Rapid Purification of Mammalian 70,000-Dalton Stress Proteins: Affinity of the Proteins for Nucleotides

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A new and rapid purification procedure has been developed for the mammalian 70,000-dalton (70-kDa) heat-shock (or stress) proteins. Both the constitutive 73-kDa protein and the stress-induced 72-kDa protein have been purified by a two-step procedure employing DE52 ion-exchange chromatography followed by affinity chromatography on ATP-agarose. The two proteins, present in approximately equal amounts in either the 12,000 × g supernatant or pellet of hypotoni cally lysed heat-shock-treated HeLa cells, were found to copurify in relatively homogenous form. The purified proteins were covalently labeled with the fluorescent dye tetramethylrhodamine isothiocyanate, and the fluorescently labeled proteins were introduced back into living rat embryo fibroblasts via microinjection. The microinjected cells maintained at 37°C showed only diffuse nuclear and cytoplasmic fluorescence. After heat-shock treatment of the cells, fluorescence was observed throughout the nucleus and more prominently within the nucleolus. This result is consistent with our earlier indirect immunofluorescence studies which showed a nuclear and nucleolar distribution of the endogenous 72-kDa stress protein in heat-shock-treated mammalian cells. The result also indicates that, for at least the 72-kDa protein, (i) the protein has been purified in apparently “native” form and (ii) its nucleolar distribution is stress dependent.

An apparent defense mechanism which cells utilize when confronted with adverse changes in their local environment has been termed the heat-shock or stress response. The response is characterized by the rapid, preferential synthesis and accumulation of the so-called heat-shock or stress proteins. This changeover in the pattern of protein synthesis is accompanied by a sharp curtailment of transcription and translation of genes which were active before the environmental insult. The exposure of cells to a number of different and seemingly unrelated agents, including heat-shock treatment, amino acid analogs, and heavy metals to name just a few, results in the synthesis and accumulation of the stress proteins. Although the function of the various stress proteins is still not clear, their accumulation in the cell collectively appears to afford the cell protection, especially upon subsequent stress situations for general reviews, see references 1, 16, and 20.

Considerable work from many laboratories has focused on the identification, characterization, and localization of the stress proteins, with the ultimate aim being to dissect the function of these proteins. In our laboratory, the proteins synthesized at elevated levels after physiological stress of mammalian cells are referred to, according to their apparent size in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as the 28-, 72-, 73-, 80-, 90-, 100-, and 110-kilodalton (kDa) proteins (20). All of these proteins, with the exception of the 72-kDa species, are present in appreciable levels in normal, unstressed 37°C cells (25). Hence, it has been suggested by us and others that the stress proteins most likely serve functions in the cell which are distinct from their participation during stress. Indeed, synthesis and post-translational modification of some of the stress proteins have been shown to be sensitive to: (i) extracellular levels of either glucose or calcium (25); (ii) viral infection (7, 11, 15); and (iii) various growth factors or mitogens (22).

Much of the work examining the structure and function of the stress proteins has focused on the ca. 70-kDa proteins. In most mammalian cells, there appear to be two forms of this protein, a constitutive form present in appreciable levels under normal conditions (the 73-kDa protein) and an induced form whose synthesis is most obvious only after physiological stress (the 72-kDa protein). Both peptide mapping and immunological studies have shown that the 73-kDa and 72-kDa proteins are similar but not identical (24). Cell fractionation and immunological methods have shown that a portion of each protein localizes to the nucleus after heat-shock treatment of various mammalian cell lines. Most interestingly, the highly induced 72-kDa protein also shows a distribution within phase-dense chromatin, including the nucleoli (24).

We previously described a procedure for the purification of the 72- and 73-kDa proteins from heat-shock-treated HeLa cells that uses a combination of ion-exchange and gel filtration chromatography (23). In the present report, we describe a new, rapid two-step purification procedure for the 72- and 73-kDa proteins based upon their ability to bind to ATP. In addition, the purified proteins have been conjugated with a fluorescent dye and reintroduced into living cells via microinjection. In the case of at least the 72-kDa protein, it is shown that the microinjected protein localizes within the nucleus and cytoplasm but not the nucleolus in cells incubated at 37°C. However, after heat-shock treatment, the microinjected protein rapidly relocates within the nucleolus, indicating that the nucleolar locale of the 72-kDa protein is heat (or stress) dependent.

MATERIALS AND METHODS

Cell culture and radioisotope labeling. HeLa cells growing in suspension were harvested by centrifugation, washed with phosphate-buffered saline, and resuspended in F13 medium containing no leucine (GIBCO Laboratories, Grand Island, N.Y.). The cells were incubated at either 37 or 42.5°C and labeled with 1 mCi of [3H]leucine (t-[4,5-3H]leucine; 50
Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 3 h. After labeling, the cells were collected by centrifugation, washed with phosphate-buffered saline, and solubilized in Laemmli sample buffer supplemented with DNase and RNase (23). Similarly, HeLa cells growing on plastic dishes (Falcon) were labeled at 37°C with [35S]methionine (New England Nuclear Corp., Boston, Mass.; specific activity, >800 Ci/mmol) in methionine-free Dulbecco modified Eagle medium (GIBCO) for 3 h. The cells were solubilized as described above.

Column chromatography, buffers, and protein purification. DEAE-cellulose (DE52) was purchased from Whatman, Inc., Clifton, N.J. ATP-agarose (linked through C-8) was purchased from Sigma Chemical Co., St. Louis, Mo. Buffers used throughout the purification included buffer B and buffer D. Buffer B contained 20 mM Tris-acetate (pH 7.5), 20 mM NaCl, 0.1 mM EDTA, and 15 mM β-mercaptoethanol. Buffer D consisted of buffer B supplemented with 3 mM MgCl₂.

For the purification of the 72- and 73-kDa stress proteins, 40 liters of suspension HeLa cells, growing in F-13 Spinner medium supplemented with 5% calf serum, were incubated at 42.5°C for 5 h. After the heat-shock treatment, the cells were collected by centrifugation, washed with phosphate-buffered saline, and resuspended in hypotonic buffer (10 mM Tris-acetate [pH 7.5], 10 mM NaCl, 0.1 mM EDTA). After maximum swelling, the cells were lysed by Dounce homogenization, and the lysate was centrifuged at 12,000 × g for 15 min. Similarly, the 12,000 × g supernatant was applied directly to a DE52-cellulose column, the column was washed with buffer B, and the proteins were eluted with a 20 to 350 mM linear gradient of NaCl in buffer B. After analysis of eluted protein by SDS-PAGE, the peak fractions containing the 72- and 73-kDa proteins were pooled and applied directly to an ATP-agarose column (1.0 by 20 cm), and the column was washed with buffer D containing 0.5M NaCl and then with buffer D alone. The column was first developed with buffer D supplemented with 1 mM GTP, resulting in the specific elution of two polypeptides of ~17 and 20 kDa (see Fig. 2). Subsequently, the column was developed with buffer D containing 3 mM ATP, resulting in the specific elution of the 72- and 73-kDa stress proteins.

The proteins present in the 12,000 × g lysed HeLa cell pellet were extracted with 10 volumes of buffer B containing 1% Triton X-100 and 0.5% sodium deoxycholate. The suspension was subjected to vigorous Dounce homogenization, and the extracted proteins were separated by centrifugation at 15,000 × g. The supernatant was then applied to a DE52-cellulose column, and the proteins were eluted exactly as described above for the 12,000 × g supernatant material. The peak fractions containing 72-, 73-, and 75-kDa proteins were pooled and applied directly to an ATP-agarose column, and the column was washed as described above. The proteins were then eluted with buffer D containing 3 mM ATP.

One- and two-dimensional gel electrophoresis. One- and two-dimensional gel electrophoresis were done as described previously (24). Two-dimensional gels employed pH 5 to 7 isoelectric focusing followed by SDS-PAGE on 12.5% slab gels. Visualization of the proteins was done by either Coomassie blue staining or fluorography.

One-dimensional peptide mapping. The [3H]leucine-labeled proteins from heat-shocked HeLa cells were separated on two-dimensional gels, the proteins were visualized by Coomassie blue staining, and the individual 72-, 73-, and 75-kDa proteins were excised. The proteins were digested with Staphylococcus aureus V8 protease (6), and the peptides were analyzed on a 17.5% SDS-polyacrylamide gel. Peptides were visualized both by silver staining of the gel (Accurate Chemical & Scientific Corp., Hicksville, N.Y.) and by fluorography.

Microinjection studies. For microinjection, the purified 72-kDa and 73-kDa stress proteins were concentrated by dialysis against 70% sucrose in buffer B, and then the sucrose was removed by dialysis against buffer B alone. Conjugation of the proteins with a fluorescent tag was done as described by Burridge and Feramisco (4). Briefly, the concentrated proteins were adjusted to pH 9.0 with 0.5 M NaHCO₃, mixed with tetramethylrhodamine isothiocyanate (20 μg of dye per mg of protein) and kept at 4°C in the dark for 4 to 8 h. The reaction was terminated by the addition of 100 mM Tris-hydrochloride (pH 6.8), and the conjugated proteins were separated from the free dye by gel filtration on a Sephadex G-50 column equilibrated in buffer B. The proteins were again concentrated by sucrose dialysis as described above and then further dialyzed against 10 mM NaPO₄ (pH 7.4)–80 mM KCl. Microinjection of the proteins into the cytoplasm of rat embryo fibroblasts was done exactly as described previously (4).

RESULTS

Two-dimensional gel analysis of the HeLa stress proteins and their relative subcellular distribution. The response at the protein synthesis level of suspension HeLa cells undergoing the stress response was analyzed by two-dimensional gel electrophoresis. Cells were incubated at either 37 or 42°C and labeled with [3H]leucine for 3 h. The cells were then collected by centrifugation, and the proteins were analyzed. Shown in Fig. 1A and B are the proteins synthesized at 37 and 42°C, respectively. The heat-shock treatment resulted in the elevated synthesis of a small number of proteins (the so-called stress proteins, indicated in the figure) and a modest decreased production of most of the other cellular proteins. A comparison of the proteins synthesized at the two temperatures shows that almost all of the stress proteins are, in fact, normal constituents of the 37°C cells. For the purpose of this report, we have focused attention on those proteins with molecular masses of ca. 70 kDa (indicated by arrows in Fig. 1). In the 37°C cells (Fig. 1A), there exist substantial amounts of the [3H]leucine-labeled 73- and 72-kDa stress proteins (upward-pointing arrows, left to right). After heat-shock treatment, both the amount and number of isoelectric variants of 73- and 72-kDa proteins increased substantially. It should be pointed out that HeLa cells are somewhat unusual in that they, unlike most mammalian cells, contain high levels of the most highly induced 72-kDa stress protein, even at normal temperatures (25). In addition, we have indicated a ca. 75-kDa protein (downward-pointing arrow) which is not induced after heat-shock treatment but, as shown later, shares some properties in common with the 73- and 72-kDa stress proteins.

Before their purification, the relative subcellular distribution of the 72- and 73-kDa proteins was analyzed. Heat-shock-treated HeLa cells were swollen in hypotonic buffer and lysed by Dounce homogenization, and the cell lysate was centrifuged at 12,000 × g for 10 min. The supernatant was removed, and the proteins present in the 12,000 × g pellet were extracted by treatment with detergents (see above). Shown in Fig. 1C and D are the proteins extracted from the 12,000 × g pellet and the proteins present in the original 12,000 × g supernatant, respectively. (Equal fractions from the supernatant and pellet were analyzed.) The
73- and 72-kDa stress proteins (upward-pointing arrows in Fig. 1) fractionated equally between the 12,000 × g supernatant and pellet. Interestingly, many of the highly [3H]leucine-labeled isoelectric variants of both the 72- and 73-kDa proteins are not readily apparent by Coomassie blue staining. In the case of some of the other stress proteins, it is worthwhile to point out that the glucose-regulated and stress-induced 90-kDa protein (indicated by b) was found predominantly in the 12,000 × g supernatant, whereas the other two glucose-regulated and stress-induced proteins of 80 and 100 kDa (indicated by c and a, respectively) fractionated primarily with the 12,000 × g pellet. Finally, a noninducible 75-kDa protein (downward-pointing arrow) was present exclusively in the 12,000 × g pellet.

**Rapid purification of the 72- and 73-kDa stress proteins.** Our fractionation studies indicated that ca. half of the 72- and 73-kDa stress proteins fractionated within either the 12,000 × g supernatant or pellet. For their purification from the 12,000 × g pellet, the proteins were extracted with detergent (Triton and deoxycholate), the chromatin was removed by centrifugation, and the extracted proteins were applied to a DE52-cellulose ion-exchange column. After extensive washing of the column, the proteins were eluted with a linear gradient of increasing NaCl in buffer B. Shown in Fig. 2A is the Coomassie blue-stained gel analyzing every other fraction eluted off the DE52 column. The 72- and 73-kDa proteins eluted somewhat early off the column, with the majority of the proteins present in fractions 26 to 50. In addition, the non-stress-induced 75-kDa protein (described in Fig. 1C) was observed to coelu e with the 72- and 73-kDa stress proteins. It should also be pointed out that minor amounts of the 72-, 73-, and 75-kDa proteins as well as the majority of the 80-kDa stress protein were present in fractions 50 to 60.

Fractions 28 to 40, containing the vast majority of the 72- and 73-kDa stress proteins as well as most of the 75-kDa proteins.
protein, were pooled, adjusted to 3 mM MgCl₂, and then applied to an agarose column containing covalently linked ATP. As is discussed later, this protocol was arrived upon after assaying a variety of different ligands attached to solid supports for their ability to bind the 72- and 73-kDa stress protein (see below). After thorough washing of the column with high- and low-salt buffer, it was then developed with 3 mM ATP. A sample from every other fraction eluting off the column was analyzed by SDS-PAGE, and the proteins were visualized by Coomassie blue staining of the gel (Fig. 2B). Both the 72- and 73-kDa stress proteins as well as the 75-kDa protein were found to coelute off the column with little or no other protein contaminants.

For the purification of the 72- and 73-kDa stress proteins present in the 12,000 × g supernatant, a similar scheme was employed. The 12,000 × g supernatant was applied to a DE52-cellulose ion-exchange column, and the proteins were again eluted with a linear gradient of increasing NaCl in buffer B (data not shown; see reference 23). The majority of the 72- and 73-kDa proteins eluting early off the column (in fractions 28 to 58) were pooled, adjusted to 3 mM MgCl₂, and applied directly to an ATP-agarose column (material applied to the column is shown in Fig. 2C, lane A). After the column was washed with low and high salt (material not retained by column shown in Fig. 2C, lane B), it was developed first with 1 mM GTP, followed by washing with

FIG. 2. (A) Rapid purification of the 72- and 73-kDa stress proteins. The HeLa heat-shocked 12,000 × g pellet was suspended in 10 volumes of buffer B containing 1% Triton X-100 and 0.5% sodium deoxycholate and subjected to vigorous Dounce homogenization. The suspension was clarified by centrifugation at 15,000 × g, the supernatant was applied directly to a DE52-cellulose column (2.0 by 20 cm), and the column was developed with a linear gradient of 20 to 350 mM NaCl. A sample from every other fraction was removed and analyzed on a 12.5% SDS-polyacrylamide gel. Shown is the Coomassie blue-stained gel. For reference, the first lane at the left (lane A) shows the proteins present in the original 12,000 × g supernatant, lane B shows the proteins extracted from the 12,000 × g pellet and applied to the column, and lane C shows the material which flowed directly through the column. Molecular weight markers are shown at the right. The peak fractions of the 72- and 73-kDa proteins were present in fractions 26 to 50. (B) Fractions 28 to 40 from the above DE52 column containing the majority of the 72- and 73-kDa proteins were pooled, adjusted to 3 mM MgCl₂, and applied directly to an ATP-agarose column (1.0 by 20 cm) equilibrated with buffer D. After washing the column extensively with buffer D containing 0.5 M NaCl and then with buffer D alone, the proteins were eluted from the column with buffer D containing 3 mM ATP, and a sample from every other fraction was analyzed by SDS-PAGE. Shown is the Coomassie blue-stained gel. Coelution of the 72- and 73-kDa stress proteins as well as a 75-kDa protein was observed. For reference, molecular weight markers are included in the first lane at the left, in the second lane (lane A) is the material applied to the column, and in lane B is the material which flowed through the column (note that the proteins present in the flowthrough material were diluted and therefore underrepresented). (C) The HeLa 12,000 × g supernatant was applied to a DE52-cellulose column, and the column was developed with a linear gradient of NaCl as described for the 12,000 × g pellet. Although not shown here (see reference 23), the peak fractions of 72- and 73-kDa proteins were pooled, adjusted to 3 mM MgCl₂, and applied to an ATP-agarose column. After the column was washed with low- and high-salt buffer D (above), it was developed first with 1 mM GTP, washed with buffer D, and then developed with 3 mM ATP. A sample from every other fraction was analyzed by SDS-PAGE, and the proteins were stained by Coomassie blue. Lane A shows the material applied to the column, and lane B shows the material which flowed through the column.
buffer D alone, and then developed with 3 mM ATP. A sample from every other fraction eluting off the column was then analyzed by SDS-PAGE (Fig. 2C). The GTP treatment resulted in the elution of small amounts of two proteins with apparent molecular masses of ca. 17 and 20 kDa. The subsequent development of the column with 3 mM ATP resulted again in the coelution of the 72- and 73-kDa stress proteins with little or no other protein contaminants.

**Two-dimensional gel analysis of the purified proteins.** To confirm the identity of the proteins purified by the procedures described above, the peak fractions eluting off the ATP-agarose columns were subjected to two-dimensional gel electrophoresis. The purified proteins were mixed with high-specific-activity $[^{35}\text{S}]$methionine-labeled HeLa cells, and the mixtures were analyzed. The gels were first stained with Coomassie blue to identify the unlabeled, purified proteins and subsequently dried and exposed to film to identify the radioactively labeled proteins. Superimposition of the stained gels with the films of the $[^{35}\text{S}]$methionine-labeled proteins allowed for the unambiguous identification of the purified proteins. A summary of these results is presented in Fig. 3. The autoradiograph of the $[^{35}\text{S}]$methionine-labeled proteins synthesized in the 37°C HeLa cells is shown in Fig. 3A. As before, upward-pointing arrows refer to the 73- and 72-kDa stress proteins (left to right, respectively), the downward-pointing arrow indicates the 75-kDa protein, and the horizontal arrow at the left of the panel indicates the location of the 80-kDa stress protein. The Coomassie blue-stained proteins purified from the 12,000 x g supernatant are shown in Fig. 3B. The two purified proteins comigrated with the two major $[^{35}\text{S}]$methionine-labeled isoforms of the 72- and 73-kDa stress proteins. In Fig. 3C are the Coomassie blue-stained proteins purified from fractions 28 to 40 off the DE52 column analyzing the HeLa 12,000 x g pellet and subsequent chromatography on ATP-agarose. Again, the 72- and 73-kDa stress proteins as well as the 75-kDa protein are apparent. Finally, in Fig. 3D are proteins which were purified from fractions 41 to 60 off the DE52-cellulose column (12,000 x g pellet) and subsequent chromatography on ATP-agarose (data not shown). The proteins purified were the 73-kDa stress protein, minor amounts of the 75-kDa protein, and, finally, large amounts of the 80-kDa stress protein.

**One-dimensional peptide maps of the 72- and 73-kDa stress proteins and of the copurifying 75kDa protein.** Because the 72- and 73-kDa stress proteins as well as the 75-kDa protein
all exhibiting binding to ATP-agarose, we examined the possible relatedness of the three proteins by one-dimensional peptide mapping. The [3H]leucine-labeled proteins synthesized in HeLa cells treated at 42°C were separated by two-dimensional gel electrophoresis, the individual 72-, 73-, and 75-kDa proteins were excised from the gels, and the proteins were mapped by proteolytic digestion with S. aureus V8 protease (6). The peptides generated were analyzed on a 17.5% polyacrylamide gel and visualized by fluorography (Fig. 4). Some peptide fragments showed similarity in size, but overall, the three proteins appeared to be distinct polypeptides.

Microinjection of the purified 72- and 73-kDa stress proteins into rat fibroblast cells. Because there exists no biochemical method to assay the “nativeness” of the purified stress proteins, we elected to examine the effects of introducing the purified 72- and 73-kDa proteins back into living cells and to determine where in the cell the proteins became localized. Our previous indirect immunofluorescence studies with an antibody specific for 72 kDa demonstrated that the protein localized within the nucleus and nucleolus in a variety of mammalian cells after heat shock (24). (In the case of the 73-kDa protein, a portion of the protein is present in the nucleus after heat shock, but we have not as yet obtained antibodies specific for the protein and therefore have not similarly determined its entire in vivo location either before or after heat-shock treatment.) To follow their distribution in the cell, the purified HeLa 72- and 73-kDa stress proteins were conjugated with the fluorescent tag tetramethylrhodamine isothiocyanate as described above. Two-dimensional gel electrophoresis of the conjugated proteins and visualization of the proteins present in the gel with UV light demonstrated the covalent incorporation of the chromophore into both proteins (data not shown). The rhodamine-conjugated proteins were microinjected into the cytoplasm of rat embryo fibroblasts growing at 37°C, and the cells were then further incubated at either 37 or 42°C. In the microinjected cells incubated at 37°C (Fig. 5A and B), most of the fluorescence was found in the nucleus, with some diffuse cytoplasmic fluorescence also being observed. In the case of the injected cells incubated at 42°C, fluorescence was found throughout the nucleus, with intense fluorescence observed within individual nucleoli (Fig. 5C and D). Interestingly, along with the nucleolar distribution of the proteins during heat-shock treatment, examination of the corresponding phase-contrast micrographs revealed increased phase-density changes in the nucleus and nucleolus of the heat-treated cells (Fig. 5C) as compared with those cells maintained at 37°C (Fig. 5A). Thus, in the case of at least the 72-kDa protein, these results indicate that the purified HeLa protein has been isolated in “native” form and is both recognized and utilized by the rat fibroblasts in a manner similar to that found for the endogenous rat 72-kDa protein.

**DISCUSSION**

We previously described a purification procedure for the mammalian 72- and 73-kDa stress proteins from the soluble fraction of stressed HeLa cells which involved a series of ion-exchange and gel filtration chromatography steps (23). As outlined in this paper, purification of the two proteins has been greatly simplified and has allowed for the purification of the proteins from either the supernatant or pellet of hypotonic lyzed cells. Purification by DE52-cellulose ion-exchange chromatography followed by affinity chromatography on ATP-agarose is rapid and results in considerable quantities of 72- and 73-kDa proteins being isolated in relatively homogenous forms.

With the 12,000 × g supernatant of hypotonically lysed heat-shocked HeLa cells as the starting material, our new purification scheme resulted in the copurification of the 72- and 73-kDa proteins. Copurification of the two proteins was also observed during our previous purification procedure (23), and to date we have not been able to purify, under nondenaturing conditions, the proteins away from one another. One-dimensional peptide mapping has demonstrated that the constitutive 73-kDa protein and the highly induced 72-kDa protein show some similarity but overall appear to be distinct polypeptides (Fig. 4). In addition, the two proteins appear to be unique immunologically since polyclonal serum specific for 72-kDa proteins shows little or no cross-reactivity with the 73-kDa protein (24). At present, we still do not understand the basis for the copurification of the two proteins. In addition to the purification of 72- and 73-kDa proteins from the soluble fraction of lysed cells, we have shown that the two proteins can be extracted from the 12,000 × g pellet of hypotonically lysed HeLa cells by treatment with nonchaotropic detergents. After their extraction, the 72- and 73-kDa proteins were found to copurify along with another protein of apparent molecular mass of 75 kDa. This 75-kDa protein, present exclusively in the 12,000 × g pellet, is not induced after stress and by peptide mapping appears to be distinct from either the 72- or 73-kDa protein. We are currently in the process of preparing polyclonal antibodies against the 75-kDa protein to facilitate the determination of its subcellular localization. Finally, from the 12,000 × g pellet we also observed fractions eluting somewhat late off the DE52 column which, after chromatography over ATP-agarose, resulted in the purification of the 73-kDa stress protein, small amounts of the 75-kDa protein, and considerable quantities of the 80-kDa stress protein (Fig. 4D). As we and others have shown, the 80-kDa stress protein is synthesized at high levels in cells either deprived of glucose (i.e., it is part of the so-called glucose-regulated protein family) or cells treated with the calcium ionophore A23187 (9, 10, 18, 25, 26). Indirect immunofluorescence studies with a polyclonal antibody raised against the 80-kDa protein have shown the
FIG. 5. Microinjection of fluorescently labeled 72- and 73-kDa stress proteins. The purified 72- and 73-kDa stress proteins were conjugated with the chromophore tetramethylrhodamine isothiocyanate as described in the text. The fluorescently labeled proteins were microinjected into the cytoplasm of 37°C rat embryo fibroblasts, and the cells were incubated at either 37°C (A and B) or 42°C (C and D). The proteins were visualized by fluorescence microscopy with the appropriate filters (see the text). (A and C) Phase-contrast micrographs; (B and D) corresponding fluorescence micrographs.

protein to be present primarily within the endoplasmic reticulum (unpublished data). Whether portions of the copurifying 73-, 75-, and 80-kDa proteins are, in fact, associated with one another as a complex in vivo or whether all individually display nucleotide binding properties is currently being examined.

The rationale for using ATP affinity chromatography as a purification step for the 72- and 73-kDa proteins was arrived upon after assessing the affinity of the two proteins for a variety of different ligands attached to solid supports. We discovered that all of the ribonucleotides, some deoxyribonucleotides, blue and red Sepharose, and NAD-agarose all were somewhat effective in binding the 72- and 73-kDa proteins. We chose to use the ATP-agarose since it showed the greatest binding of the 72- and 73-kDa proteins and the least absorption of most other cellular proteins after heat-shock treatment. Hence, because of this seemingly non-selective and general binding behavior of the two stress proteins to various nucleotides (or derivatives thereof), we do not know whether the in vitro binding to ATP-agarose actually reflects a physiological activity of the proteins in vivo. Nevertheless, as was shown in Fig. 2, the ATP-agarose affinity column is most effective in achieving the purification of the proteins, a result whose merit is obvious. It should also be pointed out that other laboratories have reported that the Escherichia coli DNA K protein, which by DNA sequence analysis has been shown to be 50% homologous to the Drosophila 70-kDa heat-shock protein (3), possesses both weak ATPase and autophosphorylating activities and appears to be involved in the replication of bacteriophage lambda DNA in infected cells (28, 29). In the case of the mammalian 72- and 73-kDa proteins, we have tested the ability of the purified proteins to either hydrolyze ATP in vitro or to autophosphorylate. Neither activity has been observed to date.

It is plausible that the binding of the two proteins to ATP-agarose (and other nucleotides) may, in fact, reflect the ability of either the 72- or 73-kDa protein to bind to nucleic acids in vivo. Many laboratories, including our own, have suggested that the family of 70-kDa stress proteins may be associated with RNA in vivo. For example, Storti et al. (19), DiDomenico et al. (8), as well as studies in our own laboratory have observed that the 70-kDa proteins tend to streak during isoelectric focusing when the cell lysates being examined are not first treated with RNase. Moreover, Kloetzl and Bautz, using in vivo UV irradiation techniques, have reported the 70-kDa stress proteins to be components of heterogenous ribonucleoprotein (RNP) complexes in heat-shock-treated Drosophila tissue culture cells (12). Consistent with these reports is our observation that the most highly induced mammalian stress protein, that of 72-kDa, is found both in the nucleus and nucleolus of a variety of heat-
shocked mammalian cells (24). Similarly, microinjection of the fluorescently labeled, purified HeLa 72- and 73-kDa stress proteins into the cytoplasm of rat embryo fibroblasts showed a heat-dependent nucleolar distribution. As we have suggested previously, we think it possible that at least the 72-kDa protein migrates to the nucleus to become assembled into some sort of RNP complex. That the site of assembly of such a putative RNP would occur within the nucleolus would not be surprising since this organelle is, in fact, the site of assembly of many RNPs, most notably ribosomes as well as other recently described small RNPs. Some of the smaller RNPs, in fact, appear to be involved in certain regulatory aspects of gene transcription, translation, or both (see references 2, 5, 13, 14, 17, 21, and 27). The questions arises, then, whether an RNP complex containing the major induced 72-kDa stress protein could be involved in the transcriptional or translational regulation or both occurring during the stress response. Studies in our laboratory, therefore, are currently in progress to isolate such a putative RNP complex containing the 72-kDa stress protein.

Finally, we should point out that our purification scheme for the HeLa 72- and 73-kDa stress proteins resulted in the isolation of the proteins in what appears to be their native form. For example, microinjection of the purified and fluorescently labeled HeLa proteins into the cytoplasm of 37°C rat fibroblasts and subsequent heat-shock treatment resulted in a migration of at least the 72-kDa protein into the nucleus and nucleolus, a result similar to that found for the endogenous rat fibroblast 72-kDa protein (Fig. 5). This result demonstrated that (i) the nucleolar distribution of the protein is heat dependent (no nucleolar fluorescence was observed in the injected cells maintained at 37°C) and (ii) the 72-kDa protein is apparently well conserved amongst different species, since the rat fibroblasts were able to both recognize and utilize the injected purified HeLa protein in a manner similar to that of the endogenous rat fibroblast 72-kDa protein. These results are encouraging since it now appears possible to rapidly purify large amounts of the 70-kDa proteins in apparently native form and to begin examining the effects of introducing large quantities of the proteins back into living cells. By using this approach, experiments addressing the possible protective role that these proteins may serve during stress as well their ability to autoregulate their own transcription, translation, or both can be pursued.

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LITERATURE CITED


