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Mammalian protein RAP46: an interaction partner and modulator of 70 kDa heat shock proteins

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A ubiquitously expressed nuclear receptor-associating protein of ~46 kDa (RAP46) was identified recently. Interaction experiments with *in vitro*-translated proteins and proteins contained in cell extracts revealed that a great variety of cellular regulators associate with RAP46. However, in direct interaction tests by the far-Western technique, only 70 kDa proteins showed up and were identified as members of the 70 kDa heat shock protein (hsp70) family. Interaction is specific since not all members of the hsp70 family bind to RAP46; interaction occurs through their ATP-binding domain. RAP46 forms complexes with hsp70 in mammalian cells and interacts with hsp70 in the yeast two-hybrid system. Consistent with the fact that hsp70 can bind a multitude of proteins, we identified heteromeric complexes of RAP46–hsp70 with some selected proteins, most notably c-Jun. Complex formation is increased significantly by pre-treatment with alkaline phosphatase, thus suggesting modulation of interactions by protein phosphorylation. We observed that RAP46 interferes with efficient refolding of thermally denatured luciferase. Moreover, ATP-dependent binding of misfolded proteins to hsp70 was greatly inhibited by RAP46. These data suggest that RAP46 functions as a regulator of hsp70 in higher eukaryotes.

Keywords: c-Jun/dephosphorylation/hsp70 heat shock proteins/nuclear receptors/protein refolding

Introduction

In recent years, the subject of protein folding within cells and in cell-free systems has evoked great interest. Appropriate folding of polypeptides and assembly of multi-subunit proteins appear to involve rather complex systems in which heat shock proteins play a pivotal role as chaperones (for reviews, see Hartl, 1996; Hartman and Gething, 1996; Frydman and Höhfeld, 1997). In this respect, members of the 70 kDa heat shock protein (hsp70) family are certainly of prime importance. They are highly homologous to each other and have a distinct domain structure: the 44 kDa amino-terminal portion binds ATP and other nucleotides while the 28 kDa carboxy-terminal domain interacts with misfolded or partially unfolded polypeptides, in particular if these contain hydrophobic regions (Boorstein *et al.*, 1994; Fourie *et al.*, 1994).

Recently, two related cDNAs have been cloned from human liver and mouse embryonal cells which code for proteins called RAP46 (Zeiner and Gehring, 1995) and BAG-1 (Takayama *et al.*, 1995). RAP46 is a nuclear receptor-associating protein of apparent mol. wt 46 kDa which was identified by screening an expression library using as probe the glucocorticoid receptor overexpressed in the baculovirus system. It also interacts with other members of the nuclear receptor superfamily (Zeiner and Gehring, 1995). Murine BAG-1 was obtained by use of a similar screening procedure in which baculovirus-expressed Bcl-2 or the hepatocyte growth factor receptor were employed to identify interacting proteins (Takayama *et al.*, 1995; Bardelli *et al.*, 1996). Subsequent studies showed (see below) that the interaction of RAP46 is not restricted to nuclear receptors but that various unrelated regulatory proteins, including Bcl-2, also react with RAP46 in pull-down experiments. This then raised the question of whether such interactions might be mediated by some common interacting protein(s). In the present study, we identified members of the hsp70 family as direct and specific interaction partners. Through binding to members of the hsp70 family, a multitude of proteins might associate with RAP46. Indeed, we detected heteromeric complexes in which hsp70 serves as adaptor. The interaction of RAP46 with hsp70s occurs via their ATP-binding domain.

Results

Proteins interacting with RAP46

Originally, RAP46 was identified by binding the activated glucocorticoid receptor (Zeiner and Gehring, 1995). In pull-down assays with RAP46 fused to glutathione-*S*-transferase (GST) and bound to glutathione (GSH)–Sepharose, we obtained similar associations with other mammalian steroid hormone receptors (Zeiner and Gehring, 1995) and with ecdysteroid, thyroid and retinoic acid receptors (data not shown). Using this technique, we now observed that several unrelated cellular regulators, most notably c-Jun, c-Fos, CREB, c-Myc, c-Mos and Bcl-2, interact similarly with GST–RAP46, although with varying efficiencies (Figure 1). In an attempt to minimize unspecific interactions, we included 1 M urea in these experiments. To our surprise, we observed that this treatment actually enhanced the interactions or, in some instances, was even required for binding. For example, CREB and Bcl-2 barely interacted if urea treatment was omitted (see Figure 6B). GST controls showed the specificity of RAP46 interactions (Figure 1, lane 1). However, several other proteins do not bind to RAP46 (see below).

Having observed that a host of proteins are retained on the GST–RAP46 affinity matrix, we wondered whether these interactions are direct or whether they might be mediated by some component(s). We used extracts of

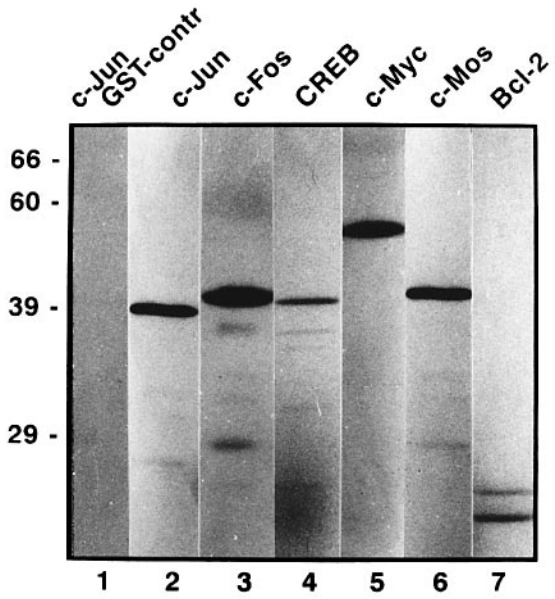

Fig. 1. Pull-down experiments with GST–RAP46 on GSH–Sepharose. [^{35}S]methionine-labeled, *in vitro*-translated proteins were assayed in the presence of 1 M urea. Various regulatory proteins were used, as indicated, and the eluted proteins analyzed by SDS–PAGE and autoradiography. GST controls for all proteins were negative; only that for c-Jun is shown in lane 1.

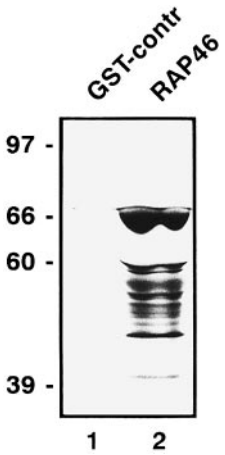

Fig. 2. Pull-down experiments with cell extracts. HeLa cells were metabolically labeled for 4 h with [^{35}S]methionine and the extract submitted to pull-down with GST–RAP46 on GSH–Sepharose. Eluted proteins were separated by SDS–PAGE; detection was by autoradiography. Lane 2 shows the pull-down with GST–RAP46, lane 1 with GST as a control.

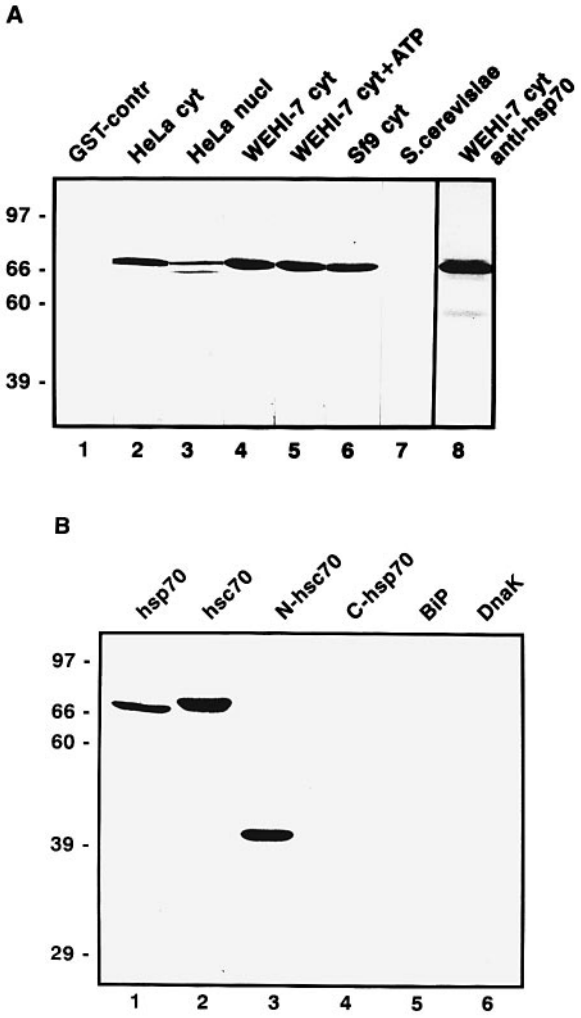

Fig. 3. Far-Western blots with GST–RAP46. (**A**) Cytosolic and nuclear HeLa cell extracts, cytosolic extracts of WEHI-7 and Sf9 cells and an extract of *Saccharomyces cerevisiae* (as indicated) were run on SDS–PAGE (100 μg each), blotted and renatured. Blots were incubated with GST–RAP46 followed by staining with antiserum Z-5 against GST. The GST control was with WEHI-7 extract. In the experiment in lane 5, ATP and $MgCl_2$ (10 mM each) were present during the interaction with GST–RAP46. Lane 8 shows a Western blot of a cell extract stained with hsp70/hsc70-specific antibody N27F3-4. (**B**) Far-Western blots were with 1 μg each of recombinant human hsp70, purified bovine hsc70, the ATP-binding domain of hsc70 (N-hsc70), the His-tagged carboxy-terminal domain of hsp70 (C-hsp70), hamster BiP and DnaK. Reactions were again with GST–RAP46 and antiserum Z-5.

metabolically labeled cells for pull-down assays with GST–RAP46. Analysis of retained material by SDS–PAGE revealed a very prominent signal at 70 kDa which was absent from the GST control (Figure 2, lane 2 versus lane 1). Several much less intense bands of lower molecular weights also showed up; these most probably represent secondary interaction partners of RAP46 (see below).

To search specifically for direct interaction partners, we used the far-Western technique. Proteins in cell extracts were separated by SDS–PAGE, blotted and renatured on the membrane by standard procedures. Interaction with RAP46 was by incubation with GST–RAP46 and detection was by staining with an antibody directed against GST. With many cellular proteins interacting directly we would expect multiple bands; instead we obtained only one prominent signal of ~70 kDa with cytosolic extracts of HeLa and WEHI-7 cells (Figure 3A, lanes 2 and 4). Nuclear extracts of HeLa cells produced two bands of slightly different mobilities (lane 3). The GST control was again negative (lane 1).

These observations show that proteins of ~70 kDa interact directly with RAP46, suggesting that they might correspond to hsp70 and the slightly larger constitutive form hsc70. Indeed, hsp70-specific immunostaining was obtained and is shown in Figure 3A (lane 8) with a cytosolic extract of WEHI-7 cells. Similarly, both bands of HeLa nuclear extracts produced hsp70/hsc70-specific immunosignals (not shown).

To ascertain the interaction between RAP46 and mem-

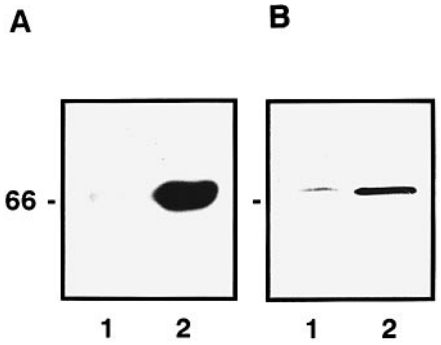

Fig. 4. Co-immunoprecipitation of RAP46 and hsp70.
(**A**) GST–RAP46 (2 μg) was incubated with reticulocyte lysate (50 μl) for 2 h at 8°C. The sample was then divided into two equal portions and reacted either with hsc70-specific antiserum K-19 (lane 2) or with non-immune serum (lane 1) in the presence of protein G–agarose overnight in the cold. Analysis was by SDS–PAGE and Western blotting with antibody C-16 that detects the carboxy-terminal portion of RAP46 but does not cross-react with hsp70, as verified in control experiments (data not shown). (**B**) WEHI-7 cell extract (500 μl each, containing 1 mg of protein) was reacted either with RAP46-specific antiserum C-16 (lane 2) or with non-immune serum (lane 1) in the presence of protein A–agarose overnight in the cold. Analysis was by SDS–PAGE and Western blotting with hsp70/hsc70-specific antibody N27F3-4 which does not cross-react with RAP46, as ascertained in control experiments (data not shown).

bers of the hsp70 family, we used specific immunoprecipitation. We incubated GST–RAP46 (apparent mol. wt in SDS–PAGE ~67 kDa) with reticulocyte lysate and carried out immunoprecipitation with hsp70-specific antiserum K-19 and protein G–agarose (Figure 4A). We observed that GST–RAP46 was co-precipitated in the presence of antibody (lane 2) but not with non-immune serum (lane 1).

Members of the hsp70 family interact directly with RAP46

In addition to crude cell extracts, we also employed pure proteins of the hsp70 family in far-Western blots (Figure 3B). The stress-inducible form, hsp70 (lane 1), and the constitutive form, hsc70 (lane 2), of mammalian origin produced distinct signals.

To investigate specificity, we used several other members of the hsp70 family in far-Western blots. Extracts of Sf9 insect cells produced a significant band at 70 kDa (Figure 3A, lane 6), while *Saccharomyces cerevisiae* (Figure 3A, lane 7) and wheat germ extracts (data not shown) yielded no signals. Also BiP, the endoplasmic form of hsp70, and DnaK, the bacterial hsp70, did not interact with RAP46 (Figure 3B, lanes 5 and 6).

Interaction occurs with the ATP-binding domain

As mentioned above, hsp70s are characterized by two major domains. The 44 kDa ATP-binding domain of hsc70 is obtained easily by chymotrypsin treatment (Chappell *et al.*, 1987). This amino-terminal fragment readily interacted with RAP46 in a far-Western blot (Figure 3B, lane 3). By contrast, the carboxy-terminal portion of hsp70 (amino acids 384–640) which was bacterially expressed as a His-tagged protein (apparent mol. wt in SDS–PAGE ~36 kDa) did not bind RAP46 (Figure 3B, lane 4).

Interaction of hsp70 with other proteins depends largely on the presence of nucleotides (Hartl, 1996). We therefore included various nucleotides in far-Western blots with WEHI-7 cell extracts. Figure 3A shows that it did not matter whether ATP was present or not (lane 5 versus lane 4). Likewise, ADP and the non-hydrolyzable ATP analog AMP-PNP did not produce any differences (not shown).

We also added several nucleotides to pull-down experiments with GST–RAP46 and radiolabeled estrogen receptor synthesized *in vitro* in the reticulocyte lysate system. The same amounts of receptor were recovered independently of whether ATP, ADP, AMP-PNP or GTP were included or not (not shown). We also checked the same samples for hsp70 and detected immunosignals of equal intensities (not shown), again proving that these nucleotides do not affect the RAP46 interaction with hsp70/hsc70.

Members of the hsp70 family are known ATPases. We therefore asked whether RAP46 might affect this enzymatic activity. Using a published protocol (Cheetham *et al.*, 1994), we did not observe any effect of RAP46 on ATPase activity (not shown).

RAP46 interacts with hsp70 in vivo

With RAP46 vividly interacting *in vitro* with hsp70 and hsc70, we wondered whether similar interactions also occur within cells. To check for complexes between endogenous RAP46 and hsp70/hsc70 in WEHI-7 extracts, we employed immunoprecipitation with RAP46-specific antiserum C-16 and protein A–agarose. The retained material was analyzed by SDS–PAGE and Western blotting with hsp70/hsc70-specific antibody. A strong hsc70 signal was obtained in the presence of RAP46-specific antibody (Figure 4B, lane 2) which was insignificant when non-immune serum was used (lane 1). This result was substantiated further by submitting WEHI-7 extracts to gel permeation chromatography on Sephacryl S-300. Analysis was by Western blotting with antisera C-16 and N27F3-4 against RAP46 and hsp70/hsc70, respectively. RAP46 eluted as large heteromeric complexes (mol. wt range 400–800 kDa) which also contained hsc70/hsp70 (not shown).

To ascertain RAP46–hsp70 interactions further *in vivo*, we employed the yeast two-hybrid system (Fields and Song, 1989) with the transcriptional activator GAL4. This system is particularly useful as yeast hsp70 does not compete due to lack of affinity for RAP46 (Figure 3A, lane 7). Rat hsc70 cDNA was fused to cDNA encoding the GAL4 DNA-binding domain and RAP46 cDNA to the GAL4-activating domain. Protein–protein contacts initiate transcriptional activation of GAL4-responsive genes, i.e. *HIS3* and bacterial *lacZ* in the strain used here. This resulted in growth in minimal medium and expression of β-galactosidase (Figure 5, line 1), thus demonstrating *in vivo* interaction between hsc70 and RAP46. Interaction occurs through the ATP-binding domain (residues 1–383 of hsc70), as exemplified by growth in the presence of the corresponding construct (Figure 5, line 2). Control experiments showed that RAP46 does not interact either with the DNA-binding domain of GAL4 (Figure 5, line 4) or with the activating domain (GAL4BD–RAP46 tested against GAL4AD; not shown).

To find out whether RAP46 is able to form multimers, we coupled its sequence to both GAL4 domains. This combination did not result in growth under selective conditions (Figure 5, line 3), suggesting that RAP46 does not dimerize. We similarly did not obtain evidence for

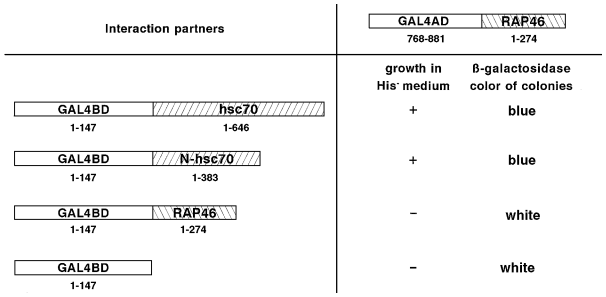

Fig. 5. *In vivo* interaction of hsc70 with RAP46. Constructs used in yeast two-hybrid experiments are described in Materials and methods. Expressed proteins are shown, with open bars for GAL4 domains and hatched bars representing sequences fused to them. Growth in deficient medium and β-galactosidase activity were scored.

homo-oligomerization in pull-down experiments with GST–RAP46 and *in vitro*-translated RAP46 (not shown).

Dephosphorylation stimulates interactions

As members of the hsp70 family are known to be phosphoproteins and phosphorylation may be important for their functions (Sherman and Goldberg, 1993; Panagiotidis *et al.*, 1994), we checked for effects of dephosphorylation. We indeed observed much stronger hsp70 signals in pull-down experiments with WEHI-7 extracts if these were pre-treated with calf intestinal alkaline phosphatase (data not shown). In order to quantify the interaction, we employed *in vitro*-synthesized and radiolabeled hsp70 (Figure 6A). We found that 45–50% of input hsp70 was retained on GST–RAP46/GSH–Sepharose, but upon prior phosphatase treatment hsp70 bound quantitatively.

Dephosphorylation of secondary partner proteins (see below) also affects their interaction with RAP46 and hsp70/hsc70. This is shown in Figure 6B for glucocorticoid and estrogen receptors, CREB and Bcl-2. With glucocorticoid receptors, we obtained at least a 20-fold increase in binding to the RAP46 affinity matrix, as judged from counting of excised bands.

In the case of c-Jun, differently phosphorylated forms are easily distinguished on SDS–PAGE (Adler *et al.*, 1992). Accordingly, dephosphorylation of *in vitro*-synthesized c-Jun resulted in a single band (Figure 6C, lane 2) produced from a triplet (lane 1). Interestingly, only the fastest moving band, i.e. dephosphorylated c-Jun, was recovered in pull-down experiments (Figure 6C, lane 3). Moreover, this signal was more intense after phosphatase treatment (lane 4 versus lane 3), and counting of excised bands showed an ~2-fold increase.

Hsc70 and RAP46 form heteromeric complexes with various proteins

The above experiments suggest that hsp70/hsc70 contained in reticulocyte lysate mediate the interactions with various proteins (see Figure 1). We therefore turned to a rather stringent approach with pure proteins and looked at their ability to interact with RAP46. For the experiment shown in Figure 7A, we used purified His-tagged c-Jun which was bacterially expressed and hence not phosphorylated properly. c-Jun was only retained on GST–RAP46/GSH–Sepharose if hsc70 was present simultaneously (Figure 7A), thus demonstrating that hsc70 is required for inter-

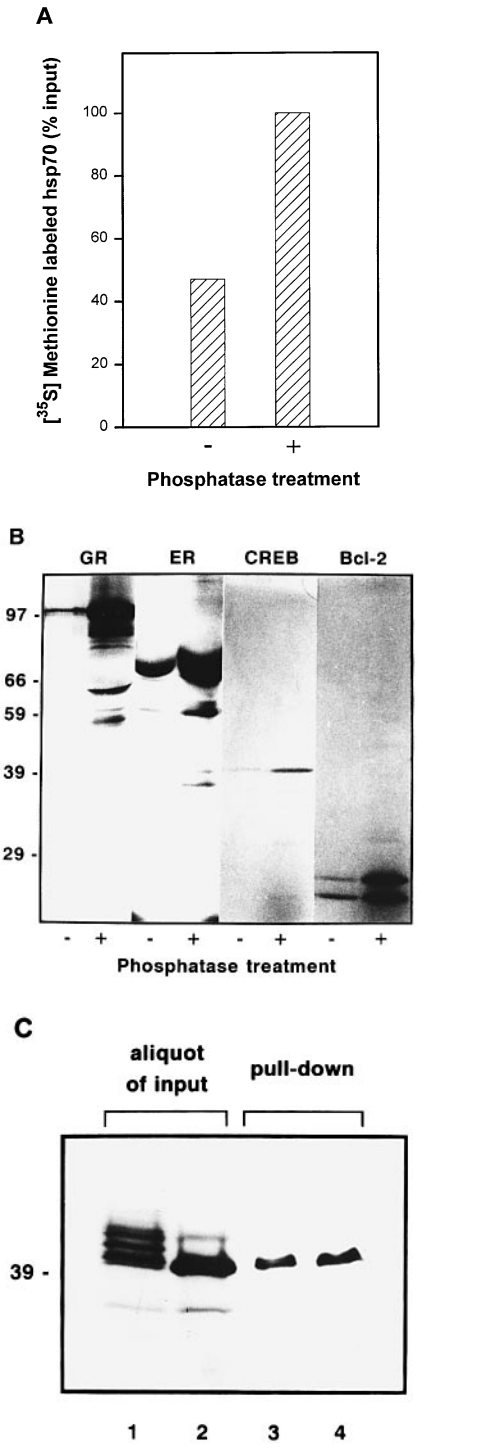

Fig. 6. Effect of dephosphorylation on RAP46 interactions. [^{35}S]methionine-labeled, *in vitro*-synthesized proteins were either treated with alkaline phosphatase (see Materials and methods) or not, as indicated, and subjected to pull-down on GST–RAP46 and GSH–Sepharose with no urea present in the reaction mixtures. Retained material was analyzed by SDS–PAGE. (**A**) Labeled human hsp70 was produced from the respective cDNA. The 70 kDa bands were detected by autoradiography, excised and radioactivity counted for quantification in relation to input [^{35}S]hsp70 similarly run on SDS–PAGE. (**B**) Labeled glucocorticoid receptor (GR), estrogen receptor (ER), CREB and Bcl-2 were produced, treated and analyzed in the same way. Autoradiographs are shown. (**C**) Labeled *in vitro*-synthesized c-Jun, either dephosphorylated (lanes 2 and 4) or not (lanes 1 and 3), was analyzed either directly or by SDS–PAGE (lanes 1 and 2) or following pull-down on GST–RAP46/GSH–Sepharose. Detection was by autoradiography.

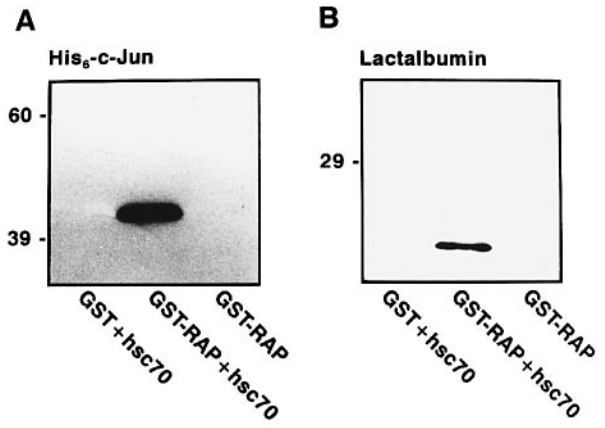

Fig. 7. Heterocomplex formation. About 10 μg of His-tagged c-Jun (**A**) or *S*-carboxymethylated α-lactalbumin (**B**) were incubated with 10 μg of hsc70 under the conditions of the pull-down assay with GST–RAP46 or GST on GSH–Sepharose in the absence of urea. Material retained on the matrix was analyzed by SDS–PAGE. Detection was by immunoblotting with antibody ^{MRGS}His in the case of His-tagged c-Jun and by Coomassie staining for α-lactalbumin.

action. This again proved to be independent of ATP or ADP (not shown).

As other proteins might interact similarly with RAP46 only in the presence of hsp70/hsc70, we used two unrelated proteins in corresponding assays. Carboxymethylated and denatured α-lactalbumin is known to bind readily to hsp70 (Cheetham *et al.*, 1994). With this model protein, we again detected binding to GST–RAP46 only when hsc70 was included (Figure 7B). A similar result was also obtained with phosphorylase b (not shown).

RAP46 affects interactions of hsc70 with various proteins

In an alternative approach, we checked for the relative amounts of proteins bound to hsp70/hsc70 in the presence or absence of RAP46. We used hsc70-specific immunoprecipitation with *in vitro*-synthesized and radiolabeled proteins (Figure 8A and B) and we pre-treated c-Jun with alkaline phosphatase and the estrogen receptor with urea. RAP46 enhanced the binding of c-Jun to hsc70 (Figure 8A, lane 4 versus lane 1) but had no effect on the binding of estrogen receptors (Figure 8B, lane 3 versus lane 1).

As interactions of hsp70/hsc70 with various proteins are influenced by nucleotides, we also included ATP in these interaction experiments. We found that ATP greatly increased the amounts of c-Jun and estrogen receptors co-precipitated with hsc70 (Figure 8A and B, lanes 2 versus lanes 1). This observation corresponds to what has been described for the binding of thermally denatured luciferase to hsc70 and hsp40 (Minami *et al.*, 1996). Most significantly, in the presence of ATP, RAP46 exerted a dramatic effect on interactions with c-Jun (Figure 8A, lane 5 versus lane 2) and estrogen receptors (Figure 8B, lane 4 versus lane 2). Binding decreased ~20-fold, as evaluated by counting of excised bands. In some assays, we included ADP but did not observe differences of any significance in the presence or absence of RAP46 (Figure 8A, lane 6 versus 3). Using thermally denatured luciferase, we observed similarly reduced binding in the presence of RAP46 and ATP (not shown).

In other experiments, we asked whether RAP46 affects

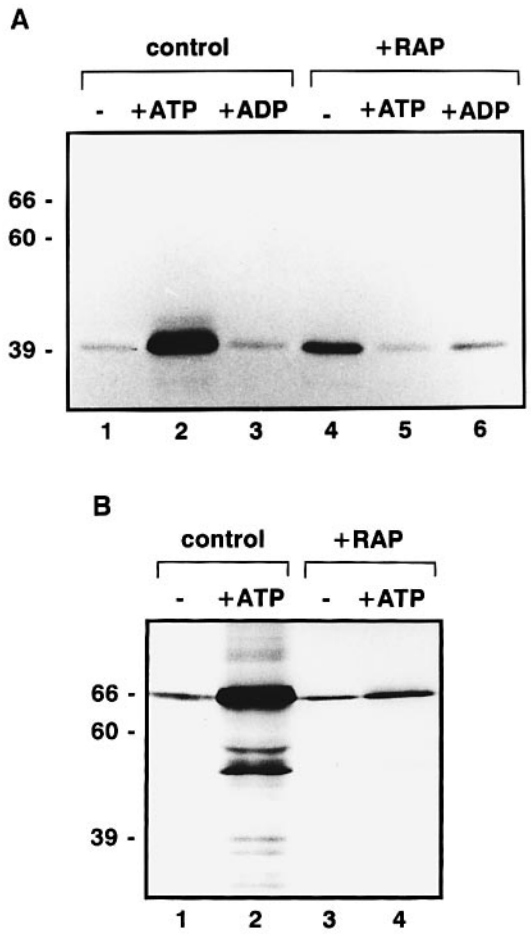

Fig. 8. Effect of RAP46 on binding of proteins to hsc70. [^{35}S]methionine-labeled, *in vitro*-translated c-Jun (**A**) and estrogen receptors (**B**) were pre-treated with phosphatase or 1 M urea, respectively, followed by 8-fold dilution. As indicated, GST–RAP46 (5 μg in 100 μl) and ATP or ADP (10 mM) were included during immunoprecipitation with hsc70-specific antiserum K-19 and protein G–agarose (as in Figure 4A). Analysis was by SDS–PAGE and autoradiography.

the interaction of the co-chaperone hsp40 with hsc70. Using hsc70-specific immunoprecipitation and His-tagged hsp40, we found that the amount of hsp40 retained, as detected by immunoblotting, was diminished 2- to 3-fold in the presence of RAP46 (three independent experiments; not shown).

RAP46 inhibits refolding of thermally denatured luciferase

Firefly luciferase has been used frequently as a model for chaperonin-dependent protein refolding. Routinely, this enzyme is either denatured by heating or unfolded by exposure to guanidinium-HCl. However, we consider thermally inactivated luciferase as the more relevant model for what happens to proteins within cells under conditions of heat and other forms of stress. We used a refolding system which employs pure hsc70 and hsp40 for maintaining thermally inactivated luciferase in a folding-competent and non-aggregating state. Refolding is then achieved by small amounts of chaperonin-containing reticulocyte lysate (Minami *et al.*, 1996). Reactivation was dependent on ATP, hsc70, hsp40 and 20-fold diluted

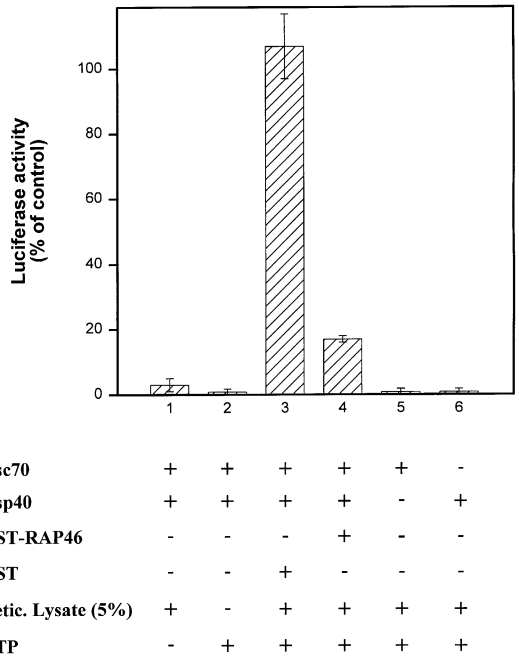

Fig. 9. Effect of RAP46 on luciferase refolding. Native luciferase (0.1 μM) was pre-incubated (5 min, room temperature) with hsc70 (5 μM), His-tagged hsp40 (3 μM) and 2 mM ATP followed by thermal inactivation (10 min, 42°C). Incubation in the presence of 5% reticulocyte lysate was for 1 h at 30°C; final volume 100 μl. Controls were without further additions and reconstituted activity was set as 100%; the level of reactivation was ~40%, as previously described (Minami *et al.*, 1996). Other assays contained GST or GST–RAP46 (10 μM each) or omissions, as indicated. All experiments were in triplicate; the average of three independent experiments is shown.

reticulocyte lysate (Figure 9, controls in columns 1, 2, 5 and 6). While GST had no effect (column 3), GST–RAP46 inhibited refolding of luciferase by 85 ± 3% (column 4; three independent experiments). Significantly, inhibition was dose dependent, with half as much GST–RAP46 causing ~65% inhibition (not shown). As RAP46 concentrations roughly equimolar to those of hsc70 are effective, it appears that it is the RAP46–hsc70 complex that causes inhibition of reactivation.

Discussion

In a previous study, we identified a novel protein, RAP46, which interacts with steroid hormone receptors (Zeiner and Gehring, 1995). We have now found that several completely unrelated regulatory proteins also bind to RAP46 in pull-down assays. However, using the far-Western blotting technique, only one major type of directly interacting protein was detected and this was identified as hsp70 (see Figure 3). In fact, we show that both the stress-inducible and the constitutive form hsc70 bind to RAP46. Hence the receptor-associating protein RAP46 should now rather be called Hap for 'hsp70/hsc70-associating protein'. Hap/RAP46 forms, together with Hip/p48 (Höhfeld *et al.*, 1995; Prapapanich *et al.*, 1996) and Hop/p60 (Chen *et al.*, 1996), a heterogeneous group of proteins which all interact with hsp70s. Recently, a reaction cycle has been proposed for hsp70 interactions with Hip/p48 and Hop/p60 (Frydman and Höhfeld, 1997) in which these interacting proteins cooperate in regulating hsp70 activity and are exchanged on hsp70/hsc70. Interestingly, Hip/p48 and Hop/p60 contain several structurally related tetratricopeptide repeats which are thought to be involved in interactions with hsp70/hsc70 (Frydman and Höhfeld, 1997). Even though Hap/RAP46 does not contain similar repeats, it will be interesting to find out how Hap/RAP46 communicates with these hsp70/hsc70-interacting proteins.

Having identified hsp70/hsc70 as direct interaction partners for RAP46 by *in vitro* techniques, i.e. pull-down and far-Western experiments, we wondered whether similar interactions might occur *in vivo*. We obtained clear evidence for the existence of such complexes in cell extracts by co-immunoprecipitation of hsc70 with a RAP46-specific antiserum (see Figure 4B). Moreover, RAP46 and mammalian hsc70 were found to cooperate in yeast (see Figure 5).

We found to our surprise that not all members of the hsp70 family are able to associate with Hap/RAP46. Endoplasmic BiP, prokaryotic DnaK, yeast and plant hsp70s did not bind to Hap/RAP46. This suggests a high specificity of interaction. Using defined fragments of hsc70, we found that it is the ATP-binding domain that accomplishes interaction with Hap/RAP46.

Our observation that a perplexing variety of regulatory proteins form complexes with Hap/RAP46 made us wonder whether such interactions require the presence of hsp70/hsc70. Indeed, we established for three unrelated proteins, c-Jun, α-lactalbumin and phosphorylase b, that they bind to RAP46 only if hsc70 is present simultaneously (see Figure 7). Thus, formation of multiple ternary complexes is probable in which hsp70 molecules mediate interactions. Such promiscuous binding is expected to occur through the carboxy-terminal domain of hsp70/hsc70 which is known for this property (Fourie *et al.*, 1994). Most probably, the multiple faint bands observed in our metabolic labeling experiment (see Figure 2) in addition to the strong hsp70/hsc70 signal are due to such ternary interactions. Our data suggest that hsc70 and Hap/RAP46 interact stoichiometrically, but complex formation with a third partner is only of the order of 5–25%, according to our rough estimates.

We suspect that most or perhaps all proteins previously detected as partners for Hap/RAP46 (Zeiner and Gehring, 1995; this study, Figure 1) do not bind by themselves but rather via hsp70s. This agrees with the technique originally used to identify and clone RAP46 by screening with a steroid receptor overexpressed in the baculovirus system. The extract used contained hsp70 of insect origin, and we now show that insect hsp70 interacts directly with Hap/RAP46. In fact, it is known that large parts of the glucocorticoid receptors produced in the baculovirus system are misfolded (Alnemri and Litwack, 1993) and thus prone to bind to hsp70/hsc70. The same may be true for other proteins like Bcl-2 and the hepatocyte growth factor receptor which were used for isolating BAG-1 (Takayama *et al.*, 1995; Bardelli *et al.*, 1996), and explains why the same technique employing completely unrelated proteins as bait resulted in identification of similar sequences. Not only nuclear receptors but also various oncoproteins and protein kinases, as well as Bcl-2, react with Hap/RAP46 in pull-down assays in which hsp70 is present (see Figure 1).

Association of Hap/RAP46 with various proteins is

favored by low concentrations of urea. This agrees perfectly with the idea that such interactions occur via hsp70s and are promoted by partial unfolding. We have now found that interactions are significantly improved by pretreating proteins with phosphatase. This was particularly evident for the glucocorticoid receptor, CREB and Bcl-2 (see Figure 6B). Even though it is unclear at present how dephosphorylation affects these interactions molecularly, a modulatory effect of phosphorylation on proteins interacting with Hap/RAP46–hsp70 needs to be considered. On the other hand, we cannot exclude the possibility that dephosphorylation of proteins results in a different folding state that interacts preferentially with Hap/RAP46–hsp70 or may even lead to partial unfolding which then increases the binding efficiency. Dephosphorylation of hsp70 itself was found to stimulate binding to Hap/RAP46 (see Figure 6A) and this in turn may aid interactions with secondary interaction partners. Nevertheless, complex formation with other proteins, e.g. the glucocorticoid receptor, is clearly favored by dephosphorylation and, in the case of c-Jun, our data prove that it is the dephosphorylated state which forms heterocomplexes with Hap/RAP46–hsp70 (see Figure 6C). The data of Figure 7A also support this notion as bacterially expressed c-Jun was used that was certainly not phosphorylated properly. Particularly with glucocorticoid and estrogen receptors (see Figure 6B), the effect of dephosphorylation is much larger than can be accounted for by that on hsp70/hsc70 contained in reticulocyte lysate.

Even though Hap/RAP46 associates with the ATP-binding domain of hsc70, we did not detect any effects of ATP or other nucleotides on this binding nor on interactions with various secondary partner proteins. Similarly, Hap/RAP46 did not affect the ATPase activity of hsc70. On the other hand, the presence of ATP, but not ADP, greatly promoted the binding of misfolded proteins to hsc70, and this interaction was inhibited strongly by Hap/RAP46 (see Figure 8). This suggests that association of Hap/RAP46 with the ATP-binding domain conformationally affects interdomain communication with the polypeptide-binding domain that is known to occur in hsp70 molecules (Buchberger *et al.*, 1995).

To investigate the effects of Hap/RAP46 on protein refolding, we used thermally denatured luciferase as a model protein. We found that refolding to the active state was inhibited strongly by Hap/RAP46 (see Figure 9). The system which we used depends on hsp70, hsp40 and ATP, as well as on diluted reticulocyte lysate (Minami *et al.*, 1996). Luciferase is inactivated readily by mild heating and does not regain activity, either spontaneously or upon exposure to hsc70–hsp40 in the presence of ATP (Frydman *et al.*, 1992; Minami *et al.*, 1996). While these heat shock proteins prevent the aggregation of partially folded polypeptides, renaturation only occurs upon subsequent incubation with diluted reticulocyte lysate that contains additional factor(s) (Nimmesgern and Hartl, 1993; Minami *et al.*, 1996). It has been suggested that the folding system used here involves a novel, as yet uncharacterized chaperonin distinct from TRiC (Minami *et al.*, 1996). Our data suggest that Hap/RAP46 interferes with efficient delivery of misfolded proteins to such chaperonin systems by locking them in a state that does not allow refolding. This view is supported by our observation that several partially denatured or misfolded proteins readily formed complexes with hsc70 in an ATP-dependent manner and such complexes correspond to the folding-competent state. Significantly, Hap/RAP46 interfered with formation of such ATP-dependent hsp70/hsc70 complexes with misfolded proteins (see Figure 8A). Moreover, Hap/RAP46 inhibited interactions of hsc70 with the co-chaperone hsp40 that is clearly required for refolding (see Figure 9). As complexes of hsp70/hsc70 and Hap/RAP46 with misfolded proteins apparently prevent these proteins from being refolded, we suppose that they eventually are transferred to the cellular degradation machinery. Within cells there are two optional pathways for partially denatured and aberrant proteins: either refolding to a native and functional state with the aid of chaperonins or intracellular proteolysis. Chaperones of the hsp70 family are known to participate in both pathways (Dice *et al.*, 1994; Hartl, 1996; Hartman and Gething, 1996; Sherman and Goldberg, 1996) and RAP46 may in fact—by way of the interactions described here—influence the decision between the alternatives of refolding or degradation.

Materials and methods

Materials

Human hsp70, bovine hsc70, hamster BiP and *Escherichia coli* DnaK were from Stressgen, firefly luciferase, rabbit muscle phosphorylase b and carboxymethylated bovine α-lactalbumin from Sigma, and [^{35}S]-methionine (45 TBq/mmol) from ICN.

Cell culture and cell extracts

HeLa, WEHI-7 and Sf9 cells were cultured and cytosolic extracts were prepared as described by Zeiner and Gehring (1995). HeLa nuclear extracts and yeast extracts were obtained by standard procedures (Ausubel *et al.*, 1995).

His-tagged proteins

His-tagged human hsp40 was expressed from plasmid pQE-9/Hsp40 (Minami *et al.*, 1996). His-tagged RAP46 was obtained by cloning the cDNA into the *Sma*I site of pQE-30 (Qiagen). Human c-*jun* cDNA was cloned into the *Bam*HI and *Kpn*I sites of pQE-31 (Qiagen). A 775 bp cDNA fragment (codons 384–640) of human hsp70 (Hunt and Morimoto, 1985) was amplified by PCR with primers 5′-aattggatccgggacaagtccgaga-3′ and 5′-aattaagcttggcccctaatctacc-3′, containing *Bam*HI and *Hin*dIII sites. The sequence was verified and cloned into pQE-32 (Qiagen). Overnight cultures of *E.coli* JM109 carrying plasmids were diluted 10-fold, cultured for 1 h, and induced with 1 mM isopropyl β-D-thiogalactopyranoside for 4 h. Bacteria were ruptured in 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris–HCl, pH 8. His-tagged proteins were bound to Ni^{2+}-NTA resin (Qiagen) and washed extensively with 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris–HCl, pH 6.3, followed by a final wash with saline and elution with 50–250 mM imidazole.

Protein interaction experiments

Pull-down assays with GST–RAP46 on GSH–Sepharose (Pharmacia) were as before (Zeiner and Gehring, 1995). In the experiments of Figure 1, *in vitro*-translated proteins were pre-treated for 60 min at 0°C with 3 M urea, diluted to 1 M urea and used for interaction with GST–RAP46. In some experiments (Figures 6 and 8), pre-treatment (60 min, 30°C) was with 20 U/ml calf intestinal alkaline phosphatase (Promega). Far-Western blotting with GST–RAP46 was essentially as described by Macgregor *et al.* (1990) using the GST-specific antibody Z-5 for detection. Proteins were analyzed by standard 10 or 12% SDS–PAGE with markers as before (Zeiner and Gehring, 1995).

Immunoblotting, immunoprecipitation and antibodies

Transfer to Immobilon-P membranes (Millipore) was as before (Zeiner and Gehring, 1995). Hsp70/hsc70 were detected by antibody N27F3-4 (Stressgen), GST–RAP46 by antibody Bag-1 (C-16) or GST-specific antibody GST (Z-5) (Santa Cruz), and His-tagged c-Jun and hsp40 with antibody ^{MRGS}His (Qiagen). Serum from an unimmunized rabbit was used as non-immune serum, and in control experiments was found not

to react with hsp70/hsc70 or RAP46. Incubation with peroxidase-conjugated second antibodies and detection with ECL (Amersham) were as before (Zeiner and Gehring, 1995). For co-immunoprecipitations with hsc70-specific antiserum HSC70 (K-19) (Santa Cruz) and with RAP46-specific antiserum C-16, we used buffer conditions as for the pull-down assays. Binding to protein A– or protein G–agarose (Boehringer Mannheim) was as described by Ausubel *et al.* (1995).

Yeast two-hybrid system

RAP46 cDNA (codons 1–274) was ligated into the *Sma*I sites of vectors pGBT9 and pGAD424 (Bartel *et al.*, 1993), resulting in plasmids pGBT-RAP and pGAD-RAP. Cells of strain HF7c (Clontech) were co-transformed (Schiestl and Gietz, 1989) with plasmid pGAD-RAP and either pGBT9, pGBT-RAP, pGBT-Hsc70 (containing the complete rat hsc70 sequence, codons 1–646) or pGBT-N-Hsc70 (codons 1–383 of rat hsc70) (Höhfeld *et al.*, 1995). Another co-transfection consisted of pGBT-RAP plus pGAD424. Growth on plates in medium lacking His, Leu and Trp was scored after 5 days at 30°C. Colonies were assayed for β-galactosidase with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma).

Protein refolding assay

Refolding experiments with thermally denatured luciferase were carried out exactly as described by Minami *et al.* (1996) with purified hsc70 and hsp40 and 20-fold diluted rabbit reticulocyte lysate (Promega). Enzyme activity was determined by the Promega luciferase assay.

In vitro translation

Reticulocyte lysate was used in a coupled transcription–translation system (TNT, Promega). All cDNAs to be transcribed were in plasmids containing T3, T7 or SP6 promoters. Standard 50 µl translation mixes were used for interaction experiments with GST–RAP46/GSH–Sepharose; for controls (for example, Figure 6C), ~1/20 of the material was used per lane of SDS–PAGE.

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We have now identified two separate sequences from a human cDNA library that interact with Hap/RAP46 independently of hsp70s.