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The heat shock cognate protein from *Dictyostelium* affects actin polymerization through interaction with the actin-binding protein cap32/34

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During isolation of the F-actin capping protein cap32/34 from *Dictyostelium discoideum*, a 70 kDa protein was copurified which by cloning and sequencing was identified as a heat shock cognate protein (hsc70). This protein exhibited a specific and MgATP-dependent interaction with the heterodimeric capping protein. To investigate the protein-protein interaction *in vitro*, we expressed all three polypeptides separately in *Escherichia coli* and performed reconstitution experiments of complete or truncated hsc70 with the 32 and 34 kDa subunits of the capping protein. Viscosity measurements and studies on the polymerization kinetics of pyrene-labeled actin showed that hsc70 increased the capping activity of cap32/34 up to 10-fold, whereas hsc70 alone had no effect on actin polymerization. In addition, hsc70 acted as a molecular chaperone by stimulating the refolding of the denatured 32 and 34 kDa subunits of the capping protein. To study the interaction of the two domains of hsc70 with cap32/34, the N-terminal 42 kDa ATPase region and the C-terminal 30 kDa tail of hsc70 were expressed separately in *E.coli*. The 32 and 34 kDa subunits were capable of associating with both domains of hsc70. The ATPase domain of hsc70, which is structurally related to actin, proved to be responsible for the increased capping activity of cap32/34, whereas the C-terminal tail of hsc70 was involved in folding of the subunits of cap32/34. Our data indicate a novel linkage between 70 kDa heat shock proteins and the actin cytoskeleton. A function of the actin-like ATPase domain appears to be the inhibition of actin polymerization in an indirect fashion via an increase in the activity of an F-actin capping protein.
Key words: actin polymerization/cytoskeleton/F-actin capping protein/heat shock proteins/molecular chaperone

Introduction

The heat shock proteins of the 70 kDa class were first identified in stressed cells (Loomis and Wheeler, 1980; Lindquist, 1986); later it became clear that they also play important roles in unstressed cells. They form a conserved protein family including constitutively expressed hsc70 as well as stress-inducible hsp70 isoforms. Members of this family are located in the cytoplasm, nucleus, mitochondria (Craig *et al.*, 1989; Mizzen *et al.*, 1989) and endoplasmic reticulum (ER) (Bole *et al.*, 1986). Proteins of the hsc70 family are composed of two domains (Milarski and Morimoto, 1989): an ATPase domain with a tertiary structure similar to actin (Flaherty *et al.*, 1990, 1991; Kabsch *et al.*, 1990) and a C-terminal domain with a predicted secondary structure similar to the peptide-binding domain of human leukocyte antigen (HLA) class I (Sadis *et al.*, 1990; Rippmann *et al.*, 1991).

Heat shock proteins have various chaperone functions in unstressed cells (Ellis, 1987; Rothman, 1989; Ellis and van der Vies, 1991; Gething and Sambrook, 1992). hsc70 transiently associates with nascent polypeptide chains (Beckmann *et al.*, 1990) and prevents premature folding or aggregation during protein synthesis (Beckmann *et al.*, 1992). hsc70 proteins are required for the translocation of precursor polypeptides through membranes of mitochondria (Deshaies *et al.*, 1988; Hartl and Neupert, 1990; Neupert *et al.*, 1990), the ER (Chirico *et al.*, 1988) and lysosomes (Chiang *et al.*, 1989). BiP, which was originally described as a protein associating with immunoglobulin heavy chains (Haas and Wabl, 1983), binds in the ER to a variety of ligands and is postulated to prevent the formation of aggregates (Pelham, 1986; Kassenbrock *et al.*, 1988; Hurley and Helenius, 1989). Furthermore, heat shock proteins are involved in the ATP-dependent dissociation of protein complexes like clathrin coats (Ungewickell, 1985; Chappell *et al.*, 1986) or a complex associated at the origin of replication of bacteriophage λ DNA (Liberek *et al.*, 1988; Dodson *et al.*, 1989; Hoffmann *et al.*, 1992). The members of the hsc70 family have different substrate specificities. For example, yeast cytosolic hsc70 and *E.coli* DnaK are not capable of replacing BiP in supporting translocation of precursor polypeptides into the ER. Vice versa, in the translocation of other proteins BiP could not replace cytosolic hsc70 (Brodsky *et al.*, 1993).

The heterodimeric F-actin capping protein cap32/34 belongs to a family of highly conserved proteins (Hartmann *et al.*, 1990). Capping proteins bind to barbed ends of actin filaments, thereby blocking the addition of further actin subunits and lowering the viscosity of F-actin solutions. Only the heterodimer is active; the single subunits exhibit no capping activity (Haus *et al.*, 1991; Hug *et al.*, 1992). The activity of cap32/34 is Ca^{2+} independent, the only regulatory mechanism known is the *in vitro* inhibition by phosphaditylinositol 4,5-bisphosphate (PIP_2) (Haus *et al.*, 1991; Heiss and Cooper, 1991). It is questionable whether inhibition by phospholipids is the only regulatory mechanism *in vivo*, since in *Acanthamoeba castellanii* most of the cellular capping protein is soluble; only a part has been found in the crude membrane fraction (Cooper *et al.*, 1984). In chicken epithelial cells, the capping protein colocalizes with components of the junctional complexes, but has also been found in the cytoplasm (Schafer *et al.*, 1992).

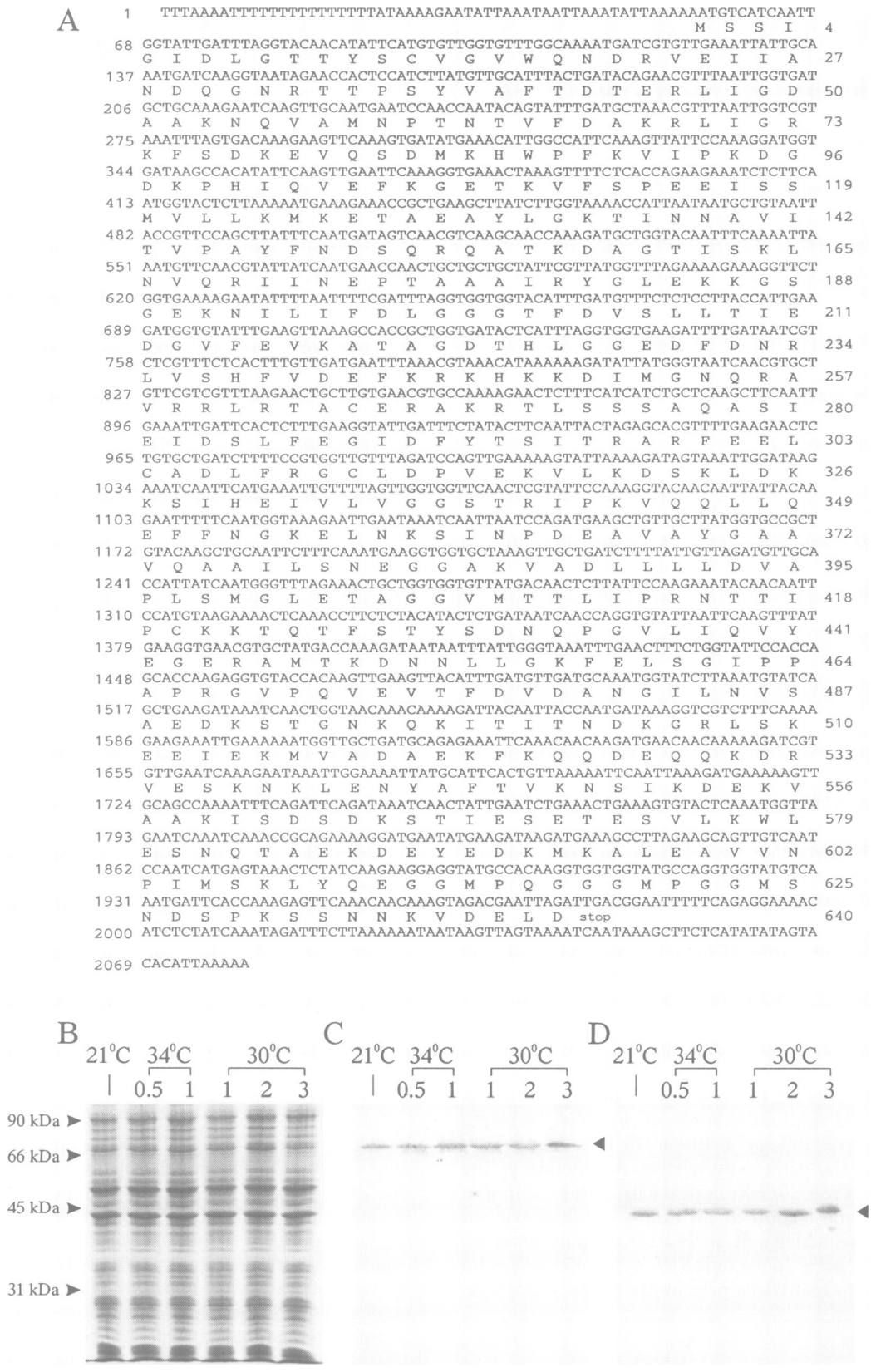

Fig. 1. Sequence of hsc70 and constitutive expression during heat shock. (**A**) cDNA and deduced amino acid sequences. Bases are numbered at the left and amino acids on the right. (**B**) Coomassie blue-stained SDS−polyacrylamide gel of *D.discoideum* homogenates derived from cells grown at 21°C, or from cells after a temperature shift to 34°C for 0.5 and 1 h, and to 30°C for 1, 2 and 3 h. (**C**) Corresponding immunoblot with hsc70 polyclonal antiserum. (**D**) Corresponding immunoblot with anti-actin monoclonal antibody for control.

Molecular chaperones are known to participate in the assembly of microtubules *in vivo*, and their involvement in the assembly of intermediate and actin filaments is conceivable (Gupta, 1990). Here we report on the cooperation of hsc70 from *D.discoideum* with cap32/34 in regulating actin polymerization. The ATPase domain of hsc70 proved to be responsible for an increase in cap32/34 activity and the C-terminal tail to be involved in refolding of cap32/34. Our data suggest a novel function of the ATPase domain in hsc70, and clearly demonstrate the proposed function of the C-terminal hsc70 domain in binding unfolded protein.

Results

A 70 kDa heat shock cognate protein is copurified with the heterodimeric actin-binding protein cap32/34

During purification of cap32/34 a 70 kDa protein was cofractionated. After raising polyclonal antibodies against this polypeptide, a λgt11 expression library (Lacombe *et al.*, 1986) was screened and several cDNA clones were isolated. The complete cDNA was 2079 bp long and harbored an open reading frame of 1921 bp. The nucleotide and derived amino acid sequences are shown in Figure 1A. Sequence comparisons showed that the 70 kDa polypeptide is a member of the 70 kDa heat shock protein family. To ensure that the isolated cDNA clones represented the 70 kDa peptide, the protein was extracted from SDS−polyacrylamide gels, digested with trypsin, and two fragments were sequenced. The obtained amino acid sequences were identical to the sequences deduced from the cDNA.

In order to test whether the cloned 70 kDa heat shock protein was heat inducible or constitutively expressed, growth-phase cells of the *D.discoideum* AX2 strain were shifted from 21 to 30 or 34°C for various times and were then analyzed on the RNA and protein levels. In Northern blots, two RNAs of 2.4 and 2.6 kb hybridized with the cDNA probe. In agreement with published data (Rosen *et al.*, 1985), the larger one was expressed during development and in higher amounts during heat shock, whereas the 2.4 kb RNA was constitutively expressed (data not shown). In immunoblots, the amount of the 70 kDa protein was not significantly increased after the temperature shift (Figure 1B−D). These data characterize the polypeptide from *D.discoideum* as a constitutively expressed heat shock protein (hsc70) and the antibodies as specific for hsc70.

The interaction of hsc70 with cap32/34 is specific and ATP dependent

In order to study the interaction between hsc70 and cap32/34, these proteins were immunoprecipitated from fractions of *D.discoideum* cell homogenates. One fraction contained hsc70 and cap32/34, the other one was free of cap32/34. Monoclonal antibodies against the 34 kDa subunit (mAb409) (Hartmann *et al.*, 1989) and the 32 kDa subunit (mAb188) were used for precipitation. hsc70 from *D.discoideum* precipitated only in the presence of cap32/34 (Figure 2A). In immunoprecipitates with mAb188, we found a 34 to 32 kDa ratio lower than the expected 1:1 ratio of a heterodimer. With mAb409, the relative amount of the 34 kDa subunit in the precipitate was >1:1. These observations indicate that

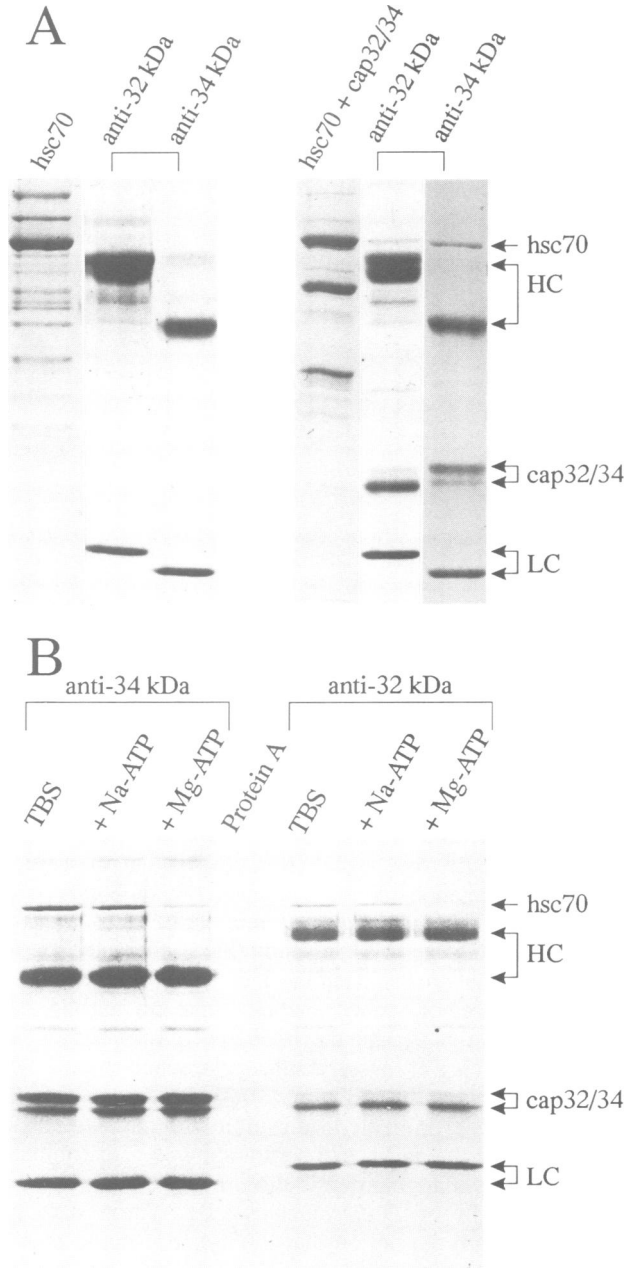

Fig. 2. Immunoprecipitation of hsc70 requires cap32/34; the complex formation is ATP dependent. (**A**) Immunoprecipitations from a *D.discoideum* hsc70 fraction free of cap32/34 (left panel) and a fraction containing hsc70 and cap32/34 (right panel) with mAb188 (anti-32 kDa) and mAb409 (anti-34 kDa) as indicated. The left lanes show the protein composition with about equal amounts of hsc70 after staining with Coomassie blue. The sizes of the IgG heavy chains (HC) and light chains (LC) differ between the two monoclonal antibodies. In most cases, the antibodies did not precipitate cap32/34 as a 1:1 complex, but favored their specific subunit. (**B**) Immunoprecipitations from *D.discoideum* fractions containing cap32/34 and hsc70 in buffer alone (TBS) or in the presence of Na^+ATP or Mg^{2+}ATP as indicated. The positions of hsc70, cap32/34, heavy chains (HC) and light chains (LC) are indicated.

the antibodies partially dissociated the subunits of the heterodimer. hsc70 was coprecipitated in both cases. Immunoprecipitations performed with increasing concentrations of cap32/34 revealed that the amount of hsc70 precipitated was dependent on the amount of sedimented cap32/34 (data not shown).

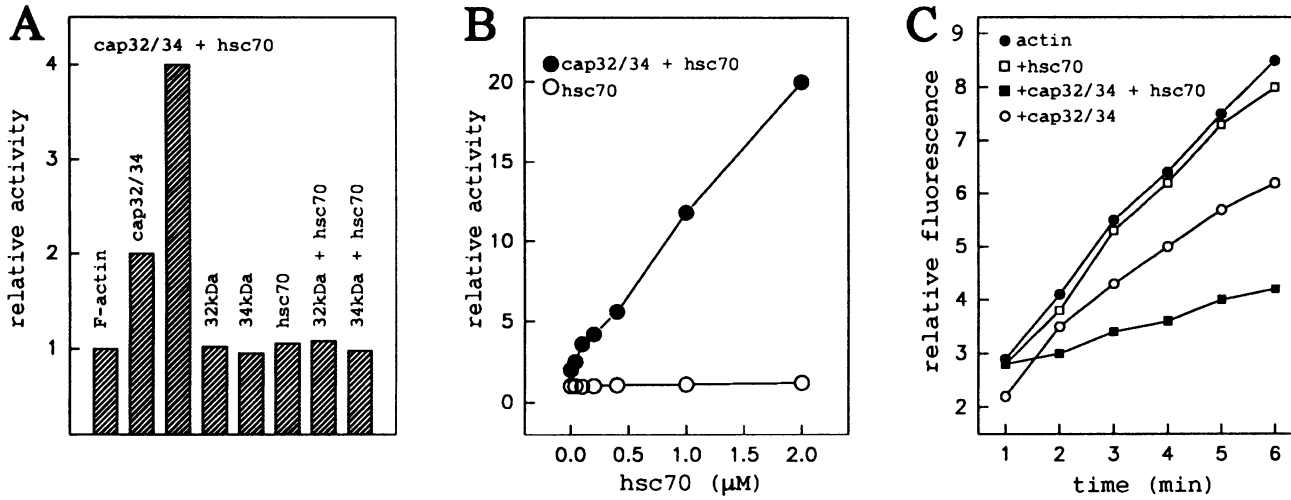

Fig. 3. F-actin capping activity of cap32/34 and hsc70. (**A**) Low-shear viscometry measurements with F-actin solutions containing 50 nM of the 32 kDa subunit, 50 nM of the 34 kDa subunit, 250 nM hsc70 or mixtures of these proteins as indicated. The viscosity of an F-actin solution was set to 1.0, a decrease of viscosity by 50% has an activity value of 2.0. (**B**) Viscosity measurements with increasing concentrations of hsc70 in the absence and presence of 40 nM cap32/34. (**C**) F-actin capping assay using pyrene-labeled actin. The elongation of F-actin seeds was measured in the presence of hsc70 (500 nM), cap32/34 (100 nM) and both proteins together.

Many heat shock protein−ligand complexes can be dissociated *in vitro* by ATP (Munro and Pelham, 1986; Flynn *et al.*, 1989). In order to check this for hsc70−cap32/34 complexes, hsc70 and cap32/34 were immunoprecipitated in the presence or absence of ATP. hsc70 was coprecipitated in the absence and in the presence of Na^+-ATP, but not in the presence of Mg^{2+}-ATP (Figure 2B). The copurification, coprecipitation and ATP-dependent binding indicate an interaction of the heat shock protein hsc70 with the actin-binding protein cap32/34 in a way that is characteristic for molecular chaperones (Gething and Sambrook, 1992).

In immunofluorescence studies using the anti-34 kDa antibody (mAb409) and the hsc70 polyclonal serum, we observed staining of several distinct cytoplasmic regions with a high intensity at the cell cortex. Both proteins were found in leading edges. Double localization with the anti-34 kDa antibody and the anti-hsc70 serum showed codistribution, or at least overlapping distribution, of the two proteins in the cytoplasm of *D.discoideum* cells. These data are consistent with the notion that hsc70 and capping protein may associate with each other *in vivo*.

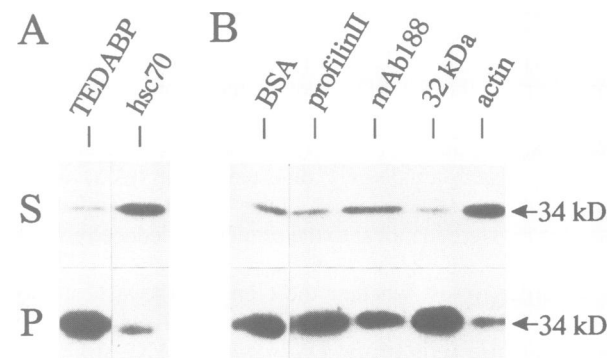

Fig. 4. Solubilization of denatured 34 kDa subunit. (**A**) Distribution of the 34 kDa subunit in supernatants (S) and pellets (P) after dilution from 6 M guanidinium chloride into buffer alone (TEDABP) or buffer containing a 5 M excess of hsc70 as detected with mAb409 in immunoblots. (**B**) Control experiments with buffer containing BSA, profilin II, immunoglobulin (mAb188), the corresponding 32 kDa subunit or actin as indicated.

Cooperation of hsc70 and cap32/34 in actin capping

For the investigation of the cap32/34 and hsc70 interaction, the three components were separately expressed in *E.coli* using the T7 RNA polymerase system (Tabor, 1990). The effect of recombinant hsc70 and cap32/34 on actin polymerization was measured in viscometry assays. The viscosity of the F-actin solution alone after 30 min of polymerization was set to 1, and the activity of any component tested was calculated relative to this basal value. The amount of cap32/34 in the assays was adjusted to give a reduction in the viscosity of 50%, resulting in a relative activity of 2. The presence of hsc70 led to an increase of the capping activity of recombinant cap32/34. Pure hsc70, mixtures of hsc70/32 kDa, hsc70/34 kDa, and also the single subunits of 32 and 34 kDa, had no detectable effect on the viscosity of the F-actin solution (Figure 3A). The viscosity-decreasing activity of the hsc70−cap32/34 complex was detected in the presence of 2 mM $MgCl_2$ or 150 mM KCl. It was independent of Ca^{2+} and EGTA. hsc70 enhanced the cap32/34 activity 2- to 10-fold in a dose-dependent manner (Figure 3B). To exclude artifacts that might result from the expression in bacteria, we routinely tested the activity of reconstituted cap32/34 and isolated hsc70 free of cap32/34 from *D.discoideum* cells. This hsc70 fraction was used in key experiments to confirm the data obtained with recombinant hsc70.

To specify the effect obtained with low-shear viscometry, the kinetics of actin polymerization were investigated by measuring the elongation of F-actin seeds with pyrene-labeled G-actin monomers. In the presence of hsc70−cap32/34 complex, a reduced elongation rate as compared with that obtained with pure capping protein was observed (Figure 3C). We conclude that hsc70 cooperates with cap32/34, since the hsc70 protein enhanced the F-actin capping activity of cap32/34, but did not influence actin polymerization. This does not exclude a direct interaction between hsc70 and F-actin. We observed a significant co-

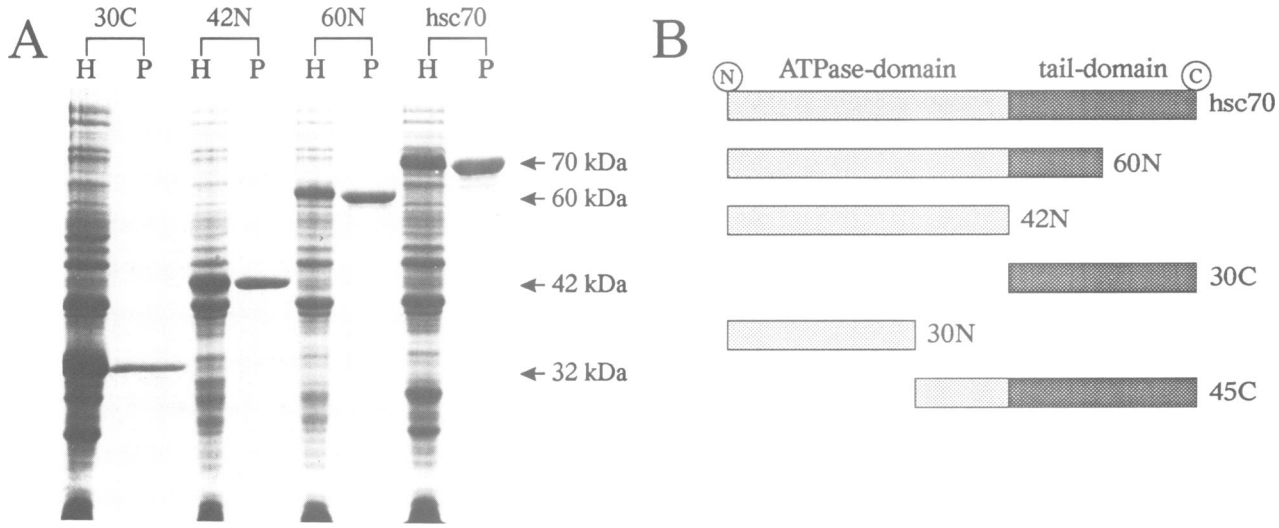

Fig. 5. Expression and purification of recombinant hsc70 and truncated molecules. (**A**) Homogenates (H) of induced *E.coli* cells and purified proteins (P) 30C, 42N, 60N and hsc70 were separated by SDS−PAGE (12% acrylamide) and stained with Coomassie blue. (**B**) Domain structure of recombinant hsc70, 60N, 42N, 30C, as well as the degradation products 30N and 45C, in a schematic overview.

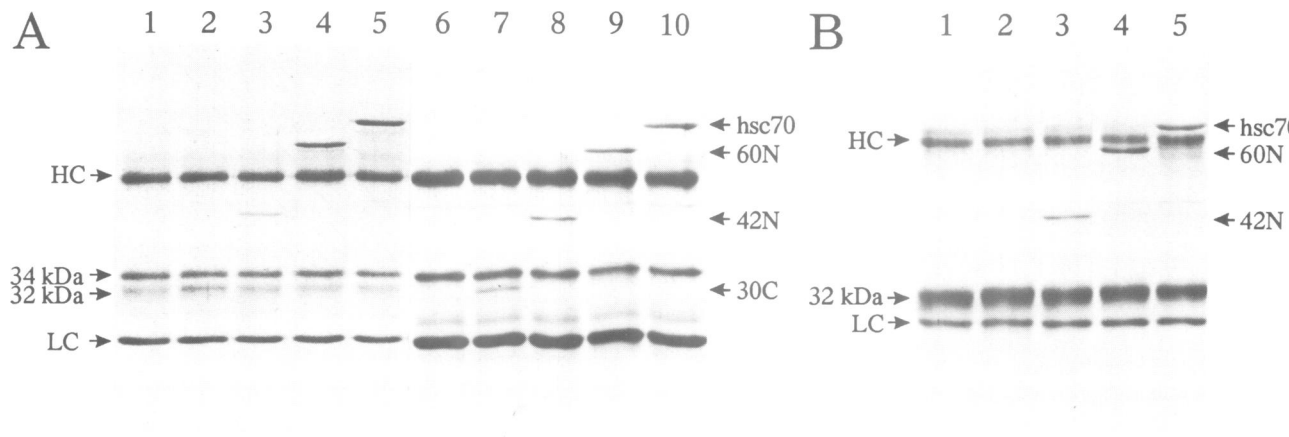

Fig. 6. Association of hsc70 derivatives with cap32/34 analyzed by immunoprecipitation experiments. (**A**) Immunoprecipitates of recombinant cap32/34 (lanes 1−5) or single 34 kDa subunit (lanes 6−10) with mAb409 (anti-34 kDa). (**B**) Immunoprecipitates of single 32 kDa subunit with mAb188 (anti-32 kDa). The immunoprecipitations were performed in the presence of recombinant 30C (lanes 2, 7), 42N (lanes 3, 8), 60N (lanes 4, 9) and hsc70 (lanes 5, 10). 30C was also coprecipitated, but is barely visible due to its migration next to the 32 kDa subunit. The positions of the proteins, heavy chains (HC) and light chains (LC) are indicated.

sedimentation of hsc70 and actin filaments in spin-down experiments (data not shown).

hsc70 affects refolding of the cap32/34 subunits

Heat shock proteins function in protein folding and assembly, and disassembly of protein complexes [for a review, see Gething and Sambrook (1992)]. The effect of hsc70 on unfolded cap32/34 was investigated by denaturing the single 32 and 34 kDa subunits and the cap32/34 complex in 6 M guanidinium chloride. Subsequently, the polypeptides were diluted in one step into buffer alone or buffer containing different proteins, followed by centrifugation. Pellet and supernatant were analyzed by SDS−PAGE and immunoblotting. Figure 4 shows an experiment with the 34 kDa subunit of the capping protein. Similar data were obtained with the 32 kDa subunit or the cap32/34 complex (data not shown). In the absence of hsc70, nearly all of the 34 kDa protein remained denatured and was sedimented. The presence of a 5 M excess of recombinant hsc70 in the dilution buffer resulted in an efficient solubilization of the polypeptide (Figure 4A). In control samples, the effect of bovine serum albumin (BSA), profilin II, immunoglobulin (mAb188), the corresponding 32 kDa subunit and of actin on the solubility of the 34 kDa subunit was determined. Surprisingly, G-actin also inhibited the aggregation of capping protein (Figure 4B). This observation raised the question whether the ATPase domain of hsc70, which is structurally related to G-actin (Flaherty *et al.*, 1991), is responsible for this effect.

Dissection of the hsc70 molecule into functional domains

In order to evaluate the functions of the individual hsc70 domains, the C-terminal hydrophobic domain (30C), the 42 kDa N-terminal ATPase domain (42N) and a 60 kDa N-terminal fragment (60N) which lacks a 10 kDa peptide of the C-terminus were expressed in pT7-7 (Tabor, 1990) and purified to homogeneity (Figure 5). Two polypeptides of 28 and 45 kDa, which accumulated during the preparation of recombinant hsc70, were studied in addition to the genetically engineered hsc70 domains. The topologies of these

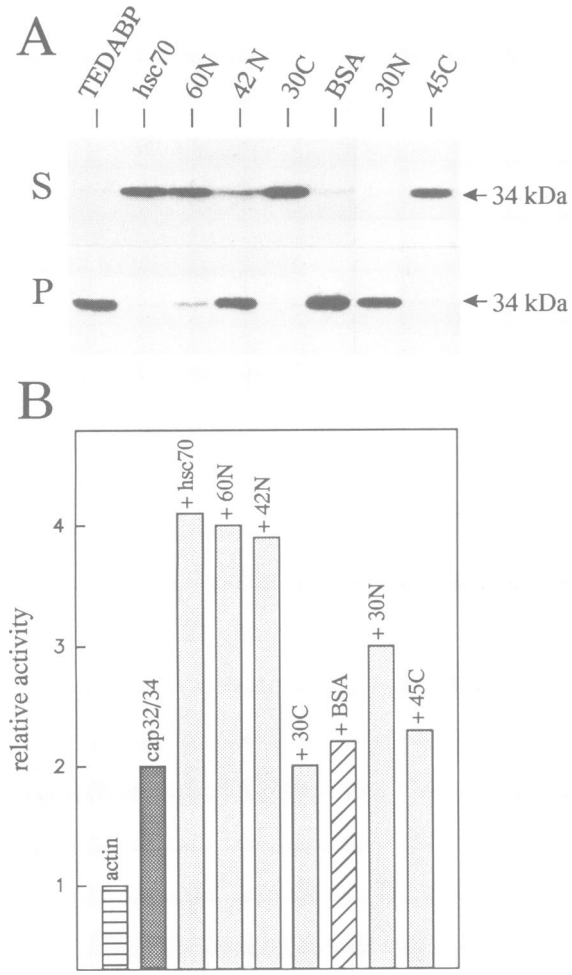

Fig. 7. Dissection of the functions of hsc70 domains. (**A**) Analysis of solubilization with mAb409. Distribution of the 34 kDa subunit in supernatants (S) and pellets (P) after dilution from 6 M guanidinium chloride into buffer alone (TEDABP) or buffer containing a 5 M excess of recombinant hsc70, 60N, 42N, 30C, of BSA, or of the hsc70 degradation products 30N and 45C. (**B**) Low-shear viscometry measurements with F-actin solutions containing 50 nM cap32/34 in the absence or presence of 250 nM hsc70, 60N, 42N, 30C, BSA, 30N or 45C as indicated. The two panels show that the C-terminal domain of hsc70 is important for refolding, but not for the increase of F-actin capping activity of cap32/34.

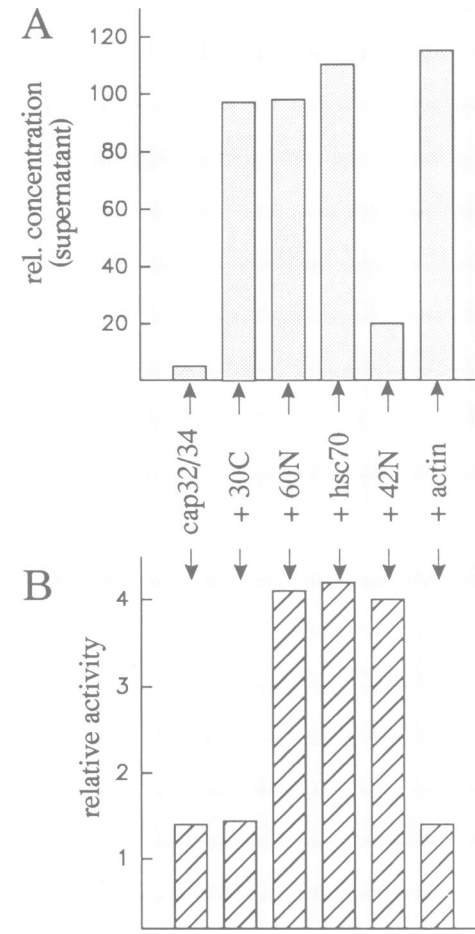

Fig. 8. Refolding and enhancement of capping activity are independent functions of hsc70. (**A**) Relative amounts of the 34 kDa subunit in the supernatants as determined by densitometry after denaturation and renaturation of cap32/34 into buffer alone (cap32/34) or buffer containing a 5 M excess of recombinant 30C, 42N, 60C, hsc70 or actin. (**B**) Low-shear viscometry measurements with the supernatants of the refolding assay containing cap32/34 and 30C, 42N, 60C, hsc70, or actin as indicated.

polypeptides were identified by determination of the N-terminal amino acid sequences. The 28 kDa polypeptide represented ~75% of the ATPase domain (30N) and the 45 kDa polypeptide the remaining C-terminal region (45C). By immunoprecipitation with the monoclonal antibodies against the 32 and 34 kDa subunits, we found that both subunits interacted autonomously with hsc70 and with its two domains 30C and 42N (Figure 6).

For identification of the domain of hsc70 that is responsible for preventing aggregation of the capping protein, reconstituted cap32/34 heterodimer was unfolded with 6 M guanidinium chloride. The degree of refolding was compared after dilution into samples that contained equal molarities of hsc70, 60N, 42N, 30C or the degradation products 30N and 45C (Figure 7A). For convenience, we routinely determined the concentration of the 34 kDa subunit in supernatants and pellets; analysis of the 32 kDa subunit showed essentially the same results. These data indicate that mainly the C-terminal 30 kDa hydrophobic domain of hsc70 is responsible for binding to the unfolded polypeptides and for preventing aggregation.

The chaperone function and the enhancement of capping activity of cap32/34 might represent completely different functions of hsc70. Therefore, we determined which domain of hsc70 was responsible for the increased capping activity of the hsc70–cap32/34 complex. In viscometry assays, we found that 42N and 60N, the two proteins containing the N-terminal ATPase domain, increased the activity of cap32/34 comparable to complete hsc70. 30N, the incomplete ATPase domain, influenced the capping activity of cap32/34 slightly (Figure 7B). In contrast, 30C had no effect on the activity of capping protein and the effect of 45C, the other C-terminal fragment, on capping activity was very low. 30N, 42N, 60N, 30C and 45C alone had no effect on the viscosity of an F-actin solution. These data indicate that the ATPase domain of hsc70 was responsible for the increased capping activity of cap32/34.

Enhancement of capping activity versus solubilization

For enhancement of the capping activity of cap32/34 by hsc70 two mechanisms are conceivable: (i) the chaperone activity of hsc70 leads to a higher concentration of properly

folded cap32/34, and this is responsible for an increased capping activity; (ii) refolding and enhancement of capping activity are independent and distinguishable functions of hsc70. A decision between these possibilities requires further studies. To provide a first approximate answer, we investigated the influence of folding on the capping activity. Different from the assay shown in Figure 7B, where purified proteins without further de- and renaturation have been assayed in viscometry, the supernatants of a solubilization assay were now analyzed directly (Figure 8). The C-terminal tail of hsc70 (30C) clearly increased the concentration of soluble capping protein, but the capping activity of cap32/34 was only marginally shifted beyond its basal activity. The N-terminal ATPase domain showed a weaker influence on refolding, but a strong increase in the capping activity. These data show that refolding and increase of capping activity are distinguishable functions of hsc70.

Discussion

The heat shock cognate protein hsc70 from *D.discoideum* is ~75% identical to the amino acid sequences of mammalian hsc70. The C-terminal domains of *D.discoideum* and human hsc70 (Dworniczak and Mirault, 1987) show 51% identity in an overlap of 117 amino acids. The most obvious similarity resides in the ATPase domain which is structurally related to actin (Flaherty *et al.*, 1990, 1991; Kabsch *et al.*, 1990). *Dictyostelium discoideum* hsc70 interacts with the heterodimeric actin-binding protein cap32/34; both proteins are localized in the cytoplasm with an increased label at the cell cortex, the area rich in actin filaments. This is in agreement with data from rat embryo fibroblasts where a 70 kDa heat shock protein was shown to be concentrated at the leading edge and to codistribute with microfilaments (La Thangue, 1984).

The two domains of *D.discoideum* hsc70 have distinguishable effects on cap32/34: the N-terminal ATPase domain is involved in increasing the capping activity, the C-terminal tail in binding to denatured cap32/34 subunits and preventing aggregation. Both effects require a molar excess of hsc70 which reflects the protein concentrations in the cell. Similar results have been obtained for the refolding of rhodanese from bovine mitochondria which needs a 10- to 20-fold molar excess of the hsc70 homolog dnaK. Only a 5-fold molar excess of dnaK is required in the presence of dnaJ (Langer *et al.*, 1992). In the absence of ATP, 20- and 100-fold molar excesses of dnaK are used to refold immunotoxins (Buchner *et al.*, 1992), and between 2- and 8-fold molar excesses of hsp90 are used to refold citrate synthase (Wiech *et al.*, 1992).

It has been shown previously that after ATP hydrolysis polypeptides are released from hsc70, a reaction that is facilitated by interaction with other cellular components (Munro and Pelham, 1986; Hoffmann *et al.*, 1992; Langer *et al.*, 1992). We detected an ATP-affected interaction between hsc70 and cap32/34 in immunoprecipitates from crude *D.discoideum* fractions. The ATP effect has also been found with recombinant polypeptides that contain the ATPase region, but has been less obvious than with native hsc70, probably as a result of slight structural changes during expression and purification.

The interaction between the actin-binding protein cap32/34 and the ATPase domain of hsc70 might be the consequence of the structural similarity between this domain and actin, but the activity of hsc70 differs from that of actin. An interaction between heterodimeric capping proteins and G actin has not been observed so far. It should be emphasized, however, that the chaperone-like activity of G-actin in the solubilization assays of renaturing cap32/34 might indicate for the first time binding of G-actin to an heterodimeric capping protein. In our studies, neither G-actin nor F-actin have been capable of displacing hsc70 from cap32/34 (data not shown), which implies distinct binding regions at the capping protein for actin and hsc70. Immunoprecipitation indicates that both subunits of cap32/34 bind separately to both domains of hsc70, suggesting not a one-point binding, but rather extended contact regions.

Most heat shock proteins are not very specific; they are known to interact with many proteins like clathrin, nucleolar proteins, p53 tumor antigen, SV40 T-antigen or calmodulin [reviewed by Schlesinger (1990)]. BiP has also been shown not to be specific for certain sequences, but to exhibit a general affinity for peptides with aliphatic residues (Flynn *et al.*, 1991). Our data on the enhancement of the capping activity suggest a novel function of hsc70: this protein specifically interacts with cap32/34 via the ATPase domain, independent of its chaperone effect. A less specific chaperone function of the C-terminal tail of hsc70 is involved in binding to denatured subunits of the capping protein. There is a growing body of evidence that the C-terminal tails of 70 kDa heat shock proteins are the peptide-binding domains (Milarski and Morimoto, 1989; for review see Schlesinger, 1990; Gething and Sambrook, 1992), and secondary structure predictions for these regions fit to the structure of the peptide binding domain of HLA class I, which also binds diverse polypeptides (Sadis *et al.*, 1990; Rippmann *et al.*, 1991). In the case of hsc70 from *D.discoideum*, only the whole protein functions both as an enhancer of the F-actin capping activity and as a chaperone.

How does hsc70, as a structural analog of actin, enhance the capping activity of cap32/34? We consider it unlikely that the heat shock protein binds via its ATPase region to the barbed ends of actin filaments like a capping protein. One would expect in this case that hsc70 alone inhibits actin polymerization. We detected neither with native nor with recombinant hsc70 any intrinsic capping activity. We wish to emphasize that, because of the comigration of hsc70 and cap32/34 in most purification steps, it is important to check the preparations used for absence of cap32/34. A straightforward interpretation of our results is that the ATPase region of hsc70 does not touch the sites of interaction between F-actin and cap32/34. hsc70 either clamps both components together, thus decreasing the off-rate of the capping protein, or it functions as an adaptor that brings cap32/34 in closer vicinity to filament ends and is then dissociated by ATP hydrolysis.

Materials and methods

cDNA cloning and sequencing

A λgt11 expression library, kindly provided to us by Drs R.Kessin and M.-L.Lacombe, Columbia University, was screened with affinity-purified polyclonal antibodies prepared against hsc70. The DNA isolated from recombinant phages was digested with *Eco*RI and the resulting fragments were subcloned into appropriately cleaved pUC19 (Yanisch-Perron *et al.*, 1985). The cDNAs were sequenced by the chain termination method (Sanger *et al.*, 1977; Chen and Seeburg, 1985) with T7 polymerase (Pharmacia, Freiburg, Germany), using the uni- and reverse-primers, and specific

oligonucleotide primers (18–21-mers). The sequence was analyzed using the Wisconsin Genetic Computer Group software (UWGCG, Devereux *et al.*, 1984). Homology searches were done in the MIPSX database (Max Planck Institute, Martinsried, Germany).

Antibodies and immunoblotting

Monoclonal antibody mAb188 was prepared after immunization of BALB/c mice with the recombinant 32 kDa subunit essentially as described previously (Schleicher *et al.*, 1984). The spleen cells were fused with $PAIB_3Ag8I$ myeloma cells using polyethylene glycol (M_r 4000, Polysciences, Warrington, PA). For production of polyclonal antibodies against the 32 kDa subunit and hsc70, the native proteins were separated by SDS–PAGE, cut from the gel and prepared for s.c. injections using one rabbit for each protein. Later boosts were done with recombinant proteins.

For immunoblotting, proteins were resolved by SDS–PAGE in 12% acrylamide gels (Laemmli, 1970), transferred to nitrocellulose by standard procedures and either incubated with directly iodinated antibody or detected indirectly with alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Inc., Avondale, PA).

Immunoprecipitation and immunofluorescence

Dictyostelium discoideum cells were cultivated, harvested, lysed and the homogenates fractionated essentially as described previously (Schleicher *et al.*, 1984; Hartmann *et al.*, 1989). Partially purified fractions enriched in cap32/34 and hsc70 were subjected to immunoprecipitation with monoclonal antibodies mAb409 (anti-34 kDa subunit; Hartmann *et al.*, 1989) and mAb188 (anti-32 kDa subunit). In control experiments, fractions were used that contained hsc70, but were free of cap32/34. For immunoprecipitations with recombinant proteins, the 32 and 34 kDa subunits were mixed with hsc70, 60N, 42N or 30C in molar ratios of 1:5. Immunoprecipitations were performed in 1% Triton X-100 in Tris-buffered saline [TBS; 10 mM Tris–HCl (pH 8.0), 150 mM NaCl], in the presence or absence of 5 mM $MgCl_2$ and 5 mM Na^+ATP. The samples were rotated overnight at 4°C and then the proteins precipitated with protein A–Sepharose (Pharmacia, Freiburg, Germany). The supernatants were removed and the pellets washed three times with TBS. Samples were analyzed by SDS–PAGE and the proteins stained with Coomassie blue.

Growth-phase AX2 cells were allowed to adhere to coverslips for 15 min at room temperature and then fixed for 30 min at room temperature in 15% picric acid/2% paraformaldehyde solution, post-fixed with 70% ethanol and washed. For double labeling, cells were incubated with polyclonal serum against hsc70 followed by fluorescein isothiocyanate (FITC; Dianova, Hamburg, Germany) conjugated goat anti-rabbit IgG and mAb409 (anti-34 kDa) followed by carboxymethylindocyanine (Cy3; Jackson Immunoresearch Inc., Avondale, PA) conjugated goat anti-mouse IgG.

Construction of expression vectors

The complete coding sequence of hsc70 was cloned into the ATG expression vector pT7-7, kindly provided by Dr S.Tabor (Tabor, 1990), using *NdeI*/*PstI* restriction sites. The ATG of the *NdeI* site is the authentic start codon of hsc70 [shown by cloning and sequencing of the 5′-terminal and upstream non-coding region after nested inverse polymerase chain reactions (PCR) of genomic DNA] and lies at an optimal distance from the T7 promoter. In this paper, we describe three constructs with truncated coding regions of hsc70. The hsc70 cDNA was amplified by PCR with specific oligonucleotide primers to obtain 60N (60 kDa N-terminal domain, amino acids 1–543), 42N (42 kDa N-terminal domain, ATPase domain, amino acids 1–379) and 30C (30 kDa C-terminal domain, amino acids 380–640).

To obtain 60N, the 70-start primer (5′-CG CTC GAG CAT ATG TCA TCA ATT GGT ATT GAT TTA GGT ACA A-3′) and 60-end primer (5′-GCG CTG CAG TTA ATA ATT TTC CAA TTT ATT CTT TGA TT-3′) were used. To obtain 42N, the 70-start and ATPase-end primer (5′-CGC TGC AGT TAT GAA AGA ATT GCA GCT TGT AC-3′) were used. To obtain 30C, the 30-start primer (5′-GCG CAT ATG AAT GAA GGT GGT GCT AAA GTT-3′) and 70-end primer (5′-GCG CTG CAG TCA ATC TAA TTC GTC TAC TTT G-3′) were used. The 5′-primers contained an additional *NdeI* restriction site and the ATG start codon, the 3′-specific primers contained a TAA stop codon, or in the case of 70-end, the naturally occurring TGA stop codon followed by a *PstI* restriction site. The PCR fragments were cloned into pT7-7 using the *NdeI*/*PstI* restriction sites. All constructs coded for no additional amino acids at the N- or C-termini. All cloning procedures were carried out according to Sambrook *et al.* (1989).

Protein purification

Expression and purification of the recombinant 32 and 34 kDa subunits of cap32/34 were done essentially as described previously (Haus *et al.*, 1991). The polypeptides were expressed in *E.coli* BL21 (Studier and Moffat, 1986) cells with the T7 RNA polymerase under the control of the IPTG-inducible lac promoter.

For expression of hsc70, 60N, 42N and 30C, recombinant *E.coli* BL21 cells were grown at 37°C to an OD_{600} of 1.0. The expression was induced with 1 mM IPTG for 2 h. The bacterial RNA polymerase was then inhibited with 200 μg/ml rifampicin and after incubation at 37°C for an additional 2 h the cells were harvested by centrifugation (20 min, 4000 *g*), resuspended in TEDABP buffer [10 mM Tris–HCl (pH 8.0), 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.02% NaN_3, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)], frozen and thawed, incubated for 1 h with 0.1 mg/ml lysozyme at 4°C, homogenized by douncing and finally completely opened by sonication. After centrifugation (20 min, 30 000 *g*), hsc70, 60N and 42N remained in the pellet, whereas 30C was soluble and found in the supernatant. The pellets were extracted in four steps with TEDABP buffer containing (i) 0.5% Triton X-100 and 10 mM EDTA, (ii) 30% sucrose, (iii) 2 M urea, and finally (iv) 8 M urea. The major amounts of hsc70, 60N and 42N were extracted with 8 M urea and immediately subjected to anion-exchange chromatography (DEAE–cellulose, 2.5 × 10 cm) in the same buffer.

Bound proteins were eluted with a linear salt gradient of 0–350 mM NaCl; if necessary the appropriate fractions were adjusted to small volumes by concentration with Centriprep concentrators (Amicon, Beverly, MA) and loaded onto a Sepharose 6BCL column (5 × 100 cm) in IEDANBP [10 mM imidazole (pH 7.6), 10 mM EGTA, 2 mM DTT, 0.02% NaN_3, 1 mM benzamidine, 0.5 mM PMSF] containing 7 M urea. For functional assays, the urea was removed stepwise by dialysis against TEDABP buffer or G-buffer [2 mM Tris–HCl (pH 8.0), 0.5 mM DTT, 0.01% NaN_3, 0.2 mM ATP, 0.2 mM $CaCl_2$]. The amount of refolding was determined by solubility assays and binding to ATP–agarose. The solubility and ATP-binding activity of hsc70, 60N and 42N were increased after mercaptolysis with 5% β-mercaptoethanol, denaturation with 6 M guanidinium chloride and slow renaturation during dialysis against TEDABP buffer. For purification of 30C, the supernatant was subjected to DEAE–cellulose and gel filtration chromatography, as described above, but without urea; the pooled fractions were dialyzed against KPDAEBP buffer [10 mM potassium phosphate (pH 6.7), 1 mM EGTA, 1 mM DTT, 0.02% NaN_3, 1 mM benzamidine, 0.5 mM PMSF], subjected to a hydroxylapatite column (2.5 × 5 cm) in the same buffer and eluted with a linear salt gradient of 0–300 mM KH_2PO_4 (pH 6.7).

Pure 30N peptide was obtained during purification of recombinant hsc70 in trailing fractions from the gel filtration column. High amounts of 45C could be obtained when recombinant hsc70 was extracted from *E.coli* inclusion bodies in the absence of EDTA or EGTA. These degradation products were identified by peptide sequencing with an Applied Biosystems gas phase sequencer (Eckerskorn *et al.*, 1988) The N-terminal amino acid of 30N is the methionine and the N-terminal amino acid of 45C is the arginine no. 259.

Low-shear viscometry and fluorescence spectroscopy

Low-shear viscometry was carried out in a falling-ball viscometer according to MacLean-Fletcher and Pollard (1980). The polymerization was started by addition of G-actin in the presence of 10 mM imidazole (pH 7.5), 2 mM $MgCl_2$, 1 mM ATP and 1 mM EGTA or 0.2 mM $CaCl_2$. The reaction mixture usually contained 0.5 mg/ml rabbit skeletal muscle actin. The data shown are the mean values of three experiments. For fluorescence spectroscopy, actin was labeled with *N*-(1-pyrenyl)iodoacetamide (pyrene) (Kouyama and Mihashi, 1981; Cooper *et al.*, 1983) and the assays were performed as described by Eichinger *et al.* (1991). Preformed unlabeled actin filaments were used as nuclei (800 nM final concentration) for polymerization of actin monomers (3 mM final concentration, 10% pyrene-labeled actin) in G-buffer containing 50 mM KCl. In the presence of capping protein, the F-actin nuclei are blocked at the barbed ends which inhibits filament elongation.

Solubilization assay

This assay was done essentially as described by Langer *et al.* (1992). Either the single subunits or the cap32/34 complex were denatured at a concentration of 10 μM in TEDABP containing 6 M guanidinium chloride for 60 min at room temperature. Unfolded capping protein was diluted 100-fold (100 nM final concentration) into TEDABP alone or TEDABP containing 500 nM hsc70, actin, BSA, 32 kDa subunit, immunoglobulin (mAb188) or profilin II. The samples were incubated for 30 min at 25°C, centrifuged (15 min, 30 000 *g*), and the distribution of the 32 and 34 kDa subunits in supernatants and pellets detected in immunoblots with mAb409 and mAb188.

Miscellaneous

Rabbit skeletal muscle actin was prepared as described by Spudich and Watt (1971). Protein concentrations were determined using Coomassie blue G according to Bradford (1976) with BSA as a standard. *N*-(1-pyrenyl)iodoacetamide was purchased from Molecular Probes (Junction City, OR), the fluorescence was measured on an SFM25 fluorometer from Kontron Instruments (Eching, Germany). All chemicals were of analytical grade.

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