Articles

Effect of Yeast and Human DnaJ Homologs on Clathrin Uncoating by 70 Kilodalton Heat Shock Protein†

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ABSTRACT: We recently found that the DnaJ homolog auxilin is required for Hsc70 to uncoat clathrin baskets. In the present study, we investigated the effect of two other DnaJ homologs, YDJ1 from yeast and HDJ1 from humans, on the uncoating activity of Hsc70. Neither YDJ1 nor HDJ1 substituted for auxilin in supporting uncoating. Rather, in the presence of auxilin, both YDJ1 and HDJ1 strongly inhibited uncoating at pH 7 and also prevented the binding of Hsc70 to clathrin baskets at pH 6. Both YDJ1, as shown previously, and HDJ1 catalytically induce polymerization of Hsc70 into large polymers in ATP, and the YDJ1 concentration required to inhibit uncoating was similar to the concentration required for polymerization. However, uncoating was almost completely inhibited even at low concentrations of Hsc70 where only partial polymerization occurs, suggesting that YDJ1 inhibits uncoating not only by polymerizing the Hsc70 but also by some other mechanism as well. The effects of YDJ1 and HDJ1 were completely reversible; when they were removed, the Hsc70 regained full activity. Since both YDJ1 and HDJ1 inhibited the uncoating of clathrin baskets by brain cytosol as well as by purified Hsc70, this could be a physiological phenomenon which could affect other activities of Hsc70 in addition to uncoating.

It is now well recognized that the 70 kDa heat shock proteins (Hsp70) and the related constitutive proteins (Hsc70)[1] often perform their various functions in the cell working in combination with partner proteins (Rassow *et al*., 1995). This was first discovered for the *Escherichia coli* Hsc70, DnaK, which was shown to carry out its functions in combination with two partner proteins, DnaJ and GrpE (Alfano & McMacken, 1989; Zylicz *et al*., 1989). DnaJ is thought to bind to protein substrates such as σ^{32} and RepA and then present them to DnaK (Wickner, 1990; Gamer *et al*., 1992; Wawrzynow & Zylicz, 1995); DnaJ also activates the DnaK ATPase activity about 5−15-fold, which may be involved in stabilizing the DnaK−substrate complex (McCarty *et al*., 1995). GrpE is thought to increase the rate of nucleotide exchange on DnaK (Liberek *et al*., 1991). Alone, GrpE has relatively little effect on the ATPase activity of DnaK, but GrpE and DnaJ together increase the ATPase activity of DnaK about 50-fold (Liberek *et al*., 1991; McCarty *et al*., 1995).

As yet, no homologs of GrpE have been identified in the cytosol of eukaryotes, although a homolog of GrpE has been identified in yeast mitochondria (Bollinger *et al*., 1994; Ikeda *et al*., 1994; Laloraya *et al*., 1994). On the other hand, eukaryotes contain a large number of proteins in the DnaJ family, but the structure of these proteins varies greatly [see review in Caplan *et al*. (1993)]. *E. coli* DnaJ has 3 specific domains: a 70 amino acid J domain generally at the N-terminal of the protein; a cysteine-rich domain in the middle of the protein; and a "G/F" domain distal to the J domain which is thought to act as a hinge region separating the J domain from the cysteine-rich domain. Some of the DnaJ homologs found in eukaryotes, such as yeast YDJ1, contain all three of the domains found in *E. coli* DnaJ (Caplan & Douglas, 1991). Others contain only one or two of the domains. For example, the human cytoplasmic DnaJ, HDJ1, contains the J domain and the "G/F" domain (Ohtsuka, 1993), while Sec63 (Sadler *et al*., 1989), an integral membrane protein of the endoplasmic reticulum which interacts with the endoplasmic reticulum Hsc70 (BiP), contains only the characteristic 70 amino acid J domain. Finally, several DnaJ homologs such as MIM44 (Rassow *et al*., 1994), which interacts with mitochondrial Hsc70, and auxilin (Ungewickell *et al*., 1995) contain only small regions of limited homology to parts of the J domain.

Several of the eukaryotic DnaJs which have only limited homology to *E. coli* DnaJ have been shown to be involved in presentation of substrates to the Hsc70s. In yeast, Sec63 appears to present proteins to BiP (Brodsky & Schekman, 1993), and MIM44 appears to present proteins to the mitochondrial Hsc70 (Rassow *et al*., 1994; Schneider *et al*., 1995). In bovine brain, auxilin seems to be involved in presenting clathrin-coated vesicles to Hsc70 (Ungewickell *et al*., 1995). On the other hand, the function of more classical DnaJ proteins like YDJ1 is unclear. Caplan and co-workers (1992) found that mutations in YDJ1 produced defects in translocation of proteins into mitochondria and

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[1] Abbreviations: Hsc70, constitutive isoform of the 70 kDa heat shock protein; AP, assembly protein; FPLC, fast protein liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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the endoplasmic reticulum similar to those observed with mutations of the yeast cytoplasmic Hsc70, SSA1. Evidence has also been obtained in yeast that YDJ1 is involved with Hsc70 and Hsc90 in their interaction with steroid receptors and tyrosine kinases (Caplan *et al*., 1995; Kimura *et al*., 1995). However, in neither case is there evidence that the YDJ1 is involved in presenting protein substrates to Hsc70. Furthermore, although mutations in the yeast mitochondrial DnaJ protein lead to misfolding of imported proteins, they appear to affect the transfer of proteins from Hsc70 to the mitochondrial chaperonin, Hsc60, rather than affecting the import of proteins into mitochondria (Rowley *et al*., 1994). This suggests that the mitochondrial DnaJ is not directly involved in presenting proteins to mitochondrial Hsc70.

The available *in vitro* data are also ambiguous in regard to the effect of DnaJ homologs on the function of Hsc70. Although there is general agreement that the DnaJ homologs activate the Hsc70 ATPase activity, there are major disagreements on whether they strengthen or weaken the binding of peptide and protein substrates to Hsc70. Cyr *et al*. (1992) observed that YDJ1 not only activated the SSA1 ATPase activity but also weakened the binding of peptide substrates to SSA1. Similar effects occur with porcine brain Hsc70 and another human homolog of DnaJ, HSJ1 (Cheetham *et al*., 1994). On the other hand, several laboratories have shown that DnaJ homologs strengthen or stabilize the binding of substrates to DnaK and Hsc70 (Langer *et al*., 1992; McCarty *et al*., 1995; Wawrzynow *et al*., 1995; Minami *et al*., 1996). It has been suggested that this is caused by DnaJ homologs stimulating the hydrolysis of ATP to ADP by Hsc70, thereby priming the Hsc70 to bind substrates. Disagreements on the basic mechanism of action of DnaJ homologs are of major functional significance since it is clear that DnaJ homologs are intimately involved in the ability of DnaK and Hsc70 to refold denatured proteins *in vitro*. The mechanisms of action of the DnaJ homologs are of particular importance given the major disagreements on whether Hsc70 and DnaJ homologs can refold various substrates such as luciferase *in vitro* and whether ATP is necessary for this refolding activity (Freeman *et al*., 1995; Freeman & Morimoto, 1996; Minami *et al*., 1996).

In an effort to better understand the interaction of DnaJ homologs with Hsc70, we recently examined the interaction of the yeast DnaJ protein YDJ1 with bovine brain Hsc70 and yeast SSA1, and found that, in the presence of ATP where Hsc70 is normally monomeric, catalytic amounts of YDJ1 induced Hsc70 to form large polymers, an effect which was reversed when either ATP or YDJ1 was removed (King *et al*., 1995). On the other hand, DnaJ does not appear to induce polymerization of DnaK. Since substrates apparently bind only to monomeric Hsc70 (Gao *et al*., 1996), the ability of DnaJ homologs to induce polymerization of Hsc70 but not DnaK could explain why they inhibit the binding of protein and peptide substrates to Hsc70 but not to DnaK *in vitro*. However, it is not clear whether such inhibition occurs *in vivo*, and in addition, it is not clear how this effect of the DnaJ homologs relates to the ability of other DnaJ-like proteins such as auxilin to present substrates to Hsc70. In particular, since it has been suggested that DnaJ might prime DnaK to strongly bind substrates before the substrate actually binds (Wawrzynow *et al*., 1995), it is possible that other DnaJ homologs, in addition to auxilin, could support the uncoating reaction by priming Hsc70 to bind to clathrin baskets along with other substrates.

To approach this problem, we investigated the effect of YDJ1 on the ability of Hsc70 to uncoat clathrin-coated vesicles and artificial clathrin baskets with and without auxilin present. Our results show that, rather than substituting for auxilin, YDJ1 strongly inhibits the ability of Hsc70 to uncoat clathrin-coated vesicles. This inhibition occurs not only with yeast YDJ1 but also with human HDJ1, and not only occurs with purified Hsc70 but also occurs with brain cytosol. Thus, there is nothing present in isolated brain cytosol which prevents the inhibitory effect of HDJ1, suggesting that under certain conditions it might occur *in vivo*. Although this effect occurs over the same range of YDJ1 concentration as induces polymerization, it also occurs at low concentrations of Hsc70 where only partial polymerization occurs, suggesting that the inhibitory effect of YDJ1 involves more than just the polymerization of Hsc70. However, it is unlikely that the Hsc70 is covalently modified since the effect of YDJ1 is completely reversed when it is separated from Hsc70.

MATERIALS AND METHODS

YDJ1 and HDJ1 were prepared from recombinant expression proteins according to the procedure of King *et al*. (1995) and Freeman *et al*. (1995), respectively. Clathrin-coated vesicles, clathrin, mixed AP, and mixed AP−clathrin baskets were made according to the procedure of Barouch *et al*. (1994). AP_{180} was expressed as a GST-fusion protein and purified according to the procedure of Ye and Lafer (1995). Auxilin, the 100 kDa cofactor needed for uncoating, was prepared according to Prasad *et al*. (1993). Hsc70 was prepared according to Greene and Eisenberg (1990), and SSA1 was prepared according to Gao *et al*. (1991). Clathrin and cytochrome *c* peptide were labeled with [^{14}C]formaldehyde (Dupont) by reductive methylation according to the procedure of Jentoft and Dearborn (1983).

The uncoating of clathrin from clathrin-coated vesicles or AP−clathrin baskets was performed in buffer A (20 mM imidazole, 25 mM KCl, 10 mM ammonium sulfate, 2 mM magnesium acetate, and 1 mM dithiothreitol, pH 7.0) in the presence of 1 mM MgATP as described by Greene and Eisenberg (1990). Routinuely, the Hsc70 was mixed with the YDJ1 and ATP, and after 5 min at 25 °C, the reaction was started by addition of substrate. The reaction mixture was then incubated for 15 min, followed by ultracentrifugation in a TL-100 ultracentrifuge at 380000*g* for 6 min to separate the uncoated clathrin from clathrin-coated vesicles and baskets. Note that under these conditions, both monomer and polymerized Hsc70 remain almost entirely in the supernatant. Polymerized Hsc70 only shows significant sedimentation when centrifuged for longer times (15−20 min) and at high concentrations (5−10 μM) of Hsc70 (King *et al*., 1995). The quantification of the released clathrin was determined either by SDS gel electrophoresis on 4−20% SDS gels (Integrated Separation Systems) followed by gel scanning on an LKB Ultroscan-XL Laser Densitometer or by liquid scintillation counting of the ^{14}C-labeled clathrin.

The binding of Hsc70 to mixed AP baskets was performed identically to the uncoating experiments, except that buffer A contained 20 mM MES, pH 6.0, instead of imidazole. After centrifugation, the Hsc70 in the supernatant was quantified

by SDS gel electrophoresis, followed by gel scanning. The polymerization of Hsc70 by YDJ1 or HDJ1 was measured by incubating Hsc70 and the DnaJ homolog in buffer A, containing 1 mM ATP for 30 min at 25 °C. The mixture was then chromatographed on a Superose 12 FPLC column (King *et al*., 1995) equilibrated in buffer A, containing 1 mM ADP and 1 mM Pi. The elution pattern was determined by running the fractions on SDS gels. When dilute samples of Hsc70 were chromatographed, the fractions were first concentrated by trichloroacetic acid precipitation prior to gel electrophoresis.

RESULTS

Hsc70 uncoats clathrin-coated vesicles and clathrin baskets in an ATP-dependent reaction consisting of a stoichiometric initial burst of uncoating followed by a much slower steady-state uncoating (Greene & Eisenberg, 1990), and we previously showed that the 100 kDa protein, auxilin, is required for the uncoating of clathrin-coated vesicles by Hsc70 (Ungewickell *et al*., 1995). Since auxilin contains a limited region which is homologous to the J region present in classical DnaJ proteins, we were interested in whether other DnaJ homologs could substitute for auxilin to support the uncoating reaction, particularly since it has been proposed that DnaJ might prime Hsc70 to bind substrates before the substrates actually bind, implying that DnaJ homologs might nonspecifically induce binding of substrates to Hsc70. We, therefore, first determined whether YDJ1 could substitute for auxilin in the uncoating reaction. The solid squares in Figure 1A show that when YDJ1 was added in the absence of auxilin to AP_{180}−clathrin baskets prepared from purified clathrin and recombinant AP_{180}, Hsc70 was unable to uncoat these baskets whereas in the presence of auxilin normal uncoating occurred (data not shown). Therefore, YDJ1 is not able to substitute for auxilin in supporting the uncoating reaction.

We next tested the effect of YDJ1 on uncoating in the presence of auxilin; in these experiments, we used either clathrin−coated vesicles or clathrin baskets prepared with mixed assembly proteins which contain auxilin. Figure 1B shows the time course of uncoating of clathrin-coated vesicles by Hsc70 at varying concentrations of YDJ1. As the YDJ1 concentration was increased, the overall time course of uncoating remained the same but YDJ1 strongly inhibited the magnitude of the initial burst of uncoating. This is the same pattern of inhibition which we previously obtained with increasing peptide concentration (Greene *et al*., 1995) or decreasing auxilin concentration (Prasad *et al*., 1993). In the experiment shown in Figure 1B, the uncoating reaction was started by adding clathrin-coated vesicles to the other components, but an identical time course was obtained when the reaction was started by adding Hsc70, which shows that the inhibition of uncoating by YDJ1 occurs essentially instantaneously. Furthermore, we obtained nearly the same time course of uncoating by YDJ1 when mixed AP baskets rather than clathrin-coated vesicles were used as a substrate (data not shown).

Figure 1A summarizes the inhibition of uncoating by YDJ1 with both clathrin-coated vesicles and mixed AP−clathrin baskets over a wide range of YDJ1 and Hsc70 concentrations. These data were obtained by measuring the extent of uncoating at 15 min after the initial burst of uncoating was

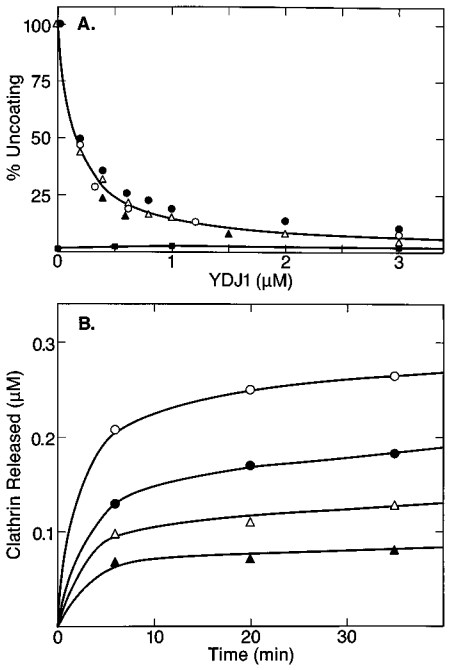

FIGURE 1: YDJ1 cannot substitute for auxilin, and it inhibits the initial burst of clathrin uncoating by Hsc70. In panel A, varying concentrations of YDJ1 (0.2−3 μM) were added to the following concentrations of Hsc70: 0.3 μM (▲), 0.9 μM (○, ●, ■), and 3 μM (△). The uncoating of clathrin by Hsc70 was performed with the following substrates: 0.3 μM clathrin-coated vesicles (▲), 0.9 μM clathrin-coated vesicles (●), 0.9 μM mixed AP baskets (○), 0.9 μM AP_{180}−clathrin baskets (■) devoid of auxilin, and 3 μM clathrin-coated vesicles (△). The addition of 0.2 μM auxilin gave stoichiometric uncoating with the AP_{180}−clathrin baskets as auxilin is necessary for these baskets to uncoat (data not shown). Each set of data was normalized by equating to 100% the uncoating obtained in the absence of YDJ1, i.e., 0.1 μM clathrin triskelions released by 0.3 μM Hsc70, 0.28 μM clathrin triskelions released by 0.9 μM Hsc70, and 0.9 μM clathrin triskelions released by 3 μM Hsc70. Panel B shows the clathrin released by Hsc70 at varying YDJ1 concentrations as a function of time. In this experiment, 0.5 μM clathrin-coated vesicles were uncoated by 0.9 μM Hsc70 in the absence of YDJ1 (○) and in the presence of the following concentrations of YDJ1: 0.1 μM (●), 0.2 μM (△), and 0.5 μM (▲). At the indicated times, the samples were centrifuged, and the clathrin released was determined.

complete. Under all conditions tested, rather than promoting uncoating like auxilin, YDJ1 profoundly inhibited the ability of Hsc70 to uncoat clathrin-coated vesicles or clathrin baskets in the presence of auxilin. Interestingly, even at 3 μM Hsc70, the inhibition of uncoating saturates at about 0.5 μM YDJ1, suggesting that YDJ1 is acting catalytically to inhibit uncoating; YDJ1 also acts catalytically when it induces polymerization of Hsc70 in the presence of ATP.

Auxilin apparently acts by inducing Hsc70 to bind to clathrin-coated vesicles in the presence of ATP (Ungewickell *et al*., 1995). Therefore, we were interested in determining whether YDJ1 inhibited uncoating by preventing this binding from occurring. This could not be investigated at pH 7 where uncoating occurs, but at pH 6, conditions that do not promote uncoating, Hsc70 binds to clathrin baskets in the presence of ATP and auxilin (Ungewickell *et al*., 1995; Barouch *et al*., 1997). As shown in Figure 2, the addition of YDJ1 causes a marked decrease in the amount of Hsc70 bound to the baskets which suggests that YDJ1 inhibits uncoating by preventing auxilin-induced binding of Hsc70 to clathrin baskets.

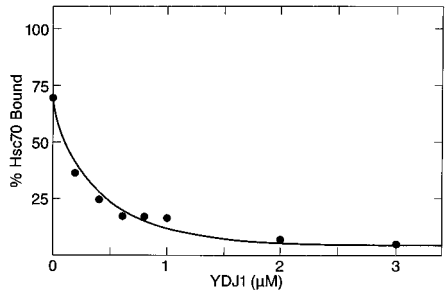

FIGURE 2: YDJ1 reversed the binding of Hsc70 to mixed AP baskets at pH 6.0. Varying concentrations of YDJ1 (0.2−3.0 μM) were added to 1 μM Hsc70 and 1 μM mixed AP baskets. After centrifugation, the amount of Hsc70 in the supernatant was quantified by SDS gel electrophoresis, followed by gel scanning.

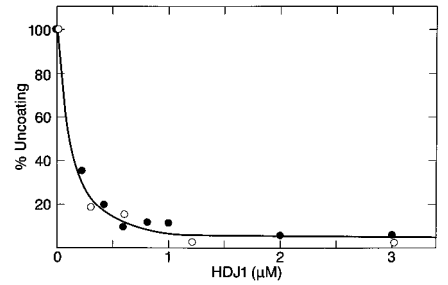

FIGURE 3: Effect of HDJ1 on the uncoating of clathrin by brain Hsc70. Varying concentrations of HDJ1 (0.2−3.0 μM) were added to 0.9 μM brain Hsc70. The substrate used was either 0.9 μM clathrin-coated vesicles (●) or 0.9 μM mixed AP baskets (○). The data were normalized as described in Figure 2.

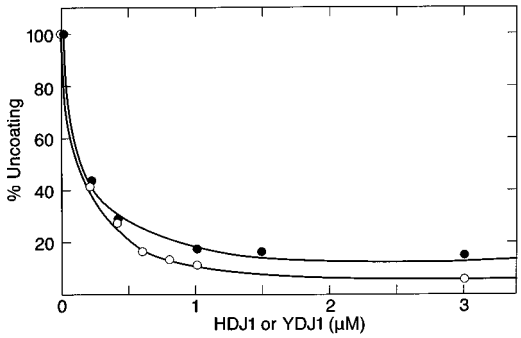

FIGURE 4: YDJ1 and HDJ1 inhibit the uncoating of mixed AP−clathrin baskets by brain cytosol. Varying concentrations (0.2−3.0 μM) of HDJ1 (○) and YDJ1 (●) were added to brain cytosol and 1 μM mixed AP baskets, composed of ^{14}C-labeled clathrin.

We next investigated whether the inhibition of uncoating is a specific effect of YDJ1, or whether it also occurs with a mammalian DnaJ homolog, HDJ1. Figure 3 shows that with both mixed AP−clathrin baskets and clathrin-coated vesicles, HDJ1 is as effective as YDJ1 in inhibiting the uncoating activity of bovine brain Hsc70. These data suggest that the inhibition of uncoating caused by the DnaJ proteins could occur physiologically since a mammalian DnaJ homolog is able to inhibit the activity of a mammalian Hsc70.

To further investigate the possibility that the inhibitory effects of YDJ1 and HDJ1 occur physiologically, we tested whether YDJ1 inhibits uncoating by the Hsc70 present in bovine brain cytosol. This experiment was performed using radioactive clathrin baskets as a substrate because the background level of clathrin present in the brain cytosol precluded quantitative determination of clathrin release using SDS gel chromatography. Figure 4 shows that the addition of either YDJ1 or HDJ1 inhibited uncoating by the Hsc70

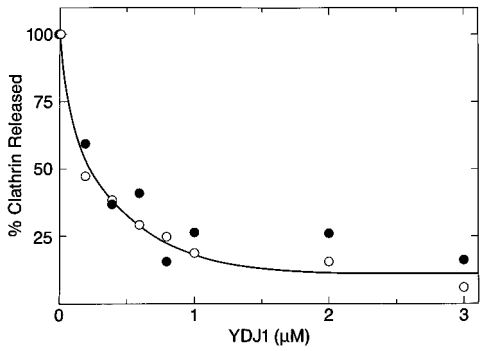

FIGURE 5: Absence of competition between auxilin and YDJ1 in the uncoating of mixed AP baskets. In this experiment, varying concentrations (0.2−3.0 μM) of YDJ1 were added to 0.6 μM mixed AP baskets and 0.9 μM Hsc70. This was done either in the absence of added auxilin (○) or in the presence of 0.4 μM added auxilin (●). The data were normalized to 100% uncoating, which was 0.33 μM in the absence of excess auxilin and 0.24 μM in the presence of excess auxilin.

present in bovine brain cytosol to the same extent as it inhibited uncoating by purified Hsc70. These data strongly suggest that there is nothing present in the isolated bovine brain cytosol which modifies either the added HDJ1 and YDJ1 or the intrinsic Hsc70s to prevent inhibition of the uncoating reaction.

We next investigated the mechanism by which YDJ1 and HDJ1 inhibit uncoating. One possible mechanism is that YDJ1 acts as a competitive inhibitor of auxilin. However, increasing the auxilin concentration had no effect on the concentration of YDJ1 required for inhibition as would have occurred if YDJ1 were acting as a competitive inhibitor (Figure 5). An alternative explanation for the YDJ1 effect is that it causes Hsc70 to polymerize and the polymerized Hsc70 is then unable to bind to the coated vesicles. This would be consistent with our previous observation that peptide substrates do not bind to the dimers, trimers, and small oligomers of Hsc70 which are present along with Hsc70 monomers in ADP (Gao *et al.*, 1996).

In support of the view that YDJ1 inhibits uncoating by polymerizing Hsc70, we found that about 0.5 μM YDJ1 almost completely polymerized 10 μM Hsc70 (King *et al.*, 1995), almost the same concentration of YDJ1 which causes complete inhibition of uncoating (Figure 1A). Similarly, 0.5 μM HDJ1 almost completely polymerized 10 μM Hsc70 (data not shown). However, YDJ1 also completely inhibited uncoating by 0.3 μM Hsc70 (Figure 1A), and examination of the magnitude of polymerization at this low Hsc70 concentration suggested that polymerization of Hsc70 could not be the complete explanation for the inhibition by YDJ1. The elution profile from a Superose 12 FPLC column obtained with 0.3 μM Hsc70 and 1 μM YDJ1 (Figure 6A) shows only about 50% polymerization under these conditions. For comparison, we also show the Hsc70 profile obtained in ATP in the absence of YDJ1 which is about 90% monomeric (Gao *et al.*, 1996) and the pattern of peptide bound to Hsc70 in ATP; since peptide only binds to monomer, this pattern clearly defines the position of monomer during column chromatography (Gao *et al.*, 1996). These data also show that the Hsc70 is not complexed with YDJ1 since its pattern of elution was not affected by the presence of YDJ1. To be certain that column chromatography is not depolymerizing the Hsc70 polymer at the low concentration of Hsc70 used in this experiment, we com-

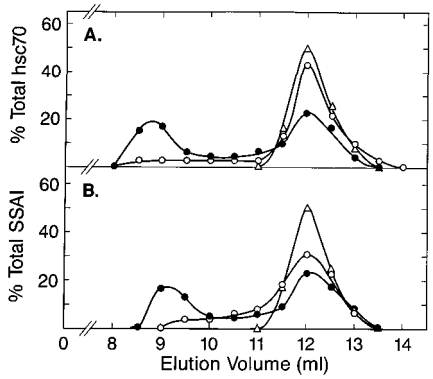

FIGURE 6: Superose 12 gel filtration profiles showing monomer−polymer distribution of Hsc70 and SSA1. In panel A, the elution patterns are of 0.3 μM Hsc70 in ATP (○), 0.3 μM Hsc70 in ATP and 1.0 μM YDJ1 (●), and of ^{14}C-labeled cytochrome *c* peptide bound to Hsc70 (△). In panel B, the elution patterns are of 3.0 μM SSA1 in ATP (○), 3.0 μM SSA1 in ATP and 3.0 μM YDJ1 (●), and of ^{14}C-labeled cytochrome *c* peptide bound to SSA1 (△). Peptide binding was measured using 50 μM peptide and 5 μM Hsc70 or SSA1 in 1 mM ADP, conditions which saturate these proteins with peptide (Greene *et al*., 1995). The extent of polymerization was quantified by measuring the fraction of Hsc70 in the polymer (elution volume 8.5−10.5 mL) and monomer peak (11.0−13.5 mL).

pletely polymerized 10 μM Hsc70 with YDJ1 and then diluted it to 0.3 μM before applying it to the column. The resulting elution pattern was nearly identical to undiluted 10 μM Hsc70; almost all of the Hsc70 was polymerized (data not shown). Therefore, the results show that at 0.3 μM Hsc70 about 50% of the Hsc70 is monomer and the remainder is polymerized, mostly in the form of large polymers.

We next examined whether the inhibition of uncoating stems solely from polymerization of Hsc70 by YDJ1. If this were the case, since about 50% of the Hsc70 occurs as monomer at 0.3 μM Hsc70, we would expect at most 50% inhibition of uncoating at this Hsc70 concentration, but in fact we obtained about 90% inhibition (Figure 1A). This suggests that not only polymerized Hsc70 but also monomeric Hsc70 is unable to carry out the uncoating reaction in the presence of YDJ1. This result was confirmed by two other experiments. Since, at pH 6, as at pH 7, the YDJ1 only partially polymerizes low concentrations of Hsc70, we first tested whether YDJ1 inhibited the binding of low concentrations of Hsc70 to clathrin baskets at pH 6. Our results showed that YDJ1 indeed caused much more inhibition of binding at 0.3 μM Hsc70 than would be expected given the low level of polymerization (data not shown). Second, we tested whether YDJ1 prevented uncoating by the yeast Hsc70, SSA1, which we previously showed is polymerized less by YDJ1 than bovine brain Hsc70 (King et al., 1995). This is confirmed in Figure 6B, which shows that about 50% of the yeast SSA1 occurs as monomer at a concentration of 3 μM. Yet as shown in Figure 7, again the YDJ1 inhibited SSA1 uncoating by more than 80%. This again strongly suggests that YDJ1 inhibits uncoating by monomeric Hsc70.

Since YDJ1 appears to prevent both monomeric and polymerized Hsc70 from uncoating, we wondered if covalent modification of the Hsc70 by YDJ1 might inhibit uncoating. In this regard, phosphorylation of BiP, the Hsc70 present in the endoplasmic reticulum, has been associated with its polymerization and inactivation (Freiden *et al*., 1992).

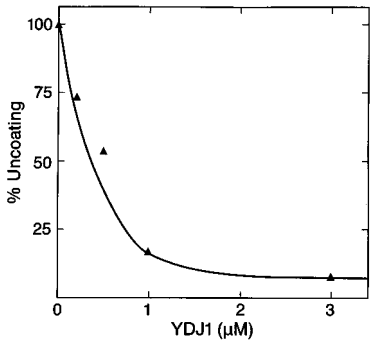

FIGURE 7: YDJ1 inhibits uncoating of clathrin-coated vesicles by SSA1. Varying concentrations (0.2−3.0 μM) of YDJ1 were added to 3.0 μM SSA1 to determine its effect on the uncoating of 1 μM clathrin-coated vesicles. In the absence of YDJ1, SSA1 released 0.3 μM clathrin triskelions, in agreement with Gao *et al*. (1991).

However, we found that the effect of the YDJ1 is completely reversible. Immediately following column chromatography which separates polymerized Hsc70 from YDJ1, the Hsc70 obtained from the polymeric peak, which is devoid of YDJ1, regains complete uncoating activity (data not shown) suggesting that YDJ1 must actually be present to prevent Hsc70 from uncoating.

DISCUSSION

DnaJ-like partner proteins of Hsc70 vary widely in their homology to the DnaJ of *E. coli*. One of the proteins we have used in this study, yeast YDJ1, has all three domains found in *E. coli* DnaJ: the N-terminal J domain, the G−F hinge domain, and the cysteine-rich domain in the center of the protein (Caplan & Douglas, 1991). A second protein we studied, human HDJ1, has only the first two domains (Ohtsuka, 1993) while a third protein we used, auxilin, has only a limited region which is homologous to the J domain (Ungewickell *et al*., 1995). In the uncoating reaction, the presentation of clathrin baskets to Hsc70 was performed by the protein auxilin (Ungewickell *et al*., 1995). Since *E. coli* DnaJ apparently presents protein substrates to DnaK, it might be expected that homologous proteins like YDJ1 could also carry out this function particularly if DnaJ homologs prime Hsc70 to bind substrates before they actually bind to Hsc70 (Wawrzynow *et al*., 1995). Our current results suggest that YDJ1 and HDJ1 cannot substitute for auxilin and, in fact, strongly inhibit uncoating by bovine brain Hsc70 in the presence of auxilin. Therefore, our data on uncoating suggest that the presentation of proteins to Hsc70 by partner proteins does not require extensive homology to *E. coli* DnaJ and, in addition, proteins which have considerable homology to *E. coli* DnaJ may be specific in the substrates which they present to Hsc70 and may also have other functions in the cell.

Similar observations have been made for the role of partner proteins in other functions of Hsc70 in the cell. Data obtained for mitochondria show that MIM44 which has only very limited homology to *E. coli* DnaJ presents substrates to mitochondrial Hsc70 (Rassow *et al*., 1994; Schneider *et al*. 1994), while the mitochondrial DnaJ homolog, MDJ1, appears to be involved in the dissociation of protein substrates from Hsc70 as they are passed to Hsc60 for folding (Rowley *et al*. 1994). In the endoplasmic reticulum, as well, the membrane protein Sec63 appears to present proteins to the endoplasmic Hsc70, BiP (Brodsky & Schekman, 1993), while the DnaJ homolog present in the lumen of the

endoplasmic reticulum, SCJ1, may play some other physiological role (Schlenstedt *et al*., 1995). Furthermore, cytoplasmic Hsc70 cannot substitute for BiP (Brodsky *et al*., 1993), again suggesting specificity in the interaction between Hsc70s and DnaJ homologs.

Our observation that YDJ1 and HDJ1 cause the formation of large polymers of Hsc70 over the same range of concentration as they inhibit the uncoating activity of Hsc70 suggested that perhaps polymerization of the Hsc70 prevented it from interacting with the clathrin-coated vesicles. However, our data clearly show that either the low concentrations of bovine brain Hsc70 or with yeast Hsc70, considerable amounts of the Hsc70 remained monomeric in the presence of YDJ1, yet the YDJ1 still almost completely inhibited uncoating. Furthermore, in the absence of clathrin baskets, auxilin itself appears to induce the polymerization of Hsc70, although unlike YDJ1 and HDJ1, it remains bound to the polymerized Hsc70 (Jiang *et al*., 1997). Therefore, although polymerization of Hsc70 may play a role in the inhibition by YDJ1, YDJ1 must be having other effects as well. One possibility is that YDJ1 is affecting the state of the nucleotide bound to monomeric Hsc70. We have shown that Hsc70−ADP does not bind to clathrin baskets in the presence of auxilin. Furthermore, although both auxilin and YDJ1 activate the rate of ATP hydrolysis by Hsc70 (Jiang *et al*., 1997), preliminary data suggest that YDJ1, at a concentration which causes marked inhibition of uncoating, appears to activate the rate of the ATP hydrolysis step of Hsc70 about 10 times more than auxilin. Therefore, by stimulating conversion of the bound nucleotide on Hsc70 from ATP to ADP more rapidly than auxilin, YDJ1 may prevent even monomeric Hsc70 from interacting with clathrin baskets. Note that, if this is the case, it implies that hydrolysis of ATP to ADP can inhibit the binding of a substrate rather than promoting it as suggested by models in which DnaJ homologs prime Hsc70 to bind substrates (Wawrzynow *et al*., 1995).

Two aspects of our data imply that the inhibitory effect of DnaJ homologs could occur *in vivo*. First, we have shown that this inhibition is caused by HDJ1, a human DnaJ protein, and second, we have shown that this inhibition occurs in a system with isolated brain cytosol and HDJ1. However, the question then arises as to why this inhibition does not occur in the isolated brain cytosol before the addition of extraneous HDJ1. One possibility is that there is relatively little HDJ1 present in cells and, therefore, it has relatively little effect on the Hsc70 activity. Alternatively, the HDJ1 may be located in such a way, e.g., attached to membranes, so that it only inhibits the Hsc70 activity under closely controlled conditions. For example, the HDJ1 may inhibit the Hsc70 unless the HDJ1 is actually bound to substrate, in which case it will present the substrate to the Hsc70. In any event, it will be interesting to overexpress either YDJ1 or HDJ1 in mammalian cells and determine its effect on the physiology of the cell.

While this paper was in the review process, Cheetam *et al*. (1996) showed that HSJ1, another human DnaJ homolog, inhibits uncoating of clathrin-coated vesicles prepared from PC12 cells by about 50%; these experiments were carried out using porcine Hsc70. Based on sedimentation experiments, it did not appear that the porcine Hsc70 was polymerized by HSJ1. Therefore, these data are consistent with our observation that DnaJ homologs inhibit uncoating by Hsc70 even under conditions where they do not induce polymerization of Hsc70. However, the HSJ1 appeared to induce the porcine Hsc70 to form an inactive complex with the clathrin-coated vesicles even though the HSJ1, itself, did not bind to the vesicles. In contrast, we did not observe binding of bovine brain Hsc70 to coated vesicles under conditions where YDJ1 or HDJ1 inhibits uncoating; as discussed under Materials and methods, in our experiments, centrifugation sedimented only clathrin-coated vesicles, not monomeric or polymerized Hsc70. Therefore, further work will be required to determine the mechanism by which DnaJ homologs inhibit uncoating by Hsc70.

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