We have studied the direct interaction of the constitutive isoform of Hsp70 (Hsc70) with the DnaJ homolog, auxilin, a cofactor that binds to clathrin-coated vesicles and is required for their uncoating by Hsc70. Auxilin caused a 5-fold increase in Hsc70 ATPase activity and a corresponding increase in steady-state levels of bound ADP; the dissociation constant for this effect was 0.6 μM. Auxilin also induced polymerization of Hsc70 and bound to the resulting polymer at a 1:1 molar ratio; here too the dissociation constant was 0.6 μM. Both this binding and polymerization required ADP; the Hsc70 depolymerized with a 4-min half-life when ATP was completely hydrolyzed to ADP. Although auxilin induces polymerization stoichiometrically and other DnaJ homologs induce polymerization catalytically, these data show that auxilin is similar to other DnaJ homologs in its ability to activate the Hsc70 ATPase activity, to polymerize Hsc70, and in the nucleotide dependence of this polymerization. Furthermore, the 70-amino acid J-domain of auxilin polymerized Hsc70 with the same nucleotide dependence as intact auxilin. Therefore, although only auxilin and not other DnaJ homologs support uncoating, our data suggest that various DnaJ homologs share a common mechanism of interaction with Hsc70, perhaps because their J-domains interact similarly with Hsc70.

Members of the 70-kDa class of heat shock proteins are not only expressed after heat shock but are also present constitutively in almost all living cells (1, 2). Both Hsp70 and the related constitutive protein, Hsc70, function as molecular chaperones in mediating biological processes such as the folding and unfolding of proteins, the formation and dissociation of protein complexes, and the translocation of proteins across membranes (3). Their basic mechanism of action is not understood although it appears to involve the binding and release of proteins in an ATP-dependent manner (4–6).

It has now become clear that the Hsp70 class of proteins does not function alone; its action depends on the presence of partner proteins (7). Much of the work on the mechanism of these partner proteins has been carried out with the Escherichia coli Hsc70 protein, DnaK, and its partner proteins, DnaJ and GrpE, which are required for numerous functions of DnaK. Thus far, GrpE homologs have only been found in eukaryotic mitochondria (8), but there are numerous eukaryotic DnaJ homologs that interact with Hsc70 (7, 9, 10). Some are members of the DnaJ class of proteins which is generally thought to function by presenting substrate proteins to Hsc70, but the mechanism of this presentation, particularly with eukaryotic Hsc70, remains unclear. Several laboratories have found that the binding of unfolded proteins and peptides to Hsc70 is inhibited by DnaJ homologs (14–16). On the other hand, studies on the refolding of several proteins by Hsc70 have strongly suggested that DnaJ homologs promote substrate binding to Hsc70 or at the very least are required for refolding to take place (8, 17, 18). Even in these studies, however, there is controversy as to whether ATP is always required for refolding as well as to whether Hsc70 and DnaJ alone are sufficient for refolding or other factors are also required. It is possible that some of these controversies stem from the fact that in almost all of the studies on the interaction of Hsc70 with eukaryotic DnaJ homologs the substrates being used are not physiological substrates for Hsc70 in vitro.

Because auxilin is one of the few eukaryotic DnaJ homologs that presents a known substrate to Hsc70 both in vivo and in vitro, we are carrying out detailed studies on the interaction of Hsc70, auxilin, and clathrin-coated vesicles. Thus far we have found that the ability of auxilin to support uncoating is not duplicated by a number of other DnaJ homologs such as the yeast DnaJ homolog, YDJ1. YDJ1 is not only unable to support the uncoating reaction itself but, in addition, it strongly inhibits uncoating and the binding of Hsc70 to clathrin baskets in the presence of auxilin (19), suggesting that the various DnaJ homologs are specific in the substrates they present to Hsc70. Nevertheless, it is still possible that the various DnaJ homologs including auxilin have similar direct effects on Hsc70 in the absence of substrate proteins. We previously showed that YDJ1 as well as DnaJ catalytically induces polymerization of Hsc70 in a reaction that requires ATP (20) and, in addition, that YDJ1 causes a rapid initial burst of ATP hydrolysis and thus increases the steady-state ATPase rate of Hsc70 (21). In the present study we investigated whether auxilin directly interacts with Hsc70 and, if so, whether this interaction is similar to the interaction of other DnaJ homologs such as YDJ1 with Hsc70.

Our results show that auxilin does indeed interact directly with Hsc70. As with YDJ1, low concentrations of auxilin cause a marked increase in the Hsc70 ATPase rate although auxilin...
do not cause as rapid an initial burst of ATPase activity as YDJ1. Furthermore, like YDJ1, auxilin appears to polymerize Hsc70 in an ATP-dependent reaction. In addition, the 70-amino acid J-domain of auxilin binds to Hsc70 with the same nucleotide dependence as auxilin itself. These data suggest that although auxilin appears to act specifically in regard to the substrate it presents to Hsc70, it closely resembles other DnaJ homologs such as YDJ1 in its direct interaction with Hsc70.

EXPERIMENTAL PROCEDURES

Proteins—Auxilin was prepared by the procedures of Prasad et al. (11) with minor modifications. After chromatography of a 0.5 M Tris extract of calf brains on Superose 6 and hydroxyapatite columns, the auxilin was further purified by FPLC on a Pharmacia Mono Q HR 5/5 column (1 ml) equilibrated in 20 mM Tris, pH 8.0. Auxilin was eluted at 0.2 ml/min with a gradient of 25–500 mM NaCl, 20 mM Tris, pH 8.0. The auxilin fraction was then collected and dialyzed against 10 mM Tris, pH 8.0, and the concentration was determined as described previously (11).

Hsc70 and clathrin-coated vesicles were prepared according to Greene and Eisenberg (22). Hsc70 and peptides were trace-labeled by 8.0, and the concentration was determined as described previously (11).

Interaction of Auxilin with Hsc70

The results clearly show that auxilin causes about 5-fold activation of Hsc70 ATPase activity, which indicates that auxilin interacts directly with Hsc70. To determine how strongly auxilin interacts with Hsc70, we determined the dependence of Hsc70 ATPase activity on auxilin concentration (Fig. 2). The double-reciprocal plot in the inset of Fig. 2 yields a dissociation constant of 0.6 µM, showing that auxilin interacts very tightly with Hsc70 in ATP.

Because the rate of ATP hydrolysis is the rate-limiting step during steady-state ATP hydrolysis by Hsc70 (25), auxilin appears to increase this rate, which is typical of the effect of DnaJ homologs on Hsc70. In addition to the steady-state activation, YDJ1 causes a rapid initial burst of Hsc70 ATP hydrolysis (21), and because the ATP hydrolysis step is much faster than the rate of ADP release, almost all of the nucleotide bound to Hsc70 is ADP during steady-state ATP hydrolysis in the presence of YDJ1. However, auxilin did not have a similar effect on the nucleotide bound to Hsc70.

FIG. 1. Time course of ATPase activity of Hsc70 in the absence and presence of 2 µM auxilin. Hsc70 (1 µM) was preincubated with 20 µM [γ-32P]ATP for 10 min at 25 °C. At zero time auxilin was added. The data of P, release versus time were obtained in the presence (●) and absence (○) of auxilin. The data were corrected for hydrolysis, which occurred during the incubation time.

FIG. 2. ATPase activity of Hsc70 in the presence of different concentrations of auxilin. The ATPase activity was measured as described in Fig. 1 using 1.0 µM Hsc70 and varied concentrations of auxilin (0.2–4.0 µM). The inset is a double-reciprocal plot of the ATPase data in which the abscissa intercept is a measure of the affinity of Hsc70 for auxilin. The data were corrected for the rate of ATP hydrolysis by the Hsc70 in the absence of auxilin.
file was not affected by the speed of elution, this method appears to be a valid measurement of the interaction of Hsc70 with auxilin. This shift in the elution profile could be due either to the auxilin catalytically inducing polymerization of Hsc70 or to complex formation between auxilin and Hsc70. To test this point, we increased the Hsc70 concentration to 10 μM and determined the elution profile of both Hsc70 and auxilin. Using 0.7 μM auxilin, only a small percentage of the 10 μM Hsc70 shifted to the void volume (data not shown), whereas at the same time almost all of the auxilin, which by itself eluted slightly faster than monomeric Hsc70, eluted in the void volume (Fig. 3B). This differs from the effect of YDJ1, which catalytically induces complete polymerization of Hsc70, so that essentially all of the Hsc70 elutes in the void volume, but is not itself bound to the resulting polymer (20).

These data suggest that, rather than catalytically inducing polymerization of Hsc70, auxilin forms a complex with Hsc70 that then elutes in the void volume of the Superose 12 column. To test this point, we determined the amount of Hsc70 that elutes in the void volume as the Hsc70 concentration is increased with the auxilin concentration held constant. With increasing Hsc70, the auxilin present shifted to the void volume along with the Hsc70 (see Fig. 3). When these data are plotted according to the Scatchard equation (Fig. 4), the results show that auxilin binds to Hsc70 at a 1:1 molar ratio with a dissociation constant of about 0.6 μM, in good agreement with the dissociation constant determined from the activation of the Hsc70 ATPase activity by auxilin.

Because Hsc70 forms a 1:1 complex with auxilin, the question arises as to whether elution in the void volume of the Superose 12 column is simply due to anomalous elution of the auxilin-Hsc70 complex or whether Hsc70 is indeed forming large polymers composed of Hsc70 and auxilin. To test this point, we examined the elution profile of Hsc70 on a Superose 6 column that has an exclusion size of about 4 \times 10^7 Da. Although not all of the Hsc70 eluted in the void volume of the Superose 6 column, the Hsc70 did elute as a very broad peak starting at the void volume, a similar elution pattern we observed for Hsc70 polymerized by YDJ1 (data not shown). Therefore, these data are consistent with auxilin inducing polymerization of Hsc70 but remaining attached to the polymer at a 1:1 molar ratio rather than acting catalytically like YDJ1.

We previously found that YDJ1 polymerized Hsc70 in the presence of ATP; in ADP no polymerization occurred, and furthermore, when all of the ATP was hydrolyzed to ADP, the polymerization was completely reversed (20). We were therefore interested in determining the nucleotide dependence of the interaction between auxilin and Hsc70. In contrast to its effect in ATP, auxilin had no effect on the elution profile of Hsc70 in ADP (data not shown). The observation that auxilin does not interact with Hsc70 in ADP might be either a kinetic or an
equilibrium phenomenon. Specifically, auxilin might bind to Hsc70 as strongly in ADP as it does in ATP but bind much more slowly in ADP, like the interaction of Hsc70 with clathrin and peptide substrates (26, 27). Alternatively, auxilin might bind much more weakly to Hsc70 in ADP than ATP. To distinguish between these possibilities, the Hsc70-auxilin complex was formed in the presence of ATP, hexokinase/glucose was added to completely hydrolyze the ATP to ADP, and then, after incubation for varied times at 25 °C, the solution was chromatographed on a Superose 12 FPLC column, and the elution profile of the Hsc70 was determined.

We next tested whether the 70-amino acid auxilin J-domain would retain the properties of auxilin in regard to its interaction with Hsc70. We have found that a recombinant GST-auxilin fusion protein acts like normal auxilin in supporting polymerization of the Hsc70. While this paper was in review, Holstein et al. (29) also presented evidence for direct interaction between auxilin and Hsc70. While this paper was in review, Holstein et al. (29) also presented evidence for direct interaction between auxilin and Hsc70.

Our results show that, like the yeast DnaJ homolog, YDJ1, very low concentrations of auxilin activated the Hsc70 ATPase activity; a double-reciprocal plot yielded a dissociation constant of auxilin from Hsc70 of about 0.6 μM. In addition, like YDJ1, it increased the amount of ADP bound to Hsc70 during steady-state ATP hydrolysis. However, because it did not increase the rate of the ATP hydrolysis step as much as YDJ1, which caused a rapid initial burst of ATP hydrolysis (21), only 65% of the bound nucleotide occurred as ADP with auxilin compared with almost 100% with YDJ1. Finally, like YDJ1 and DnaJ, auxilin appeared to induce polymerization of Hsc70. However, it did not do so catalytically because FPLC showed that auxilin remained bound to polymerized Hsc70 at a 1:1 molar ratio of auxilin to Hsc70. We do not yet understand why auxilin remains bound to the polymerized Hsc70 whereas YDJ1 dissociates from it; much more work is required to determine the role of Hsc70 polymerization in the cell and how this polymerization is related to the interaction of Hsc70 with various DnaJ homologs.

In addition to their similarity in polymerizing Hsc70 and activating the Hsc70 ATPase activity at very low concentrations, YDJ1 and auxilin also show a striking similarity in the nucleotide dependence of their interaction with Hsc70. Like

**Fig. 7. Interaction of auxilin J-domain with Hsc70 in ATP.** Hsc70 (10 μM) was incubated in the absence (○) and presence (●) of 7 μM auxilin J-domain for 1 h at 25 °C in 1 mM ATP with an ATP regenerating system (30 units/ml creatine kinase, 15 μM creatine phosphate). The solutions were then chromatographed on a Superose 12 column. Fig. 5 shows that the Hsc70-auxilin complex dissociated in ADP with a half-life of about 4 min and completely dissociated in about 20 min. Therefore, the absence of auxilin binding to Hsc70 in ADP is not due to a slow rate of binding; rather ATP must be present to maintain complex formation between auxilin and Hsc70.

We next tested whether an even smaller fragment of auxilin would resemble auxilin in its interaction with Hsc70. Tsai and Douglas (28) recently suggested that a 20-amino acid peptide, corresponding to positions 21–40 of the J-domain of YDJ1 and containing the highly conserved HPD sequence at positions
other DnaJ homologs, auxilin requires ATP to polymerize Hsc70 and to bind to the resulting polymer (20). Furthermore, as soon as the ATP is hydrolyzed to ADP, the Hsc70 polymer dissociates, and the auxilin dissociates from the Hsc70. Therefore, in contrast to the interaction between peptide substrates and Hsc70, which is weak in ATP and strong in ADP (27), auxilin, like YDJ1, shows no interaction with Hsc70 in ADP but binds strongly in ATP. Interestingly, this same nucleotide dependence is observed for the auxilin-induced binding of clathrin baskets to Hsc70 at pH 6 where uncoating does not occur (12). Here, too, when ATP is converted to ADP, Hsc70 dissociates from the clathrin baskets. Furthermore, Hsc70 dissociates from the baskets with about the same 4-min half-life as it dissociates from auxilin when ATP is converted to ADP, and the dissociation constant of Hsc70 from clathrin baskets at saturating auxilin is 0.6 μM (30), the same value as the dissociation constant of Hsc70 from auxilin. It is, therefore, tempting to speculate that the nucleotide dependence of the auxilin-induced binding of clathrin baskets to Hsc70 is related to the nucleotide dependence of the binding of auxilin to Hsc70. However, auxilin is not simply forming a bridge between clathrin baskets and Hsc70 because auxilin acts catalytically rather than stoichiometrically in inducing the binding of Hsc70 to clathrin baskets at pH 6.5 (12).

The observation that the auxilin J-domain shows the same nucleotide dependence as auxilin in its interaction with Hsc70 suggests that this J-domain may be crucial in establishing this nucleotide dependence. This is consistent with our previous observation that auxilin still binds to clathrin baskets after its J-domain is removed, suggesting that auxilin has separate clathrin-binding and Hsc70-binding domains (12). However, the auxilin J-peptides containing the HDPK region do not show the same nucleotide dependence as auxilin in their interaction with Hsc70; in contrast to a similar peptide from the J-domain of YDJ1 (28), they appear to interact with Hsc70 like any other peptide substrate. Therefore, more work is required to determine exactly which portions of the J-domain are crucial to its proper interaction with Hsc70.

Although there are quantitative differences in the interactions of auxilin and YDJ1 with Hsc70, it is surprising how qualitatively similar these interactions are considering that YDJ1 cannot substitute for auxilin in supporting the uncoating of clathrin-coated vesicles by Hsc70 (19). Because YDJ1 cannot substitute for auxilin, our results do not support a model where DnaJ homologs nonspecifically prime Hsc70 to bind substrates, particularly a model where the DnaJ homolog dissociates from Hsc70 before the substrate binds (31). Rather our results support a model where DnaJ homologs only present specific substrates to Hsc70; perhaps the substrates that bind to them. On the other hand, the similarities in the nucleotide dependence of the direct interaction of YDJ1, auxilin, and the J-domain of auxilin with Hsc70 suggest that various DnaJ homologs may act similarly in presenting bound substrates to the Hsc70 molecule. Future comparative studies on the interaction of DnaJ homologs and their J-domains with various Hsc70s may clarify the common elements that are involved in the mechanism of action of DnaJ homologs.

Acknowledgments—We thank Dr. Ernst Ungewickell for the pQE30 expression vector containing the auxilin gene product and the anti-auxilin monoclonal antibody. We also thank Angela Murphy for synthesis and analysis of the auxilin J-peptide domains.

REFERENCES

1. Pelham, H. R. B. (1986) Cell 46, 959–961
2. Ellis, R. J., and Van Der Vies, S. M. (1991) Annu. Rev. Biochem. 60, 321–347
3. Hendrick, J., and Hartl, G. (1993) Annu. Rev. Biochem. 62, 349–384
4. Hartl, F.-U., Holdan, R., and Langer, T. (1994) Trends Biochem. Sci. 19, 21–25
5. Sturti, R. A., Cyr, D. M., Craig, E. A., and Neupert, W. (1994) Trends Biochem. Sci. 19, 87–92
6. Rassow, J., and Pfanner, N. (1995) Rev. Physiol. Biochem. Pharmacol. 126, 199–264
7. Rassow, J., Roos, W., and Pfanner, N. (1995) Trends Cell Biol. 5, 207–212
8. Minami, Y., Hohfeld, J., Ohnuka, K., and Hartl, F. (1996) J. Biol. Chem. 271, 19617–19624
9. Caplan, A. J., Cyr, D. M., and Douglas, M. G. (1993) Mol. Biol. Cell 4, 555–563
10. Cyr, D. M., Langer, T., and Douglas, M. G. (1994) Trends Biochem. Sci. 19, 176–181
11. Prasad, K., Barouch, W., Greene, L., and Eisenberg, E. (1993) J. Biol. Chem. 268, 23758–23761
12. Ungewickell, E., Ungewickell, H., Holstein, S., Lindner, R., Prasad, K., Barouch, W., Martin, B., Greene, L., and Eisenberg, E. (1995) Nature 377, 632–635
13. Ahs, S., and Ungewickell, E. (1996) J. Cell Biol. 111, 19–29
14. Cyr, D. M., Lu, X., and Douglas, M. G. (1992) J. Biol. Chem. 267, 20927–20931
15. Cheetham, M., Jackson, A., and Anderton, B. (1994) Eur. J. Biochem. 226, 299–307
16. Cyr, D. M., and Douglas, M. G. (1994) J. Biol. Chem. 269, 9798–9804
17. Freeman, B. C., Myers, M. P., Schumacher, R., and Morimoto, R. I. (1995) EMBO J. 14, 2281–2292
18. Freeman, B. C., and Morimoto, R. I. (1996) EMBO J. 15, 2969–2979
19. King, C., Eisenberg, E., and Greene, L. (1997) Biochemistry, in press
20. King, C., Eisenberg, E., and Greene, L. (1995) Mol. Biol. Cell 6, 49 (abstr.)
21. Greene, L., and Eisenberg, E. (1990) J. Biol. Chem. 265, 6682–6687
22. Jentoft, N., and Dearborn, D. G. (1983) Trends Biochem. Sci. 8, 9–17
23. Cyto, S., and Eisenberg, E. (1979) J. Biol. Chem. 254, 22635–22640
24. Barouch, W., and Eisenberg, E. (1997) J. Biol. Chem. 272, 9347–9354
25. Holstein, S., Ungewickell, H., and Ungewickell, E. (1996) J. Cell Biol. 4, 925–937
26. Prasad, K., Heuser, J., Eisenberg, E., and Greene, L. (1994) J. Biol. Chem. 269, 8091–8099
27. Greene, L., Zinner, R., Naify, S., and Eisenberg, E. (1995) J. Biol. Chem. 270, 2987–2997
28. Tan, J., and Douglas, M. G. (1996) J. Biol. Chem. 271, 9347–9354
29. Holstein, S., Ungewickell, H., and Ungewickell, E. (1996) J. Cell Biol. 4, 925–937
30. Barouch, W., Prasad, K., Greene, L., and Eisenberg E. (1997) Biochemistry, in press
31. Wawrzynow, A., Banecki, B., Wall, D., Liberek, K., Georgopoulos, C., and Zylicz, M. (1995) J. Biol. Chem. 270, 19307–19311