Selective action of uncoating ATPase towards clathrin-coated vesicles from brain

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SUMMARY

Clathrin-coated vesicles from brain are primarily involved in synaptic vesicle recycling and are substrates for the constitutively expressed heat shock cognate hsc70 protein (uncoating ATPase). To investigate the regulation of clathrin coat turnover in other tissues the activity of hsc70 towards coated vesicles from other sources was examined. Concentrations of hsc70 which caused near-complete removal of clathrin from brain coated vesicles effected only partial uncoating of vesicles prepared from other tissues. The selective action of hsc70 could not be accounted for by tissue or species specificities of hsc70, but rather reflected differences in coat structure. Selective action was associated with two differences in the hsc70-dependent ATPase cycle. Firstly, uncoating of brain, but not placental vesicles, could occur under circumstances where ATP hydrolysis was prevented. Secondly, only brain coated vesicles could support multiple rounds of hsc70-dependent ATP hydrolysis. Implications of these findings for the mechanism of hsc70-dependent vesicle uncoating in non-neuronal cells and the organisation of the endocytic pathway in the axon are discussed.

Key words: hsp70, clathrin-coated vesicles, ATPase, coatomer

INTRODUCTION

The transport of proteins between compartments within the cell is mediated by coated vesicles. Assembly of the coat structure at the donor compartment followed by membrane invagination and scission leads to the formation of a transport vesicle, which contains the information required to target and fuse with its acceptor membrane (Palade, 1975). Prior to fusion with the acceptor organelle the protein coat is removed.

To date, two principal classes of vesicles have been identified. COP (COatomer Protein)-coated vesicles mediate the bulk flow transfer of proteins between several successive compartments on the secretory pathway (Malhotra et al., 1989). The coats of these vesicles comprise subunits derived from soluble COPs (Waters et al., 1991) which interact with the vesicle membrane via the small GTP-binding protein, ADP ribosylation factor (ARF: Serafini et al., 1991; Palmer et al., 1993). In contrast to COP coats, clathrin coats mediate the enrichment of specific proteins into transport vesicles to enable selective transfer of proteins. Formation of clathrin-coated vesicles occurs at the cell surface, where specific receptors are internalised (Goldstein et al., 1985), and also in the trans-Golgi network (TGN: Griffiths et al., 1985; Tooze and Tooze, 1986), where proteins are sorted from the constitutive secretory pathway into the endosomal/lysosomal pathway. Although clathrin is the major structural component of vesicles derived from both the cell surface and the TGN, the two populations of vesicles are distinguished by their complement of associated adaptor complexes, which bind specifically to the cytoplasmic domains of receptors in vitro (Pearse, 1988) and are responsible for the enrichment of particular receptors in coated vesicles (Chen et al., 1990; Trowbridge, 1991). AP-1, consisting of 100 kDa γ- and β′-adaptns, as well as 47 and 20 kDa polypeptides, associates with the Golgi complex (Ahle et al., 1988). AP-2, which is localised to the plasma membrane (Robinson, 1987), is composed of α- and β-adaptns, and polypeptides of 50 and 17 kDa. Formation of clathrin-coated vesicles is not as well understood as formation of COP-coated vesicles. However, GTP-binding proteins appear to play a role; binding of AP-1 complexes to the TGN requires ARF (Stamnes and Rothman, 1993), while formation of coated vesicles at the cell surface requires a number of GTP-binding proteins (Carter et al., 1993), most notably dynamin (Herskovits et al., 1993; van der Bliek et al., 1993).

The soluble pool of clathrin exists as a complex (triskelion) of three heavy chains (180 kDa) and three light chains (33-36 kDa), bound at the apex (Pearse, 1975; Ungewickell and Branton, 1981). Within a vesicle coat each triskelion can bind to three neighbours, so that interaction between triskelias produces a lattice (Keen et al., 1979). Hence, clathrin-clathrin interactions contribute primarily to the structure of the coated vesicle, though other interactions play a role in coat stability; clathrin will self-assemble into a heterogeneous population of baskets at low pH, but inclusion of adaptors allows assembly of a more homogeneous population of baskets at physiological pH (Pearse and Robinson, 1984; Zaremba and Keen, 1983).

In the case of endocytic coated vesicles, contents are delivered to the next compartment on the pathway, the
endoosome, by a specific membrane fusion event (Woodman and Warren, 1991). Since the coat proteins would prevent close apposition of the vesicle membrane with the endosome, for fusion to proceed it is crucial that at least part of the coat is removed. The coated vesicle is a relatively stable intermediate in solution, so uncoating must be catalysed. Additionally, to avoid the futile removal of proteins from the developing coated pit, any uncoating reaction must be regulated to ensure that proteins are removed only from the mature vesicle.

The major contributions to studies on vesicle uncoating have been derived from a simple biochemical assay, based on the transfer of coat proteins from a high-speed pellet (vesicular) to a supernatant fraction (Patzer et al., 1982). This assay enabled the isolation of a soluble activity, termed uncoating ATPase, which coupled ATP hydrolysis to the dissociation of clathrin triskelions from bovine brain coated vesicles in vitro (Schlossman et al., 1984; Braem et al., 1984). Uncoating ATPase was subsequently identified as heat shock cognate 70 (hsc70), a 70 kDa member of the family of heat-shock proteins (Ungewickell, 1985; Chappell et al., 1986). Hsc70 is believed to function in a variety of contexts within the cell, including catalysis of protein folding (Beckmann et al., 1990), passage of cytosolic proteins into lysosomes (Chiang et al., 1989) and nuclei (Shi and Tomas, 1992), translocation of secretory and mitochondri-
ammonium molybdate in 15% (w/v) \( \text{H}_2\text{SO}_4 \). Molybdato-phosphate was extracted with 200 µl n-butanol/toluene (1:1, v/v) by vortexing. Phases were separated by centrifugation for 1 minute at 13,000 rpm in a microfuge. The organic phase was transferred to a separate vial, and dried further by centrifugation for 5 minutes; 150 µl of this phase was assayed for radioactivity. For experiments using ATP\( \gamma \text{S} \) the validity of this extraction assay for PO\( \gamma \text{S}^35 \) was verified by incubating ATP\( \gamma \text{S} \) with 2 M \( \text{H}_2\text{SO}_4 \) at 95°C to hydrolyse the terminal thio-phosphate. An extraction efficiency of approximately 60% was obtained.

RESULTS

Hsc70 removes clathrin selectively from brain coated vesicles

Coated vesicles were incubated with or without hsc70 in the presence of an ATP regenerating cocktail, and the extent of uncoating was assayed by following the transfer of clathrin heavy chain from the pellet to the supernatant fraction after sedimentation of the vesicles. Incubation of brain coated vesicles with a small amount of hsc70 resulted in the appearance of clathrin in the high-speed supernatant, in combination with the amount of clathrin associated with the pellet (Fig. 1, lanes 1-4), similar to that described before (Schlossman et al., 1984; Greene and Eisenberg, 1990). The reaction was specific for clathrin, since removal of adaptor complexes did not occur, as assessed by examination of 100 kDa adaptins in agreement with previous authors (Schlossman et al., 1984; Greene and Eisenberg, 1990). In contrast, incubation with the same amount of hsc70 with coated vesicles prepared from human placenta resulted in the transfer to the supernatant of a much smaller fraction of clathrin, and no reduction in the amount of clathrin in the pellet could be detected (Fig. 1, lanes 5-8).

This selective action is demonstrated more clearly by titrating the amount of hsc70 used in each reaction (Fig. 2). With increasing quantity of hsc70 the efficiency of uncoating of sheep brain coated vesicles rose sharply, reaching a plateau of almost 90% of the total clathrin in the coated vesicle preparation, in agreement with the results of Greene and Eisenberg (1990) using coated vesicles from bovine brain. The ability of hsc70 to remove clathrin from placental coated vesicles increased far less with increasing hsc70 quantity, such that at low ratios of hsc70 to coated vesicles the amount of hsc70 required to achieve the same extent of uncoating differed at least 5-fold according to the vesicle preparation. Even at high concentrations of hsc70 the extent of uncoating was limited (see also Fig. 1A). Vesicles derived from sheep liver were also poor substrates for hsc70, with clathrin removal reaching only 35% of the total clathrin at a molar ratio of hsc70:clathrin of 2.5:1. Preparation of brain coated vesicles with 1% Triton X-100 did not affect their sensitivity towards hsc70, demonstrating that resistance of liver coated vesicles to hsc70 was not due to the presence of detergent in these preparations (not shown; see also Schlossman et al., 1984).

Selectivity of hsc70 action is not due simply to matching components of the reaction from different species or tissues. Hsc70 from human placenta could uncoat sheep brain coated vesicles with an efficiency similar to sheep brain hsc70, yet was unable to remove clathrin efficiently from placental coated vesicles (Fig. 3A). Indeed, hsc70 prepared from mung bean uncoated brain vesicles efficiently (not shown). Furthermore, placental hsc70 could not uncoat vesicles derived from sheep liver. It has been suggested that millimolar levels of calcium ions might promote vesicle uncoating by exposing cryptic sites within the clathrin lattice for hsc70 attachment (DeLuca-Flaherty et al., 1990). Therefore, it might be supposed that such sites are more deeply buried in coated vesicles derived from tissues other than brain, and that exposure of these sites could be effected by inclusion of calcium ions in the incubation. However, inclusion of 1 mM CaCl\( _2 \) did not stimulate uncoating of vesicles from any source (Fig. 3B).
Relative efficiencies of uncoating could be found by comparing the protein. To exclude these possibilities, uncoating of brain LCb overlaps with placental LCa, which competed with clathrin for hsc70 binding or inactivated by a fixed amount of hsc70 in separate incubations was approximately 8:1. When the coated vesicles from different sources were combined the ratio was approximately 6.5:1.

Selective activity of hsc70 towards coated vesicles from brain could be due to extensive aggregation of coated vesicle preparations from other sources. This seemed unlikely, since negative staining of placental coated vesicle preparations by Pearse (1982) revealed discrete coats with no evidence of extensive aggregation. However, to confirm this vesicles derived from brain or placenta were analysed for their sedimentation properties and were also visualised by negative staining.

Fig. 5 demonstrates that both vesicle preparations contained a fast migrating component (peak 2). In each case some size heterogeneity was apparent, as shown by the nonsymmetrical nature of the peak. However, placental coated vesicles migrated largely as a clearly defined peak with a sedimentation coefficient \( s_{20,w} \) of approximately 157 S (Fig. 5B). Coated vesicles from brain migrated as a somewhat broader peak, which could be separated into at least two different species further into the run, with sedimentation coefficients of 124 and 144 S, respectively (Fig. 5B). Hence, coated vesicles from brain were somewhat smaller, and more heterogeneous in size than vesicles from placenta. Further confirmation of these findings were provided by electron microscopy. Negatively stained images of both preparations of vesicles showed essentially separate and intact vesicles, and also demonstrated the smaller size and greater size heterogeneity of brain-derived vesicles (Fig. 5C and D).

In both preparations a relatively slow migrating species was identified which appeared unrelated to the coated vesicles and probably represents a high molecular mass component remaining from the Ficoll gradients (Ficoll 400-DL has a molecular mass of approximately 400,000).
Specificity of clathrin uncoating by hsc70

Uncoating of non-brain vesicles occurs at elevated pH

Given the difference in coated vesicle size, it seemed possible that the differential activity of hsc70 could be ascribed to differences in coat structure between the vesicle preparations. One condition which influences coat structure is pH (Woodward and Roth, 1978), and it has been shown previously that coated vesicles from brain are poor substrates for hsc70 at low pH (Schlossman et al., 1984), where the coat is stabilised. Therefore, the action of hsc70 on brain or non-brain coated vesicles was examined using pH as a variant. As demonstrated in Fig. 6, coated vesicles from brain were resistant to the action of hsc70 at low pH, but were increasingly susceptible to uncoating as the pH rose to between pH 6.4 and pH 7.0 as described earlier (Schlossman et al., 1984). At pH 8, the extent of clathrin removal by hsc70 was greater still, but was accompanied by a greater increase in hsc70-independent uncoating (data not shown) as reported by Schlossman et al. (1984). Coated vesicles from placenta or sheep liver remained relatively stable at pH 7.0, but hsc70-dependent uncoating activity towards both these preparations of coated vesicles rose steadily under increasingly alkaline conditions between pH 7 and pH 8.

Fig. 5. Characterisation of coated vesicle preparations from brain and placenta. (A) Sedimentation analysis of brain coated vesicles. (1) Coated vesicle preparations were centrifuged in an analytical centrifuge and sedimentation of particles was recorded as Schlieren patterns. (2) Migration of each the arrowed peaks was assessed at various times to obtain an estimate for its sedimentation coefficient. (B) Sedimentation analysis of placental coated vesicles. (C) Negative staining of brain coated vesicles. ×120,000. Bar, 0.1 µm. (D) Negative staining of placental coated vesicles.
Placental coated vesicles drive only a single round of ATP hydrolysis by hsc70

We examined whether differences in susceptibility of coated vesicles to hsc70 could be attributed, in whole or in part, to respective abilities to stimulate hsc70 activity. Reconstituted clathrin cages are able to accelerate the ATPase activity of hsc70 in a clathrin light-chain-dependent manner (Schmid et al., 1984). However, high levels of endogenous ATPase activities within coated vesicle preparations have prevented analysis of the effect of intact vesicles on hsc70-dependent ATPase activity. We overcame this difficulty by inclusion of vanadate ions, which reduced the endogenous ATPase activity of brain coated vesicles substantially. ATPase activity of placental coated vesicles was reduced to a lesser extent and was resistant to other inhibitors including NO$^-$, SCN$^-$ and N-ethylmaleimide (not shown).

Hsc70 alone had a low endogenous ATPase activity (Fig. 7A) as reported by others (Flynn et al., 1989; Sadis and Hightower, 1992). Addition of brain coated vesicles increased the rate of hydrolysis significantly in a biphasic reaction. The first, rapid, phase we ascribe to a single round of ATP hydrolysis. The second, somewhat slower, phase could be attributed to subsequent rounds of ATP hydrolysis, where the exchange of ADP for ATP becomes rate limiting (Greene and Eisenberg, 1990). Coated vesicles from placenta effected a quite different pattern of ATP hydrolysis (Fig. 7B). The first phase occurred, though somewhat slower than that found with brain coated vesicles. The second phase, however, was absent, with the rate of hydrolysis falling to that of free hsc70 itself, indicating that further rounds of stimulated hydrolysis were blocked. This is seen more clearly when background values are subtracted from the vesicle-dependent stimulation of hsc70 ATPase activity (Fig. 7C). The differential effect was not due to depletion of ATP by endogenous ATPases within placental vesicles, since hsc70 ATPase activity supported by brain vesicles remained high during prolonged incubations (not shown) or during incubations lacking vanadate (not shown), where greater than 50% of the ATP was hydrolysed.

Uncoating of brain vesicles occurs without ATP hydrolysis

Although the entire catalytic cycle of hsc70 has not been elucidated it has been found that partial reactions including substrate dissociation may occur without ATP hydrolysis (Palleros et al., 1993). The role of ATP hydrolysis during vesicle uncoating has been a matter of some dispute (Braell et al., 1984; Heuser and Steer, 1989). In order to clarify this debate and to provide additional information to dissect the uncoating reactions of brain and non-brain vesicles we examined uncoating activity towards both vesicle preparations under conditions where ATP hydrolysis was blocked.

It has been reported that the non-hydrolysable analogue of ATP, ATPγS, could not elicit hsc70-dependent uncoating (Schlossman et al., 1984). However, recent data have shown limited activity of ATPγS in the uncoating reaction (Heuser and Steer, 1989). These apparently conflicting results may be reconciled by examining the ability of ATPγS to promote uncoating at different ratios of hsc70 to clathrin triskelia. When ATP was present, hsc70 removed clathrin effectively from brain coated vesicles even at low ratios of hsc70 to clathrin (Fig. 8A). When ATP was substituted by ATPγS low quantities of hsc70 were relatively ineffective in the uncoating reaction, consistent with an earlier study (Schlossman et al., 1984). As the concentration of hsc70 was increased, however, uncoating with ATPγS occurred with increasing efficiency, rising to approximately the same level as ATP-dependent uncoating. In contrast to brain-derived vesicles, vesicles from placenta were resistant to ATPγS-dependent uncoating even at the highest ratios of hsc70 to clathrin (Fig. 8B).

Uncoating of brain-derived vesicles was still ATP-dependent, since replacement of nucleotides by an ATP-depleting cocktail of hexokinase and glucose abolished activity (Fig. 9A). The effect of ATPγS could not be attributed to contamination of the hsc70 or coated vesicle preparations with ATP, since little activity was observed in the absence of added nucleotide. AMP-PNP was partially effective in the uncoating reactions under these conditions. The speed of uncoating in the presence of ATPγS was indistinguishable from that in the presence of ATP, being completed within 2 minutes at 25°C.
Specificity of clathrin uncoating by hsc70

ATPγS contains a substantial amount of ADP as a contaminant, and it was possible that vesicle-associated adenylate kinase could convert it to ATP. This appeared unlikely, however, since ADP did not support uncoating and the adenylate kinase inhibitor diadenosinepentaphosphate (100 µM; Lienhard and Sikomski, 1973) did not affect the ATPγS-dependent reaction (data not shown). ATPγS is hydrolysed by hexokinase (Eckstein, 1983), so it was important to establish that hsc70 could not hydrolyse ATPγS during the uncoating reaction. This was confirmed (Fig. 9B).

The ability of hsc70 to uncoat brain-derived vesicles without hydrolysing ATP was confirmed by examining the temperature dependence of the two reactions. Vesicle-dependent ATP hydrolysis by hsc70 was temperature dependent (Fig. 10) with no release of orthophosphate detected at 4°C. Despite this, uncoating of brain-derived vesicles continued on ice (Fig. 11A), although less efficiently than at 25°C. This contrasted with the inability of hsc70 to remove clathrin from placental vesicles at this temperature. The apparent reduction in efficiency reflected a reduction in rate, since near-optimal uncoating could be obtained after longer incubation on ice (Fig. 11B).

DISCUSSION

This paper is concerned with the clathrin-uncoating ATPase activity of the abundant, constitutively expressed heat-shock cognate protein, hsc70. Previous studies have outlined possible mechanisms for the action of hsc70 on clathrin coats from brain (Rothman and Schmid, 1986; Greene and Eisenberg, 1990), but have not addressed two features that would be required of an uncoating reaction linked to vectorial protein transport. Firstly, the uncoating reaction must be prevented at

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Fig. 7. Brain, but not placental, coated vesicles elicit multiple rounds of ATP hydrolysis. (A) ATPase activity of hsc70 alone, pig brain coated vesicles alone, or both combined, was measured as described. (B) As A, but using placental coated vesicles. (C) Background ATPase activity from the coated vesicle preparations was subtracted to obtain the specific, vesicle-dependent stimulation of hsc70 ATPase activity. Values are means (± s.e.m. shown only for C) from a representative experiment performed in triplicate.
the site of coat production. Secondly, the reaction must be regulated to ensure that it occurs preferentially at, or in the vicinity of, the target organelle. Since coated vesicles from brain are predominantly involved in a highly specialized transport pathway (see below), we sought to determine if clathrin coats from other sources behaved in a similar, or different, fashion.

The principal new finding is the selective action of hsc70 towards brain coated vesicles. Selective action is not due to trivial species or tissue specificities of hsc70, and is not influenced by inclusion of calcium ions, which may affect clathrin structure (DeLuca-Flaherty et al., 1990). Other trivial explanations, such as the presence of inhibitors in some vesicle preparations or proteolysis of clathrin light chains, were ruled out by using mixed incubations. Similarly, vesicle aggregation does not appear to play an important role in hsc70 specificity, since placental coated vesicle preparations sediment as a single species, though somewhat larger than brain coated vesicles. Sedimentation analysis of coated vesicles did reveal structural differences between vesicles derived from brain and those from placenta. Vesicles from brain were heterogeneous in size, separating into at least two defined species. Moreover, these vesicles were somewhat smaller than those from placenta. It has been noted previously that coated vesicle preparations from brain contain a range of coat sizes, including small cages which

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**Fig. 8.** ATPγS supports uncoating of brain, but not placental, vesicles. Coated vesicles were incubated with increasing amounts of hsc70 in the presence of 1 mM MgATP or 1 mM MgATPγS, and the extent of uncoating measured. (A) Brain vesicles, means ± s.e.m. from three experiments. (B) Placental vesicles, means from two experiments.

**Fig. 9.** Binding but not hydrolysis of nucleotide is essential for uncoating. (A) Brain vesicles (20 µg) were incubated with hsc70 (100 µg) and the indicated nucleotide additions. Values for the extent of uncoating are means ± s.e.m. from the stated number of experiments. (B) [35S]ATPγS was incubated with hsc70, brain vesicles, or a combination of these. At the indicated time the incubations were assayed for release of radiolabelled thiophosphate as described. Values are means ± s.e.m. from a representative experiment, performed in triplicate.
Specificity of clathrin uncoating by hsc70

apparently lack internal membranes (Heuser and Kirchhausen, 1985), and it has been suggested that formation of these microcages may be related to the postmortem acidification of neurones (Heuser and Anderson, 1989). Such empty cages might account for differential uncoating activity if they were the preferred substrates of hsc70. This seems unlikely though, since adaptins are also abundant in the preparations and previous studies have identified membrane vesicles as the products of uncoating reactions (Heuser and Steer, 1989). However, the apparent difference in structure of coats derived from brain compared with those from placenta indicates that coat structure might be linked to hsc70 action. This conclusion is supported by the finding that increase in pH, which affects coat structure (Keen et al., 1979), can overcome the differential activity of hsc70.

Fig. 10. Temperature dependence of vesicle-stimulated ATPase activity. The indicated mixtures of hsc70 and brain coated vesicles were incubated with [32P]ATP and release of radiolabelled phosphate after various times was measured. (A) 25°C. (B) 4°C. Values are means ± s.e.m. from a representative experiment.

The specificity of uncoating is emphasised by the ability of hsc70 to uncoat brain, but not placental, vesicles without hydrolysing ATP. Uncoating of brain vesicles occurs to virtually the same extent when the non-hydrolysable analogue of ATP, ATPγS, is substituted for ATP as long as a sufficient molar excess of hsc70 over clathrin is provided. Uncoating also occurs at 4°C, though somewhat more slowly than at 25°C, despite abolition of clathrin-dependent ATP hydrolysis at this temperature. These results extend a previous observation of partial uncoating activity in the presence of ATPγS (Heuser and Steer, 1989), but are difficult to reconcile with the results of Schmid and Rothman (1985), who observed no ATPγS-dependent disassembly of clathrin cages even when hsc70 was
Hsc70 is necessary for a range of cellular activities. Therefore, discussion of its action during vesicle uncoating should be coupled with an examination of its activities in other contexts (for review, Gething and Sambrook, 1992). Hsp70 was originally identified as a protein induced after heat shock, which migrated to the nucleus and nucleolus, and bound to aggregated proteins. It was released from these aggregates in vitro by ATP, but not ATP\(\gamma\S\) (Lewis and Pelham, 1985), supporting early models which coupled ATP hydrolysis by hsp70 to a conformational change in the target protein, leading to unfolding (Lewis and Pelham, 1985; Pelham, 1986). This activity has never been demonstrated in vitro, though it is consistent with a model for the uncoating reaction where hsc70 utilises the energy from ATP hydrolysis to break clathrin-clathrin interactions (see Rothman and Schmid, 1986).

According to more recent models designed to explain hsc70 action during protein folding and translocation of polypeptides across membranes, hsc70 acts by stabilising the unfolded peptide rather than possessing an unfolding activity itself (Rothman, 1989; Neupert et al., 1990). Thus during protein folding hsc70 binds to nascent cytosolic polypeptide chains to prevent rapid mis-folding. The hsc70-polypeptide complex (or DnaK-polypeptide in prokaryotes) then interacts with a ‘folding complex’ (TCP-1 in eukaryotes, Cpn60/Cpn10 [GroES/GroEL] in prokaryotes) (Lewis et al., 1992; Langer et al., 1992) where folding is catalysed by a series of partly defined co-operative interactions. For mitochondrial and chloroplast proteins the appropriate Cpn60 is located within the target organelle so that regions of the polypeptide remain unfolded until they are translocated.

Although ATP is essential for hsc70 function, the mechanism by which ATP allows hsc70 to influence the conformation of its substrate is not known. Early models suggested that slow hydrolysis of ATP, thought to occur before hsc70 dissociates from the peptide, would provide ‘timed protection’, for a nascent conformation to prevent mis-folding (Rothman, 1989). More recent data imply that ATP-binding, rather than hydrolysis, causes sufficient conformational change in hsc70 for it to release its substrate peptide (Palleros et al., 1993). Similarly, the functionally related (though structurally distinct) Cpn60 changes conformation upon ATP binding to eject peptide. A non-hydrolysable ATP analogue will also elicit this change, though it binds less tightly than ATP to Cpn60 and thus ejects peptide less efficiently (Jackson et al., 1993). In a similar way binding of ATP\(\gamma\S\) to hsc70 is relatively weak, which would explain the low efficiency of ATP\(\gamma\S\)-dependent uncoating compared with ATP-dependent uncoating.

Temperature-dependence studies show that, for brain coated vesicles at least, removal of clathrin has a low activation energy compared to that of ATP hydrolysis. This indicates that partial completion of the hsc70 ATPase cycle is sufficient to elicit release of clathrin, though hydrolysis makes the reaction more efficient. It has been reported that ATP hydrolysis causes further changes in DnaK conformation (Banecki et al., 1992), though the importance of similar changes in hsc70 for clathrin release are not known. Clathrin release from placental coated vesicles shows a greater dependency on ATP hydrolysis, and even then release is not complete. The simplest interpretation of these results is that placental coated vesicles have a more constrained structure than brain coated vesicles so that more energy is required to break the lattice. Efficient release might require additional factors working co-operatively with hsc70, as DnaJ and GrpE co-operate with DnaK (Ospiuk et al., 1993), to overcome these constraints. Alternatively, it might be argued that hsc70 does not play an active role in the uncoating reaction but merely binds to arms of clathrin triskelia transiently displaced from the lattice. By protecting these displacements, hsc70 would shift a dynamic equilibrium towards uncoating in a reaction not dependent on ATP hydrolysis. Conditions that favour transient dislocations, such as a looser structure in brain vesicles or elevated pH, would therefore affect the ability of hsc70 to uncoat. Efficient release of clathrin from placental vesicles at neutral pH might require more energy input, perhaps from another ATP\(\gamma\S\).

A second difference between coated vesicles from brain and those from placenta is their ability to stimulate hsc70-dependent ATP\(\gamma\S\)ase activity. Whilst brain-derived vesicles can catalyse multiple rounds of ATP hydrolysis, placental coated vesicles stimulate hydrolysis of only a single mole of ATP per mole of hsc70, then reduce the ATP\(\gamma\S\)ase activity of hsc70 to control levels. This inability to recycle active hsc70 may reflect the inability of hsc70 to dissociate from its substrate. Again, this might be due to constraints on hsc70-substrate movements imposed by the lattice structure, which could be overcome by the presence of ‘release’ co-factors allowing the ATP\(\gamma\S\)ase cycle to be completed. Localisation of these factors at, or close to, the target membrane would ensure that uncoating only occurs in the correct environment.

Alternatively, components of the vesicle could themselves influence the surface of the coat, so that their removal or modification would affect the ability of hsc70 to act as an efficient uncoating agent. Candidate proteins include GTP-binding proteins. Formation of coated vesicles at the cell surface involves the action of several GTP-binding proteins (Carter et al., 1993), and ADP Ribosylation Factor (ARF) is required for formation of clathrin-coated vesicles at the TGN (Stamnes and Rothman, 1993; Traub et al., 1993). Although the role of GTP-binding proteins in clathrin coat stability has not been established, comparison with COP-coated vesicles is informative. Here, coat stability has been linked intimately to the status of ARF. It is believed that ARF-GTP complexes are sufficient to stabilise COP coat assembly at the budding membrane (Serafini et al., 1991; Palmer et al., 1993; Orci et al., 1993), and hydrolysis of this GTP at the target membrane is necessary to drive coat disassembly (perhaps in conjunction with hsc70) (Tanigawa et al., 1993) and allow membrane fusion. Hence, if hydrolysis of GTP were required to destabilise coats sufficiently to allow hsc70 to act, then localisation of specific GTPase-activating proteins at target membranes could provide a means of directing the vectorial action of uncoating to the correct location within the cell.

Differences between components of brain and non-brain clathrin-coated vesicles that might account for their different susceptibilities to hsc70 are apparent. Neurone-specific forms of α- and β-adapts have been identified (Robinson, 1989; Ponnambalam et al., 1990), and light chains from brain vesicles possess insertions due to alternative splicing events (Jackson et al., 1987). Whilst these insertions do not contain
the binding sites for hsc70 that exist on clathrin light chains (Schmid et al., 1984; DeLuca-Flaherty et al., 1990) they might influence the structure of the coat. Furthermore, other neuron-speciﬁc coat components exist (Ahle and Ungewickell, 1986, 1990). These may simply inﬂuence the coat structure to make uncoating more favourable or they may play a more direct role in the uncoating reaction as endogenous ‘release’ co-factors. Recently, a 100 kDa component of clathrin coats has been identified that appears essential for the efﬁcient uncoating of brain-derived vesicles (Prasad et al., 1993), and preliminary evidence would indicate that this is the brain-speciﬁc protein auxillin (Prasad et al., 1993).

Although differences in coat structure and susceptibility to hsc70 could be caused simply by changes arising during isolation of the vesicles, they are more likely to be a consequence of the different coat components described above, and could be linked to physiological differences between the populations of vesicles. Since coated vesicles isolated from brain are almost solely involved in the recycling of synaptic membrane (Maycox et al., 1992), which occurs extremely quickly compared to the rates of receptor recycling in non-neuronal cells (Betz and Bewick, 1992), these vesicles may be specialized in their function. Although neuronal cells contain a classical endosomal compartment which can be labelled with ﬂuid-phase endocytic markers (Parton et al., 1992), they also possess a specialized endosomal compartment (Mundigl et al., 1993; Feany et al., 1993; Kelly, 1993). This consists of small vesicles, is labelled preferentially with synaptic vesicle proteins and predominates in the axonal regions of cells. This compartment may also exist in other specialized cells, including those that express the GLUT4 glucose transporter (Cain et al., 1992). Perhaps it is possible that synaptic vesicles are transformed from neuronal endocytic vesicles without transfer through a classical endosomal compartment, rendering a regulated uncoating reaction coupled to vesicle fusion unnecessary.

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