Inhibition of hsc70-catalysed clathrin uncoating by HSJ1 proteins

Michael E. CHEETHAM*, Brian H. ANDERTON† and Antony P. JACKSON‡§

*Department of Pathology, Institute of Ophthalmology, Bath Street, London, EC1V 9EL, U.K., †Department of Neuroscience, Institute of Psychiatry, De Crespigny Park, London SE5 8AF, U.K., and ‡Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

The uncoating of clathrin-coated vesicles can be mediated *in vitro* by the ‘uncoating ATPase’ that has been identified as the constitutive 70 kDa heat shock protein (hsp70), hsc70. It is now established that the activity of hsp70 proteins can be regulated by another family of molecular chaperones, the DnaJ family. In this study, we have investigated the effects of DnaJ-like proteins (the human neuron-specific proteins HSJ1a and HSJ1b) on clathrin uncoating. In order to measure the kinetics of clathrin release from coated vesicles, we have developed a quantitative, two-site ELISA for clathrin triskelions and demonstrated that stoichiometric amounts of HSJ1 proteins inhibit the initial burst of hsc70-mediated clathrin uncoating by over 40%. This inhibition

is not a consequence of ADP binding by hsc70 or the aggregation of hsc70, but correlates with an increase in the hsc70 associated with the coated vesicle fraction, suggesting that the inhibition is a consequence of a non-productive stabilization of hsc70 with a component of the coated vesicle fraction. These results strongly suggest that HSJ1 proteins interfere with an endogenous DnaJ-like protein that is involved in uncoating. Recent evidence suggests that the brain-specific vesicle-associated protein auxilin could play such a role. Although we find no evidence for auxilin in our coated vesicle preparation, our results predict that an auxilin-like protein will be a general factor in clathrin uncoating.

INTRODUCTION

The 70 kDa heat shock protein (hsp70) family of proteins fulfil many essential cellular functions in normal and stressed cells, including facilitating protein folding, translocation and protein complex assembly [1,2]. Many of these functions appear to be mediated by hsp70 proteins binding to exposed hydrophobic surfaces, thereby preventing aberrant inter- or intramolecular interactions [3]. Nonetheless, hsp70 proteins do not only interact with unfolded proteins but also associate with folded proteins and can mediate protein complex disassembly. The first documented function of hsp70 proteins, *in vitro*, was the ability of the mammalian constitutive hsp70, hsc70, to remove clathrin from clathrin-coated vesicles [4,5]. Clathrin-coated vesicles are responsible for a number of receptor-mediated transport events within the eukaryotic cell, and are composed of discrete layers of protein components. The coat comprises of inner layer of adaptors that interact with receptors and an outer layer of clathrin triskelions [6]. The physiological relevance of hsc70-catalysed uncoating is supported by the inhibition of receptor-mediated endocytosis by micro-injected antibodies to hsc70 [7]. However, the regulation of uncoating and the mechanism by which hsc70 strips clathrin from coated vesicles are poorly understood and may vary from tissue to tissue [8].

Analysis of the mechanisms of hsp70 action in other systems has suggested that when hsp70 proteins are involved in binding to folded proteins and complex disassembly events, they act in concert with at least one other stress protein. In *Escherichia coli*, hsp70(DnaK) is a host protein involved in bacteriophage replication. It binds lambda P protein and dissociates the pre-initiation complex, allowing replication to begin [9]. However, other stress proteins are required for correct positioning of hsp70(DnaK) and replication. Mutations in another gene, DnaJ, similarly suppress lambda replication, and *in vitro* reconstitution

of lambda replication shows that DnaJ protein interacts with hsp70(DnaK) to facilitate replication [9]. Furthermore, *in vitro* studies have shown that DnaJ stimulates the hydrolysis of ATP by hsp70(DnaK), whereas another protein that participates in lambda replication, GrpE, stimulates hsp70(DnaK) nucleotide exchange [10]. The interaction of DnaJ and hsp70(DnaK) has been demonstrated in several other processes, such as sigma 32 regulation [11,12], protein folding [13] and repair of heat-induced protein damage [14,15]. From these studies it appears that DnaJ is particularly important for hsp70(DnaK) function when binding to folded proteins (or intermediate conformational states) and/or protein complex disassembly is required.

Recently, a large family of proteins related to DnaJ has been described in eukaryotes [16]. These proteins are characterized by the presence of a highly conserved 70-amino-acid domain (the J domain), and we have suggested that the conserved function of DnaJ-like proteins is to activate hsp70 into an altered substrate-binding state, such that binding to folding intermediates and folded proteins would be preferred [17]. The role of hsc70 in binding assembled clathrin triskelions and removing them from coated vesicles appears to be more closely related to native protein binding and protein complex disassembly than the straightforward binding of exposed hydrophobic domains. Therefore, it would appear that a DnaJ-like protein is likely to participate in hsc70-catalysed clathrin uncoating.

HSJ1 proteins are human neuronal members of this DnaJ family [18,19] that can stimulate the ATPase activity of porcine brain hsc70 and regulate hsc70 substrate binding [17]. In this study we have analysed the effects of HSJ1 proteins upon hsc70-catalysed uncoating *in vitro*, and have shown that they modulate the activity of hsc70, inhibiting the initial burst of uncoating, apparently by the non-productive stabilization of the hsc70-clathrin complex. Sequence comparison has revealed that a previously described endogenous coated vesicle protein, auxilin,

Abbreviations used: hsp70, 70 kDa heat shock protein; hsc70, constitutive hsp70; CSP, cysteine string protein.

§ To whom correspondence should be addressed.

contains a J domain at its extreme C-terminus. We propose that HSI1 proteins are inhibiting uncoating by interfering with the normal interaction between an endogenous vesicle-associated DnaJ-like protein, such as auxilin, and hsc70.

MATERIALS AND METHODS

Purification protocols

Purification of pig-brain hsc70 and the DnaJ homologues HSI1a and HSI1b were as described previously [17]. Clathrin-coated vesicles were partially purified from PC12 neuroendocrine cells growing in tissue culture flasks. Typically, about 4×10^6 cells were removed from flasks by brief treatment with trypsin/EDTA. The suspension was centrifuged at 1000 *g* for 10 min and the cell pellet resuspended in PBS containing 0.01 % (w/v) PMSF. The cells were recentrifuged and the washing step repeated three more times. Cells were lysed by thorough mixing in 40 mM Hepes, pH 7.4/75 mM KCl/4.5 mM $MgCl_2$ /1.5 mM $CaCl_2$ /0.5 % (v/v) Nonidet P-40/0.01 % (w/v) PMSF (buffer A). The mixture was left on ice for 10 min and centrifuged at 1000 *g* for 10 min. The detergent treatment dissolves uncoated or partially coated membranes and also removes a contaminating ATPase otherwise found associated with clathrin-coated vesicle preparations [20]. The post-nuclear supernatants were centrifuged at 100 000 *g* for 20 min in a bench-top ultracentrifuge and the pellet was resuspended in 1 ml of 40 mM Hepes, pH 7.4/75 mM KCl/4.5 mM $MgCl_2$ /1.5 mM $CaCl_2$ /0.01 % PMSF (buffer B). After 15 min on ice, to remove any loosely associated clathrin, the mixture was recentrifuged at 100 000 *g* for 20 min and resuspended in 0.12 ml of buffer B. The protein concentration of such material was about 100 μ g/ml [20]. Clathrin triskelions used as standards in the ELISA were purified from porcine coated vesicles according to standard methods [21].

Uncoating assay

The uncoating assays were performed at room temperature in the wells of a 96-well tissue culture plate as follows. Two adjacent rows of five wells each were used in a typical experiment. Each well of the first row of the plate contained 0.03 ml of crude coated vesicle preparation (2 μ g total protein), creatine kinase (0.1 unit) and creatine phosphate (30 mM) in a total volume of 0.03 ml of buffer B. Each well of the second row contained hsc70 (2 μ g), ATP or ADP as appropriate (2 mM) and buffer B or buffer B containing 1.5 μ g of HSI1a or HSI1b, in a total volume of 0.03 ml. After allowing temperature equilibration (2–3 min), the reactions were initiated simultaneously using a multichannel pipette to transfer the contents of the second row wells to the first row wells and mixing thoroughly. After the appropriate time, the reactions in individual wells were stopped by transferring the whole mixture (0.06 ml) into tubes containing 0.06 ml of pre-cooled buffer B on ice. At the end of the experiment, all tubes were centrifuged at 100 000 *g* for 20 min at 4 °C. The supernatants were assayed for released triskelions as described below. In some experiments the pellets were resuspended in 0.12 ml of buffer B and duplicate samples were processed with the supernatants by Western blotting for clathrin, hsc70 and HSI1. Total clathrin in the crude coated vesicle sample was assayed as described in [22]. Briefly, 0.05 ml of the crude coated vesicle preparation was adjusted to pH 9.5 with 1–2 μ l of 1 M $NaCO_3$ and left for 1 h at 4 °C. The mixture was centrifuged at 100 000 *g* for 20 min. The supernatant was neutralized with 2 μ l of 1 M KH_2PO_4 and measured in the clathrin ELISA.

Clathrin ELISA

The two-site ELISA for free clathrin triskelions contained the anti-(clathrin) monoclonal antibody X22 [23] as the solid-phase capture reagent. The solution antibody was a polyclonal rabbit antiserum raised against an evolutionarily conserved sequence of clathrin light-chains [24]. The wells of a flat-bottomed 96-well enzyme immunoassay plate were coated overnight with 5 μ g/ml X22 in 0.05 M $NaCO_3$, pH 9.5. The following day, wells were washed and blocked with PBS containing 1 % (w/v) BSA (for 1 h), aspirated and incubated with 0.05 ml of sample or clathrin standard in buffer B. After 3 h at 4 °C, the wells were washed three times with PBS containing 0.1 % (v/v) Triton X-100 and incubated at 4 °C for 3 h with a 1/4000 dilution of the rabbit anti-(light-chain) sera in PBS containing 0.1 % (w/v) BSA. Wells were washed as described above and incubated for a further hour at 4 °C with peroxidase-labelled sheep anti-(rabbit IgG) sera diluted 1/10000 in PBS with 0.1 % (w/v) BSA. The wells were again washed as described above, warmed to room temperature and incubated with 0.2 ml of 0.4 mg/ml *o*-phenylenediamine dihydrochloride in 0.05 M phosphate/citrate buffer with 0.03 % (w/v) sodium perborate. The plates were left for 20–30 min and the absorbancies were measured at 450 nm on a Titerex Multiskan plate reader. Intra-assay coefficients of variation (CV) were estimated from a standard curve in which each concentration was determined from the means and standard deviations of four replicates [25].

Western blotting

Western blots of pellet and supernatant fractions from the uncoating assays were carried out for clathrin as described in [20], for hsc70 as described in [17] and for HSI1b as described in [19].

RESULTS

Inhibition of hsc70-catalysed uncoating by HSI1 proteins

Initially, we used a Western blot [20] to measure the effect of HSI1a on hsc70-catalysed uncoating of a rat (PC12) neuroendocrine cell coated vesicle preparation. Surprisingly, HSI1a (at equimolar concentrations with hsc70) inhibited uncoating (Figure 1). Being qualitative, this assay did not allow accurate analysis of the kinetics of this inhibition. To investigate this in a more quantitative manner we developed a sensitive two-site ELISA for free triskelions (see the Materials and methods section). The standard curve for the assay is shown in Figure 2.

Figure 1 Effect of HSI1a on clathrin uncoating measured using a qualitative blotting assay

Uncoating, in the presence and absence of HSI1a, was carried out as described in the Materials and methods section. Supernatant fractions were assayed for released clathrin by Western blotting with an anti-clathrin monoclonal antibody TD1 [20] and detected by incubation with ^{125}I -labelled rabbit anti-(mouse IgG) antibody.

Figure 2 Standard curve for the clathrin triskelion ELISA

Each point is the mean of four replicates with standard deviations indicated by error bars.

The intra-assay coefficient of variation over the entire range of clathrin concentrations measured was less than 4%. Practical considerations dictated that porcine brain triskelions were used as standards in the assay; however, serial dilution of the PC12 coated vesicle extract diluted out in parallel with the standard curve, and thus showed no species difference in immunoreactivity (Figure 2).

Previous studies have examined uncoating at relatively high concentrations of clathrin with purified components, such as cages. These highly purified vesicle preparations may not contain components involved in uncoating *in vivo*, as they may have been

Figure 3 Typical time course for hsc70-catalysed uncoating with (○) and without (●) the presence of an equimolar concentration of HSJ1b measured with the clathrin ELISA

Points are means of duplicates (variation less than 10%).

Figure 4 Stoichiometry of HSJ1b-mediated inhibition of hsc70-catalysed uncoating

Uncoating assays were performed as above in the presence of different amounts of HSJ1b. Percentage inhibition was calculated as the ratio of the initial velocities in the presence and absence of HSJ1b $\times 100$. Points are means of duplicates (variation less than 10%).

dissociated during purification. The high precision and sensitivity of our ELISA allowed the accurate quantification of uncoating kinetics at relatively low concentrations of clathrin in structurally complete coated vesicles that are likely to contain loosely associated proteins that may include additional necessary uncoating co-factors [26].

We used the two-site ELISA to follow the effect of HSJ1b on hsc70-catalysed uncoating, as our previous studies have shown that HSJ1b is a slightly more effective modulator of hsc70 than HSJ1a, though there is no mechanistic difference between the interaction of these isoforms with hsc70 [17]. hsc70-mediated uncoating was characterized by an initial burst of clathrin release, followed by a lower rate. The absolute value of these rates varied in experiments between different coated vesicle preparations, although the relative effect of HSJ1 proteins on a given coated vesicle preparation in the same experiment was always consistent. HSJ1b mainly effects the initial burst, causing more than a 40% inhibition (Figure 3) at the highest concentration of coated vesicles measured. The biphasic pattern of uncoating observed with our structurally complete vesicle preparations closely mirrors that first described by Greene and Eisenberg [27] with purified coated vesicles, in which the initial burst has been interpreted as corresponding to the first round of uncoating; the lower rate then represents the rate-limiting dissociation of hsc70 from free triskelions. Therefore, pre-incubation of HSJ1b with hsc70 appears to be modifying the action of hsc70 in the first round of uncoating. Our previous studies [17] have shown that HSJ1 proteins exert maximal effects on hsc70 ATPase activity and substrate binding at equal stoichiometric amounts; the partial inhibition of the initial burst of uncoating is also maximal at stoichiometric amounts and is not catalytic (Figure 4).

Mechanisms of inhibition

HSJ1 proteins enhance the hydrolysis of ATP by hsc70, but do not alter the rate of nucleotide exchange [17], such that the hsc70 would be predominantly in the ADP-bound form. Greene and Eisenberg [27] demonstrated that pre-incubation of hsc70 with ADP and hexokinase overnight could lower the rate of the initial burst of uncoating, thereby inhibiting uncoating. In order to show that the HSJ1b-mediated inhibition was not merely a consequence of ADP binding, we mimicked the effects of HSJ1b on hsc70 ATP hydrolysis by pre-incubating the hsc70 with ADP for sufficient time to allow ADP binding. The results show

Figure 5 Time course for hsc70-catalysed clathrin uncoating

hsc70 was preincubated in the presence of ATP (●) or ADP (■) both at 2 mM. Points are means of duplicates (variation less than 10%).

Figure 6 Partitioning of HSJ1b between assembled and unassembled clathrin fractions during the uncoating assay

Uncoating reactions were initiated as described in the Materials and methods section in the presence and absence of HSJ1b. Samples from indicated time points were centrifuged at 100 000 *g* to separate the pelleted assembled clathrin (P) from the released clathrin in the supernatant (S). Aliquots of the P and S fractions were probed by Western blotting for the presence of clathrin and hsc70, followed by incubation with peroxidase-labelled secondary antibody and developed using enhanced chemiluminescence [19].

(Figure 5) that under our conditions (high levels of creatine phosphate and creatine kinase) there is no difference between pre-incubation with ADP or ATP. So, HSJ1 proteins are modulating hsc70 function by another mechanism other than merely stimulating the ATPase activity of hsc70, as hsc70-ADP either undergoes rapid nucleotide exchange or does not inhibit uncoating under these conditions.

DnaJ-like proteins have been shown to affect hsp70 substrate binding to cause both release and stabilization [11–13,16,17]. Whether release or stabilization is stimulated appears to be dependent on the conformation of the substrate. Since hsc70 has been shown to bind triskelions [28], we analysed the partitioning of hsc70 between the assembled and unassembled clathrin fractions in our uncoating assay (Figure 6). The Western blotting of clathrin heavy-chain qualitatively confirmed the lower rate of uncoating with HSJ1b. In the absence of HSJ1b the majority of hsc70 was in the supernatant, with a small proportion co-localizing with the assembled vesicle fraction. In contrast, the presence of HSJ1b causes an increase in the proportion of the hsc70 associated with the assembled clathrin fraction. The HSJ1b,

Figure 7 HSJ1 does not induce aggregation of hsc70

hsc70 (2 μ g) was incubated with HSJ1b (1.5 μ g) in a total volume of 60 μ l of buffer B with ATP or ADP (1 mM) as indicated and incubated for 0 and 10 min at room temperature. Samples were diluted into 60 μ l of ice-cold buffer B and centrifuged at 100 000 *g* for 20 min. Pellets (P) were resuspended in 0.12 ml of buffer B. Supernatant (S) and (P) pellet fractions, 0.1 ml, were examined by SDS/PAGE. The bands at 70 and 42 kDa correspond to hsc70 and HSJ1b respectively.

however, remained largely in the supernatant at all time points (results not shown).

In the absence of coated vesicles, hsc70 remains in the supernatant in the presence of HSJ1b with both ATP and ADP (Figure 7). Therefore, the alteration in hsc70 partitioning with assembled clathrin induced by HSJ1b is not due to non-specific sedimentation of hsc70 under the conditions of our assay, but a more specific association with the assembled vesicle. This suggests that HSJ1 proteins inhibit hsc70-catalysed uncoating by stabilizing a non-productive association between hsc70 and an undefined component of the coated vesicle preparation, most probably clathrin.

Auxilin as a candidate endogenous DnaJ-like protein in uncoating

These results imply that HSJ1 proteins could be exerting their effect by interfering with the normal interaction between hsc70 and an endogenous DnaJ-like protein. Although the DnaJ family shows a great deal of sequence variation, all DnaJ-like proteins possess a highly conserved 70-amino-acid domain, the J domain. We have compared the published sequence of known vesicle-associated proteins with the J domain of HSJ1 proteins and a consensus J domain derived from the ProDom family 217 (an alignment of 16 DnaJ-like proteins and 25 viral T antigens). This comparison highlighted the presence of a J domain at the C-terminus of the uncoating cofactor auxilin (Figure 8). BLAST database comparison shows that the degree of sequence similarity between the putative J domain in auxilin and the consensus sequence ($P = 0.018$) is of the same magnitude as that between bacterial DnaJ proteins and the consensus sequence ($P = 0.021$).

This similarity has also been documented by Ungewickell et al. [29] who have elegantly demonstrated that the J domain of auxilin is required for the hsc70-mediated uncoating of reconstituted clathrin baskets. Auxilin would thus appear to be an ideal candidate for the endogenous DnaJ protein in our assays. However, auxilin is believed to be brain specific [30] and we have

HSJ1	2	YEILDVPRSSADDIKKAYRRKALQWHPDKNPDN..KEFAEKKFKEVAEAYEVLSDKHKKREIY 62
AUXILIN	848	WKPVGMDLVTPEQVRKVRKAVLVVHPDKATGQPEYQYAKMIFMELNDAWSEFENQGRPLY 910
'J' CONSENSUS	1	EMKKAYRKCKKYPHPDKGDD....EERMQELNELWKKM...EKEVYYARY 43
HSJ1	15	DIKKAYRRKALQWHPDKNPDNKEFAEKKFKEVAEAYEVLSDKHKKREIYDRY 65
'J' CONSENSUS	1	EMKKAYRKCKKYPHPDKGDDDEEMQ.....ELNELWKKMEVYYARY 43
AUXILIN	861	QVKKYVRKAVLVVHPDKATGQPEYQYAKMIFMELNDAWSEFENQGRPLY 910

Figure 8 Auxilin has a J domain

BESTFIT (GCG) comparison of auxilin and HSJ1 J domains and comparison with a consensus J domain (ProDom family 217). Identical residues are marked with a dash and semi-conservative changes by (.). Residue 910 is the C-terminus of auxilin.

been unable to detect auxilin in our PC12 coated vesicle preparations using anti-auxilin antibodies (gift from Dr. S. Morris, UCSF).

DISCUSSION

The interaction of DnaJ-like proteins and hsp70 has been demonstrated in several model systems [13–15,17]. In this study we examined the effect of human DnaJ-like proteins (HSJ1a and HSJ1b) on a physiological function of hsc70, i.e. the uncoating of clathrin-coated vesicles. Although the role of hsc70 in uncoating clathrin-coated vesicles is well established [5,6,24,27,28], the mechanisms of hsc70-catalysed uncoating are poorly understood. As DnaJ-like proteins enhance hsp70-mediated protein folding and complex disassembly [13–15], we believed that they may have a similar role in clathrin uncoating. Surprisingly, we find that HSJ1 proteins inhibit the initial burst of uncoating by more than 40% and this is not caused by the stimulation of hsc70 to an ADP-bound state.

A recent study has suggested that the yeast DnaJ-like protein YDJ1 can cause the polymerization of hsp70 in the absence of substrate [31] and used a sedimentation assay to show that YDJ1 can cause hsp70 'aggregation'. However, we have shown that under the conditions of our assay, HSJ1b does not cause the sedimentation of hsc70 in the absence of substrate, in the presence of either ATP or ADP (Figure 7). In contrast, when clathrin-coated vesicles are the substrate, HSJ1 enhances the sedimentation of hsc70 with the assembled clathrin vesicle fraction. Therefore, inhibition does not appear to be caused by HSJ1-induced polymerization or 'aggregation', but by the non-productive stabilization of an hsc70–protein complex.

It does not appear that HSJ1 proteins interact with coated vesicles directly, as they do not associate with the coated vesicle fraction at any time points observed and antigenically related proteins in the PC12 cell extracts do not appear to be enriched in the vesicle fraction (results not shown). Consequently, it would appear that HSJ1 proteins activate hsc70 into an altered substrate binding form prior to incubation with the coated vesicles. In this activated state, hsc70 could bind components of the clathrin-coated vesicle in a non-productive complex due to the premature activation of hsc70.

Therefore, we believe that HSJ1 proteins are not the correct DnaJ-like components in the physiological uncoating reaction. The coated-vesicles preparations used in this study are likely to contain many vesicle-associated proteins, including additional uncoating cofactors. Another endogenous DnaJ-like protein may be present in our vesicle preparations that activates hsc70 in the correct locale, thereby stimulating uncoating. The hsc70

uncoating enzyme has been shown to bind at the vertices of clathrin triskelions [32] and it has been suggested that clathrin light chains may facilitate this [24,33]. However, we believe it is more likely that a DnaJ-like protein is involved in the correct positioning of hsc70 on clathrin. HSJ1 proteins may, therefore, be interfering with the endogenous vesicle-associated DnaJ-like protein and cause the incorrect positioning of hsc70 on the coated vesicle, leading to the formation of a non-productive complex.

One possible candidate endogenous DnaJ-like protein is the cysteine string protein (CSP). CSP is found on the cytoplasmic face of synaptic vesicles [34] and is therefore a likely component of coated vesicles from neuroendocrine cells. However, the phenotype of CSP null mutant in *Drosophila* suggests that CSP is more likely to be involved in the fusion of synaptic vesicles at the pre-synaptic membrane rather than membrane recycling and uncoating [35].

A better candidate is the coated-vesicle-associated protein auxilin. This protein was first identified from brain coated vesicles as a factor that promoted the assembly of purified triskelions into baskets [30], and has been shown to promote the uncoating activity of hsc70 in a catalytic manner [36], a role consistent with our proposed model for an endogenous DnaJ-like protein in uncoating. Indeed, sequence comparison shows that auxilin has a region at its C-terminus that contains the J domain obligate conserved HPDK motif and the overall similarity is on a par with other DnaJ-like proteins of known importance. Recent functional studies on auxilin have shown that this J domain stimulates hsc70-mediated clathrin basket uncoating, whereas other regions of the molecule appear to be important for the association of auxilin with the vesicle [29] such that the proposed role of auxilin is to prime the clathrin basket and facilitate the correct positioning of hsc70 on the triskelion vertex.

So, auxilin would appear to be an ideal candidate for the endogenous DnaJ-like protein that HSJ1 proteins inhibit. However, auxilin is believed to be brain-specific, and as we cannot detect auxilin in our coated vesicle preparations, auxilin may not be present in PC12 cells. Nonetheless, our data provide strong evidence that an endogenous DnaJ-like protein is present in coated vesicles from these cells. So auxilin may represent a brain-specific isoform of a more general coated vesicle DnaJ-like protein, and may be another example of the evolution of structurally distinct isoforms of coated-vesicle-associated proteins (e.g. clathrin light chains, AP180) in brain.

The involvement of DnaJ-like proteins in the exocytic/endocytic pathway highlights the evolutionary diversification within the DnaJ family and the specialized roles that molecular chaperones play in cellular metabolism in addition to their fundamental functions in protein folding. This further illustrates that DnaJ-like proteins are not merely non-specific enhancers of hsp70 activity, but can serve to direct hsp70 function to specific cellular requirements.

The hsp70 chaperone machinery not only consists of DnaJ and hsp70 but also includes GrpE. Analyses of DnaJ/hsp70 interactions with protein complexes suggest that GrpE-like proteins will play an essential role in completing the cycle of hsp70 substrate binding and release initiated by DnaJ [9,13,17]. If coated vesicles have a specialized DnaJ-like protein to direct hsc70 function, then it is likely that a specialized GrpE-like protein will exist to further catalyse uncoating.

We thank Dr. Frances Brodsky for the anti-clathrin antibodies and Helen Coombs for technical assistance. This work was funded by MRC project grant G8931770CB to A.P.J. and the Wellcome Trust grant 036267 to M.E.C. and B.H.A.

REFERENCES

- 1 Gething, M.-J. and Sambrook, J. (1992) *Nature (London)* **355**, 33–45
- 2 Becker, J. and Craig, E. A. (1994) *Eur. J. Biochem.* **219**, 11–23
- 3 Hartl, F.-U., Hlodan, R. and Langer, T. (1994) *Trends Biochem. Sci.* **19**, 20–25
- 4 Ungewickell, E. (1985) *EMBO J.* **4**, 3385–3391
- 5 Chappell, T. G., Welch, W. J., Schlossman, D. M., Palter, K. B., Schlesinger, M. J. and Rothman, J. E. (1986) *Cell* **45**, 3–13
- 6 Brodsky, F. (1988) *Science* **242**, 1396–1402
- 7 Höning, S., Kreimer, G., Robenek, H. and Jockusch, B. M. (1994) *J. Cell Sci.* **107**, 1185–1196
- 8 Buxbaum, E. and Woodman, P. G. (1995) *J. Cell Sci.* **108**, 1295–1306
- 9 Georgopoulos, C., Ang, D., Liberek, K. and Zylicz, M. (1990) in *Stress Proteins in Biology and Medicine* (Morimoto, R. I., Tissieres, A. and Georgopoulos, C., eds.), pp. 91–221, Cold Spring Harbor Laboratory Press, New York
- 10 Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C. and Zylicz, M. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2874–2878
- 11 Gamer, J., Bujard, H. and Bukau, B. (1992) *Cell* **69**, 833–842
- 12 Liberek, K. and Georgopoulos, C. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11019–11023
- 13 Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K. and Hartl, F.-U. (1992) *Nature (London)* **356**, 683–689
- 14 Schroder, H., Langer, T., Hartl, F.-U. and Bukau, B. (1993) *EMBO J.* **12**, 4137–4144
- 15 Ziemienowicz, A., Skowrya, D., Zeilstrayalls, J., Fayet, O., Georgopoloulos, C. and Zylicz, M. (1993) *J. Biol. Chem.* **268**, 25425–25431
- 16 Caplan, A. J., Cyr, D. M. and Douglas, M. G. (1993) *Mol. Biol. Cell* **4**, 555–563
- 17 Cheetham, M. E., Jackson, A. P. and Anderton, B. H. (1994) *Eur. J. Biochem.* **226**, 99–107
- 18 Cheetham, M. E., Brion, J.-P. and Anderton, B. H. (1992) *Biochem. J.* **284**, 469–476
- 19 Cheetham, M. E., Brion, J.-P. and Anderton, B. H. (1994) in *Heat-shock Proteins in the Nervous System* (Mayer, R. J. and Brown, I. R., eds.), pp. 169–190, Academic Press, London
- 20 Acton, S. L., Wong, D. H., Parham, P., Brodsky, F. M. and Jackson, A. P. (1993) *Mol. Biol. Cell* **4**, 647–660
- 21 Jackson, A. P. (1993) in *Methods in Molecular Biology*, vol. 19, *Biomembrane Protocols I. Isolation and Analysis* (Graham, J. M. and Higgins, J. A., eds.), pp. 83–96, Humana Press Inc, Totowa, NJ
- 22 Goud, B., Huet, C. and Louvard, D. (1985) *J. Cell Biol.* **100**, 521–527
- 23 Nathke, I. S., Heuser, J., Lupas, A., Stock, J., Turck, C. W. and Brodsky, F. M. (1992) *Cell* **68**, 899–910
- 24 De-Luca Flaherty, C., McKay, D. B., Parham, P. and Hill, B. L. (1990) *Cell* **62**, 875–887
- 25 Ekins, R. P. (1983) in *Immunoassays for Clinical Chemistry*, 2nd edn., (Hunter, W. M. and Corri, J. E., eds.), pp. 76–105, Churchill Livingstone Press
- 26 Prasad, K., Barouch, W., Greene, L. and Eisenberg, E. (1993) *J. Biol. Chem.* **268**, 23758–23761
- 27 Greene, L. and Eisenberg, E. (1990) *J. Biol. Chem.* **265**, 6682–6687
- 28 Prasad, K., Heuser, J., Eisenberg, E. and Greene, L. (1994) *J. Biol. Chem.* **269**, 6931–6939
- 29 Ungewickell, E., Ungewickell, H., Holstein, S. E. H., Lindner, R., Prasad, K., Barouch, W., Martin, B., Greene, L. E. and Eisenberg, E. (1995) *Nature (London)* **378**, 632–635
- 30 Ahle, S. and Ungewickell, E. (1990) *J. Cell Biol.* **111**, 19–29
- 31 King, C., Eisenberg, E. and Greene, L. E. (1995) *J. Biol. Chem.* **270**, 22535–22540
- 32 Heuser, J. and Steer, C. J. (1989) *J. Cell Biol.* **109**, 1457–1466
- 33 Schmid, S. L., Braell, W. A., Schlossman, D. M. and Rothman, J. E. (1984) *Nature (London)* **311**, 228–231
- 34 Mastrogiacomo, A., Parsons, S. M., Zampighi, G. A., Jenden, D. J., Umbach, J. A. and Gundersen, C. B. (1994) *Science* **263**, 981–982
- 35 Zinsmaier, K. E., Eberle, K. K., Bucher, E., Walter, N. and Benzer, S. (1994) *Science* **263**, 977–980
- 36 Barouch, W., Prasad, K., Greene, L. E. and Eisenberg, E. (1994) *J. Biol. Chem.* **269**, 28563–28568