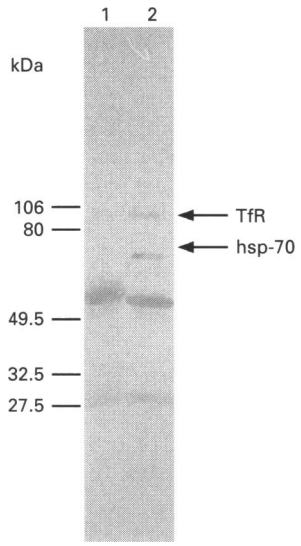


**Figure 8 Co-immunoprecipitation of TFR with anti-(hsp-70)-immobilized hsp-70**

Triton X-100 extracts of exosomes were applied to Protein A–Sepharose-immobilized monoclonal mouse anti-(human hsp-70) (lane 2) or preimmune mouse IgG (lane 1) and incubated overnight at 4 °C. After elution with SDS/ME the bound proteins were subjected to reducing SDS/PAGE and transferred to nitrocellulose. The bands at 45 kDa and 27 kDa are the subunits of IgG. The samples were probed with anti-hsp-70 after which the blot was reprobed with anti-TFR antibody. The band at ~ 70 kDa appeared after blotting with anti-hsp-70 and that at 94 kDa after the blotting with anti-TFR antibody.

the presence of $Ca^{2+}$ and $Mg^{2}$ (Figure 7, lanes 3 and 5), there is clear retention of hsp-70 by immobilized TFR. In the absence of TFR (lane 1) no hsp-70 is retained by anti-TFR. In the experiments shown in Figure 7, membrane-free red cell lysate was used as a source of hsp-70 (as in Figure 6). Although stained for total proteins, it is evident that only a peptide of ~ 70 kDa, from the many peptides in the lysate, associates with the immobilized TFR (compares lane 2 to 3 and 4 to 5 in Figure 7). If the TFR association with hsp-70 were non specific, a variety of other peptides would be expected to adhere to the immobilized TFR. Clearly, in the presence of cations and at 30 °C, only the 70 kDa peptide adheres to immobilized TFR and no adherence is seen in the absence of bivalent cations or at 4 °C.

### Co-precipitation of TFR with ligands which bind hsp-70

(A) TFR binding to anti-hsp-70 antibody

Anti-hsp-70 antibody can be used to co-immobilize TFR if hsp-70 is present. Exosome extracts (Figure 8) were incubated with anti-hsp-70 or non-immune mouse IgG bound to Protein A–Sepharose. The results show that no exosomal proteins are retained by the non-immune beads (Figure 8, lane 1). Peptides of both 70 and 94 kDa from exosome extracts are retained by anti-hsp-70. After SDS/PAGE of the retained peptides and sequential immunoblotting with anti-hsp-70 and anti-TFR, the presence of both hsp-70 and TFR was detected. Similar results (not shown) were obtained with exosomes from a differentiating chicken erythroleukaemic cell line (HD3 cells) where immobilized anti-hsp-70 also adsorbed both hsp-70 and TFR.

(B) TFR binding to immobilized ATP

Hsp-70 is known to bind to ATP–agarose [23]. The results show

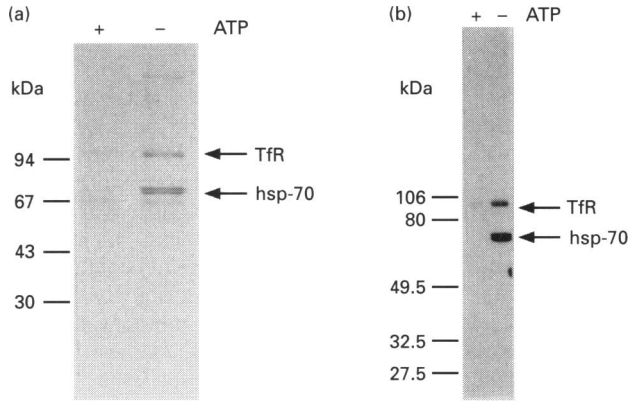


**Figure 9 Co-precipitation of TFR with ATP–agarose-immobilized hsp-70**

(**a**) Triton X-100 extracts of exosomes were applied to ATP–agarose in the presence and absence of excess ATP (20 mM) and incubated for 15 min on ice. The agarose was washed and the agarose-retained material eluted with SDS/ME sample buffer before being subjected to SDS/PAGE. Coomassie Blue-stained gels of the eluted proteins are shown. Left lane, with 20 mM ATP in solution; right lane, without ATP in solution. (**b**) After transfer of the samples in (**a**) to nitrocellulose, the blots were sequentially probed with anti-hsp-70 and anti-$TFR_{CD}$. Left lane, with 20 mM ATP in solution; right lane, without ATP in solution.

(Figure 9a) that when TX-100 extracts of exosomes are passed through ATP–agarose, both hsp-70 and TFR are retained. If the binding is carried out with excess ATP, there is clear reduction in the retention of both TFR and hsp-70. Sequential immunoblotting, after SDS/PAGE of the peptides retained, shows that ATP–agarose bound both TFR and hsp-70 (Figure 9b) and the binding of both was reduced with excess ATP. TFR itself is not known to bind ATP. All the data obtained point to a close physical association between hsp-70 and TFR in exosomes.

### Stoichiometry of binding between hsp-70 and TFR

To assess the stoichiometry of hsp-70 binding to TFR, total amino acid analyses were carried out for TFR and hsp-70 regenerated from the ~ 240 kDa cross-linked complex. Since the molecular masses of the unglycosylated peptides are known, the moles of the individual peptides could be estimated. Comparison of the moles thus estimated, yielded a ratio of 1.5 mol of TFR monomer to 1 mol of hsp-70 in the cross-linked complex.

In addition, silver stains of the regenerated peptides after SDS/PAGE (for example see Figure 2b, lane 1) were subjected to laser scanning to obtain the relative amounts of TFR and hsp-70 in the complex after reduction. This procedure gave a similar result (1.6 mol of TFR monomer to 1 mol of hsp-70). Such data suggest that hsp-70 need not bind to each monomer of the dimeric TFR, but that at least 1 mol of hsp-70 is associated with dimeric TFR. Bioimage scans of Coomassie Blue-stained gels of the native (uncross-linked) exosomes gave ratios of TFR monomer to hsp-70 of 1.3–1.4.

## DISCUSSION

It is well known that in mature red cells many enzyme activities are reduced compared with earlier precursor cells. During maturation of reticulocytes a population of vesicles (exosomes) is formed which contains a variety of membrane proteins lost from the cell during maturation. The near quantitative recovery in exosomes of the lost TFR [31] and nucleoside transporters [28], suggests that exosome formation is an important route for removal of specific membrane proteins.

In addition to TFR, exosomes from several species including avian red cells and an avian erythropoietic cell line (HD3), show the presence of a 70 kDa protein, identified as the hsp-70. In sheep exosomes, hsp-70 is found in the lumen of the exosomes, since surface iodination of intact exosomes does not label hsp-70. In TX-100-solubilized exosomes, hsp-70 is iodinated [27]. To date few soluble cytosolic proteins have been demonstrated in exosomes, the major soluble proteins (i.e. lactate dehydrogenase) being absent [13]. Hsp-70 [13,14] and *rab* proteins [32], however, have been detected in exosomes. We have recently confirmed the presence of *rab* proteins and GTP-binding activity in sheep exosomes (J. Paiement and R. M. Johnstone, unpublished work).

The TFR released in sheep exosomes is in the same orientation as in the original plasma membrane and has the same molecular mass and iodotyrosyl peptide map as the native cellular TFR [12,27]. The TFR in exosomes differs from the cellular TFR in a single aspect identified to date. Immunoprecipitates of TFR from sheep exosomes unlike those from the plasma membranes do not act as substrates for phosphorylation by exogenous protein kinase C [33]. Thus, some subtle change in the receptor has occurred during the course of externalization or in preparation for externalization of TFR in exosomes.

Given the properties of hsp-70 and its ability to associate with partly unfolded proteins [16], the possibility was considered that the subtle changes in TFR during maturation might enhance hsp-70 binding to TFR, the complex then acting as a signal for exosome formation or externalization during maturation.

Such a mechanism would predict a close physical association between TFR and hsp-70 in exosomes. The results presented here argue that such is indeed the case. Three different types of experiments show that in exosomes, hsp-70 is closely associated with TFR without covalent linkage. (1) TFR can be cross-linked to hsp-70 in intact or detergent-solubilized extracts of exosomes; (2) hsp-70 can be bound to immobilized TFR, and (3) TFR can be bound to immobilized hsp-70.

The selective affinity of TFR for hsp-70 is noteworthy. Of the myriad of proteins in a red cell lysate, only hsp-70 is retained by an immune complex of TFR with anti-TFR (Figure 6a). The data also show that per unit amount of TFR or hsp-70, cross-linkage is preferentially obtained in exosomal preparations, indicative of either closer proximity or preferential association. The data suggest that there is preferential association of hsp-70 with TFR in the exosomes since more hsp-70 is bound per unit amount of TFR.

Preliminary experiments to assess whether *rab* proteins are also associated with the TFR–hsp-70 complex showed no evidence for cross-linkage of Rab to the complex (A. Mathew, J. Paiement and R. M. Johnstone, unpublished work). The ability to cross-link TFR and hsp-70 in extracts of the plasma membrane itself does not eliminate the possibility that hsp-70 is involved in targeting for externalization. A sizeable percentage of the total cellular TFR is intracellular, some of which is clearly in the multivesicular sacs bearing exosomes prior to externalization [21,34–36]. The receptor in these multivesicular sacs may already have bound hsp-70, in preparation for release from the cells. However, since some of the TFR in the plasma membranes is probably still in its native state, and not bound to hsp-70, it is anticipated that not all the available hsp-70 or TFR in the membranes would become cross-linked.

The consistent association of hsp-70 with TFR in exosomes also suggests that the association has some physiological significance (Figure 4). The simultaneous presence of both hsp-70 and TFR in exosomes in four species (sheep, rat, pig and chicken) as well as a haematopoietic chicken cell line (HD3) suggest that the association of hsp-70 with TFR in exosomes is not a fortuitous occurrence. When the chicken erythroblast cell line HD3 is induced to differentiate, exosome formation begins [29] and hsp-70 is released in conjunction with TFR from the earliest stages of differentiation. Thus, there is both direct and indirect evidence for a close, specific association of hsp-70 and TFR in exosomes from maturing red cells.

Given the nature of many of the processes in which hsp-70 is involved, the consistent and close association of hsp-70 with TFR in exosomes argues that hsp-70 may have a role in TFR (and other protein) targeting for externalization via the exosome route.

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