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Cell Biology

Coprecipitation of heat shock proteins with a cell surface glycoprotein

(monoclonal antibody/plasma membrane/3T3 cells/cytoskeleton)

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ABSTRACT **Monoclonal antibodies recognizing a mouse cell surface glycoprotein of M_r 90,000 were found to coprecipitate the M_r 70,000 and 72,000 heat shock-induced proteins of NIH/3T3 cells. These two smaller proteins were among the most abundant components of heat-treated NIH/3T3 cells. The M_r 70,000 component was not detected in normal cells whereas there was a low rate of incorporation of [^{35}S]methionine into the M_r 72,000 polypeptide in the absence of heat shock. Tryptic peptide mapping and two-dimensional gel electrophoresis indicated that the coprecipitated and heat shock-induced polypeptides were identical and that the M_r 70,000 and 72,000 components contained homologous peptides. Also, the heat shock proteins had extensive structural homology with a cytoskeleton-associated protein of HeLa cells. The results suggest that the M_r 90,000 cell surface glycoprotein and the M_r 70,000 and 72,000 heat shock-inducible proteins mediate an association between the plasma membrane and the cell cytoskeleton.**

The remarkable changes in the protein composition of cells induced by sudden increases in temperature include the increased synthesis of a limited set of gene products and a concomitant decrease in the rate of synthesis of most cellular proteins (1, 2). This phenomenon has been observed with cells from various species (3–10). A similar response was also observed with other experimental regimens that disturb normal cell metabolism (11–13). Recent studies of the chemical properties of avian and mammalian heat shock proteins (8, 14) showed that the major heat-induced protein, M_r 68,000, was a methylated cell component identical to a highly conserved polypeptide previously shown to copurify with intermediate filaments (15) and microtubules (16, 17). The association of this M_r 68,000 heat shock protein with myofibrils, microtubules, and intermediate filaments suggested a possible cytoarchitectural role for the polypeptide (14).

We have found that six independently derived monoclonal antibodies recognizing a mouse cell surface glycoprotein of 90,000 daltons (gp90) coprecipitated the major heat shock-induced proteins of M_r 70,000 and 72,000 (HSP 70/72) from NIH/3T3 cells. The peptide map of HSP 70/72 closely resembled that of a reported 68,000-dalton cytoskeleton-associated protein of HeLa cells (16, 17). The data provide evidence for an association between the 90,000-dalton major plasma membrane glycoprotein and the cell cytoskeleton.

METHODS

All cells were grown without antibiotics in Dulbecco's modified Eagle's medium with glucose (GIBCO) at 4.5 g/liter and 10% (vol/vol) heat-inactivated fetal calf serum (Sterile Systems, Logan, UT) in 5% CO_2/95% O_2 at 37°C. The anti-mouse fibroblast (AMF) monoclonal antibodies (18) used for these studies were anti-p220 (AMF-1), anti-gp110 (AMF-9), anti-gp90 (AMF-4, AMF-5, AMF-6, AMF-7, AMF-17, and AMF-18), anti-gp80 (AMF-12), and anti-p72 (AMF-13).

RESULTS

Monoclonal Antibodies Recognizing a Major 90,000-Dalton Cell Surface Glycoprotein. Monoclonal antibodies recognizing mouse cell surface antigens were obtained from hybridomas of mouse myeloma cells and spleen cells of rats immunized with NIH/3T3 cells or plasma membranes (18). One group of these monoclonal antibodies immunoprecipitated a M_r 90,000 cell surface glycoprotein (gp90) that was a major plasma membrane constituent expressed by 18 different mouse cell lines (18). The gp90 was specifically immunoprecipitated from cells externally labeled with ^{125}I and from cells biosynthetically labeled with [^{3}H]fucose or [^{35}S]methionine (Fig. 1). The control immunoprecipitate with preimmune rat serum contained a M_r 65,000 polypeptide, a nonspecific precipitate of [^{35}S]methionine-labeled cells commonly observed with preimmune sera and many different monoclonal antibodies.

Induced Proteins of Heat Shock Cells Coprecipitated with gp90. It has been suggested that heat shock-induced glycoproteins of chicken embryo fibroblasts were major proteins of the plasma membrane (8). Thus, in the course of characterizing the glycoproteins of NIH/3T3 fibroblasts, the response of these cells to heat shock was analyzed.

Monolayer cultures were propagated at 37 or 44°C, and the cell proteins were then biosynthetically labeled with [^{35}S]methionine at 37°C for 30 min. The effect of heat shock on plasma membrane glycoproteins was examined by immunoprecipitation with the different monoclonal antibodies. As expected, the anti-gp90 monoclonal antibody specifically immunoprecipitated the M_r 90,000 glycoprotein from extracts of [^{35}S]methionine-labeled, uninduced cells. The new finding was that, after heat shock, the major labeled proteins of the immunoprecipitate were a polypeptide doublet of M_r 70,000/72,000, HSP 70/72 (Fig. 2). The polypeptides appeared as a single band, but with reduced exposure during autoradiography or upon two-dimensional electrophoresis, as shown below, there clearly were two components. As expected, because of the markedly diminished rate of synthesis of most proteins after heat shock, the [^{35}S]methionine-labeled M_r 90,000 glycoprotein was greatly decreased in the immune complex from the heat-treated cells. It should be noted, however, that the immune complexes of both the uninduced and heat-treated cells were composed chiefly of the unlabeled gp90 present in the cells prior to the addition of the [^{35}S]methionine-labeled substrate; pulse–chase kinetic analysis showed that the gp90 had a cellular

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Abbreviations: AMF, anti-mouse fibroblast; HSP 70/72, heat shock-induced proteins of M_r 70,000 and 72,000; gp90, M_r 90,000 cell surface glycoprotein.

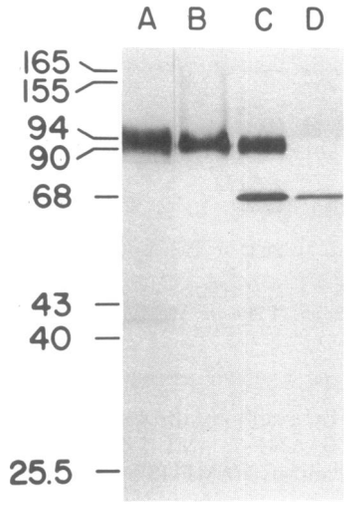

FIG. 1. Immunoprecipitation of a specific cell surface glycoprotein by anti-gp90 monoclonal antibody. Subconfluent monolayers of NIH/3T3 cells were biosynthetically labeled by incubation for 4 hr at 37°C with 4 ml of glutamine-free Eagle's minimal essential medium containing 50 μCi of [^{3}H]fucose per ml (30 Ci/mmol; Amersham; 1 Ci = 3.7×10^{10} becquerels) or for 2 hr with 4 ml of methionine-free Eagle's minimal essential medium containing 125 μCi of [^{35}S]methionine per ml (1,000 Ci/mmol; Amersham). Cell surface proteins were labeled with ^{125}I (carrier-free NaI; Amersham) by the lactoperoxidase/hydrogen peroxide system (19). Cell proteins were extracted with Nonidet P-40, immunoprecipitated, and resolved by NaDodSO$_4$/polyacrylamide gel electrophoresis (20). Lanes: A, ^{125}I-labeled NIH/3T3 cell extract (5×10^5 cpm) immunoprecipitated with AMF-5 monoclonal antibody; B, [^{3}H]fucose-labeled NIH/3T3 cell extract (1×10^6 dpm) immunoprecipitated with AMF-5 antibody; C, [^{35}S]methionine-labeled NIH/3T3 cell extract (5×10^6 dpm) immunoprecipitated with AMF-5 antibody; D, [^{35}S]methionine-labeled NIH/3T3 cells immunoprecipitated with preimmune rat serum. ^{125}I-Labeled polypeptides were detected by autoradiography for 3 days with Kodak X-Omat XR film and a Du Pont Cronex intensifying screen. [^{3}H]Fucose-labeled and [^{35}S]methionine-labeled proteins were detected by fluorography for 10 or 8 days, respectively (21). Molecular weights were calculated by reference to the mobilities of the standard proteins: *Escherichia coli* RNA polymerase, β' and β subunits (165,000, 155,000), phosphorylase a (94,000), RNA polymerase σ subunit (90,000), bovine serum albumin (68,000), ovalbumin (43,000), RNA polymerase α subunit (40,000), chymotrypsinogen (25,500), and cytochrome c (11,700).

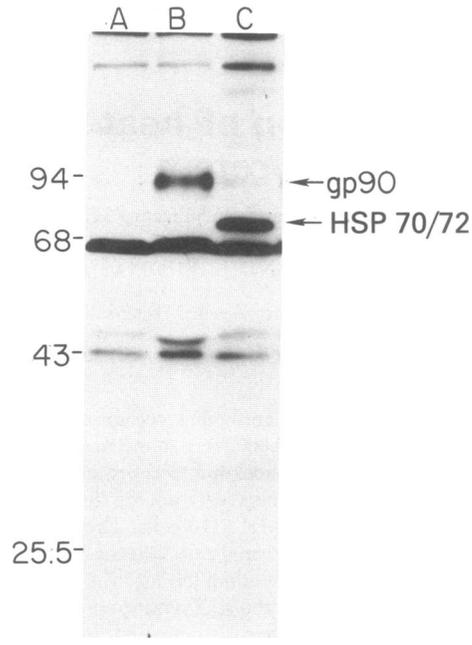

FIG. 2. Immunoprecipitation of [^{35}S]methionine-labeled proteins from uninduced and heat shock-induced NIH/3T3 cells by anti-gp90 monoclonal antibody. Confluent NIH/3T3 cells were maintained at 37°C or incubated at 44°C for 30 min. The cultures were washed once with warm Earle's balanced salt solution (GIBCO) and incubated for 2 hr at 37°C with 10 ml of Eagle's minimal essential medium (GIBCO) lacking methionine and fetal calf serum. Proteins were isotopically labeled by removing the medium and adding 4 ml of Eagle's minimal essential medium lacking methionine but containing [^{35}S]methionine at 250 μCi/ml (1,000 Ci/mmol; Amersham) for 30 min. Equivalent aliquots (5×10^6 dpm of acid-insoluble radioactivity) from Nonidet P-40 lysates were immunoprecipitated and analyzed on 10% polyacrylamide gels (20). Radiolabeled proteins were detected by fluorography for 7 days. Lanes: A, heat shock-induced NIH/3T3 cells and preimmune rat serum; B, normal uninduced NIH/3T3 cells and AMF-5 antibody; C, heat shock-induced NIH/3T3 cells and AMF-5 antibody. Identical results were obtained with the five other monoclonal antibodies recognizing gp90.

turnover time of at least 20 hr (unpublished data).

Coprecipitation of HSP 70/72 and gp90 from heat shock cells was specific. Five other independently derived monoclonal antibodies recognizing gp90 also precipitated HSP 70/72 whereas four other monoclonal antibodies that reacted with NIH/3T3 cell surface polypeptides of M_r 220,000, 100,000, 80,000, and 72,000 did not.

The following experiments showed that the immunoprecipitation of HSP 70/72 was due to an association with gp90, not to a shared antigenic determinant. A detergent extract of heat shock-induced NIH/3T3 cells labeled with [^{35}S]methionine was absorbed with an excess of anti-gp90 monoclonal antibody. Proteins of the supernatant and immunoprecipitate were resolved by NaDodSO$_4$/polyacrylamide gel electrophoresis. The gp90 and the HSP 70/72 were eluted from the gel and the radioactivity was measured. All of the gp90 but only 2–3% of the HSP 70/72 was immunoprecipitated by the monoclonal antibody. Furthermore, repeated immunoprecipitation of the supernatant with the monoclonal antibody did not yield additional gp90 or HSP 70/72.

Characterization of HSP 70/72. The two-dimensional electrophoretic pattern of [^{35}S]methionine-labeled polypeptides from the heat shock-induced cells showed that proteins HSP 70/72 were major polypeptides of the heat-treated cell (Fig. 3). Synthesis of most other cell proteins was decreased after heat shock. There was no obvious counterpart of the M_r 70,000 component in the normal cell whereas M_r 72,000 polypeptide was synthesized in the absence of heat shock. This M_r 72,000 component was also observed as a coprecipitate with gp90 in the absence of heat shock, but only with cells that had been biosynthetically labeled with [^{35}S]methionine for 12 hr and not from cells labeled only for 30 min.

That the M_r 70,000/72,000 polypeptides coprecipitated by the anti-gp90 monoclonal antibody were HSP 70/72 was confirmed by two-dimensional gel electrophoresis and by tryptic peptide mapping. The two-dimensional electrophoretic pattern of the immunoprecipitated polypeptide doublet from heat shock-induced cells was exactly the same as that of HSP 70/72 shown in Fig. 3 and the peptide map of HSP 72 was identical to the map of the M_r 72,000 polypeptide coprecipitated by the anti-gp90 monoclonal antibody (Fig. 4). Likewise, the tryptic peptide maps of the HSP 70 and the coprecipitated M_r 70,000 polypeptide were identical to each other. The peptide patterns of the M_r 70,000 and 72,000 proteins were similar except that the M_r 70,000 component lacked a distinct set of peptides present in the M_r 72,000 component.

Cellular Concentration of HSP 70/72. The relative concentration of HSP 70/72 in the cell was estimated by direct staining of proteins resolved by two-dimensional gel electrophoresis

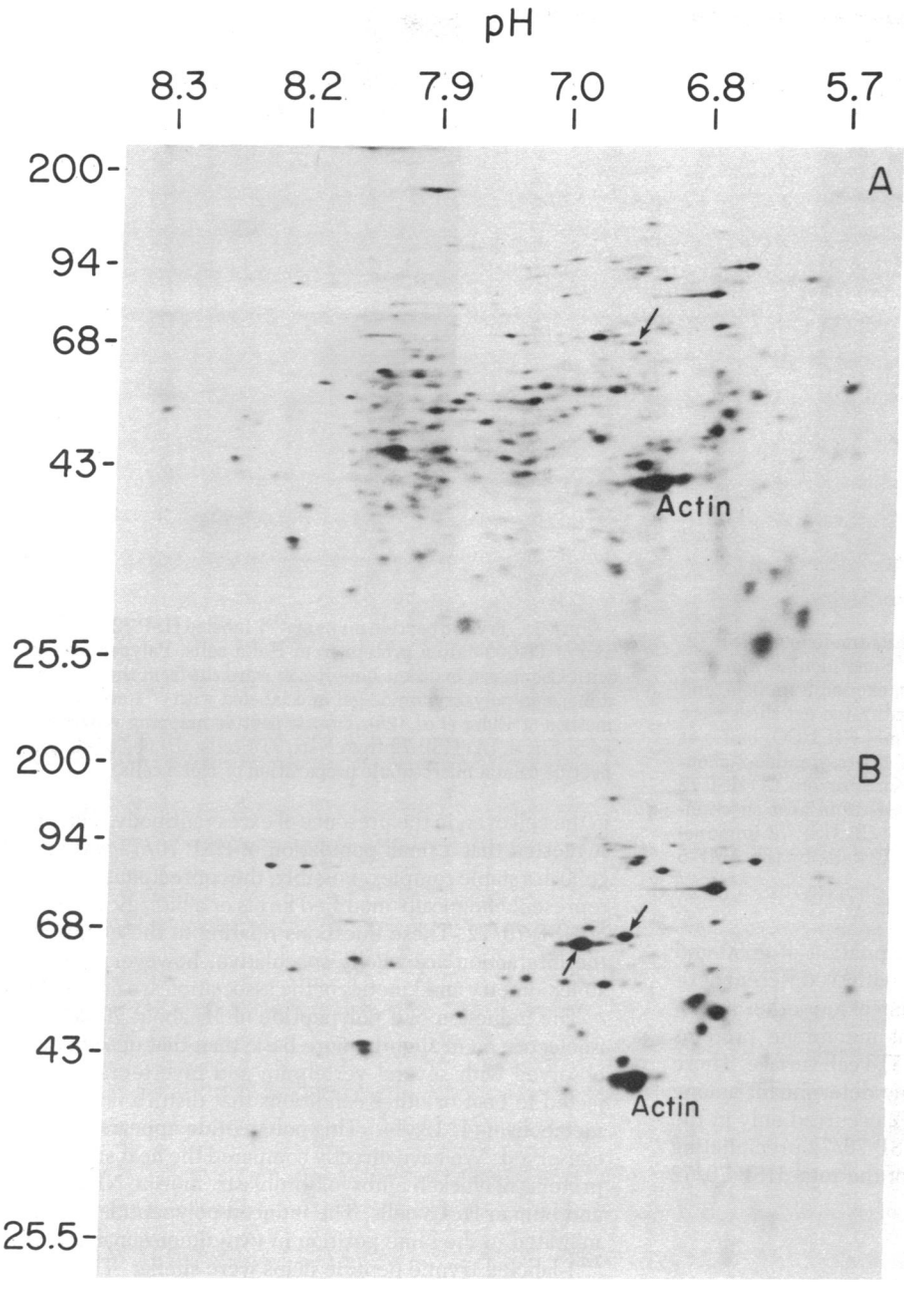

FIG. 3. Two-dimensional gel autoradiograms of [^{35}S]methionine-labeled polypeptides from uninduced and heat shock-induced NIH/3T3 cells. Cells were maintained at 37 or 44°C and labeled with [^{35}S]methionine for 30 min, as in Fig. 2. Cell proteins were extracted with a high pH, high ionic strength lysis buffer containing Nonidet P-40 (22). Two-dimensional gel electrophoresis was carried out according to O'Farrell (23). After isoelectric focusing, the samples were analyzed by electrophoresis on 10% slab gels (20). Radiolabeled proteins were detected by autoradiography. (*A*) Cells continuously grown at 37°C (4×10^5 dpm in 5 μl of extract). (*B*) Heat shock-treated cells (4×10^5 dpm in 5 μl of extract). Autoradiography was for 8 days. Actin served as an internal marker of 43,000 daltons, pI 6.85. Arrows, major heat shock-induced proteins, HSP 70/72 and the 72,000-dalton counterpart in the uninduced cell.

(Fig. 5). The heat-induced culture was incubated for 2 hr at 44°C, and both cultures were then maintained at 37°C for 20 hr. HSP 70/72 were among the most abundant components of heat-treated NIH/3T3 cells. As estimated by staining with Coomassie brilliant blue, the amount of induced HSP 70 was similar to that of actin, indicating that HSP 70/72 may comprise about 10% of the total protein of the heat-treated cell.

Structural Relationships Among Heat Shock-Proteins and a Cytoskeleton-Associated Protein from HeLa Cells. Several of the polypeptides synthesized at high rates after heat shock appeared to be components of the cell cytoskeleton. This, and the high concentration of the heat shock proteins in the cell, suggested to us that the HSP 70/72 might have a cytoarchitectural role in the cell. Such a possibility was consistent with the findings in several laboratories of polypeptides of M_r about 70,000 associated with microtubules (16, 17, 25, 26), microfilaments (27), and intermediate filaments (15).

One of these cytoskeleton-associated proteins, a 68,000-dalton component of HeLa cells (16, 17), was directly compared with the HSP 70/72. The 68,000-dalton polypeptide, isolated by repeated cycles of polymerization-depolymerization of HeLa cell microtubules, was provided by James Weatherbee and Ronald Luftig as a polyacrylamide gel-purified fraction. After elution from the gel and labeling with ^{125}I, this protein was similar to the major heat shock proteins of NIH/3T3 cells. It had the same apparent molecular weight and isoelectric point as HSP 72 and a similar tryptic peptide map (Fig. 6).

DISCUSSION

The coprecipitation, by monoclonal antibodies, of gp90 expressed on the plasma membrane of most murine cells (18) and HSP 70/72 from NIH/3T3 cells subjected to heat shock treatment provides strong evidence for the physical association of these polypeptides. Similar immunochemical techniques have been used to demonstrate association of a small fraction of a heat shock protein of M_r 89,000 with pp60^{src} (29), the Rous sarcoma virus transforming protein having the properties of an integral

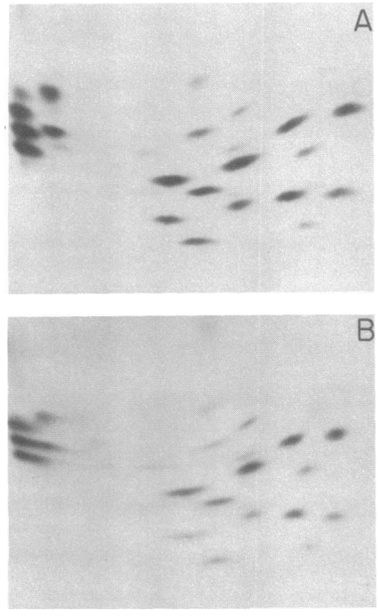

FIG. 4. Tryptic peptide maps of [^{35}S]methionine-labeled HSP 72. Polypeptides biosynthetically labeled with [^{35}S]methionine were isolated by two-dimensional gel electrophoresis, excised from gels, and analyzed by tryptic peptide mapping (24). Triturated gel slices were incubated at 25°C for 18 hr with 0.5 ml of 50 mM NH_4HCO_3 buffer at pH 8.0 containing 25 μg of trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (232 units/mg; Worthington). (*A*) HSP 72 isolated by two-dimensional gel electrophoresis from heat shock-induced NIH/3T3 cells radiolabeled for 30 min; (*B*) HSP 72 immunoprecipitated from heat shock-induced NIH/3T3 cells by the AMF-5 monoclonal antibody.

membrane component (30). The coprecipitation of gp90 and HSP 70/72 was specific: it was observed with six different anti-gp90 monoclonal antisera and not with any of four other monoclonal antibodies precipitating different membrane proteins expressed in equivalent amounts on the 3T3 cell surface. There was no evidence for a common antigenic determinant among the proteins: precipitation of HSP 70/72 occurred only in the presence of gp90, and the amount of HSP 70/72 precipitating with gp90 represented only a fraction of the total HSP 70/72

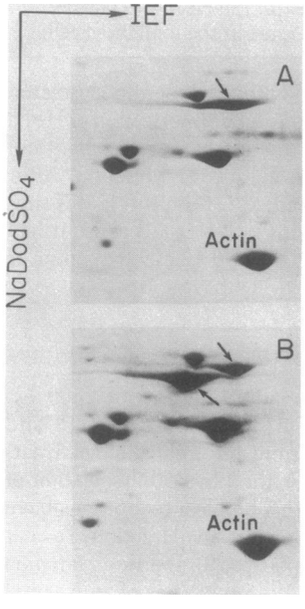

FIG. 5. Analysis of concentration of HSP 70/72 in uninduced and heat shock-induced NIH/3T3 cells by two-dimensional gel electrophoresis. Confluent monolayers were maintained at 37°C or subjected to heat shock at 44°C for 2 hr. The cultures were then maintained at 37°C for 20 hr. Extracts were prepared, and the proteins were analyzed by two-dimensional gel electrophoresis as in Fig. 3. The polypeptides were stained with Coomassie brilliant blue R-250. Relevant sections from the gels are shown. (*A*) Normal uninduced NIH/3T3 cell (425 μg of protein). Arrow, M_r 72,000 polypeptide. (*B*) Heat shock-induced NIH/3T3 cell (400 μg of protein). Arrows, HSP 70/72.

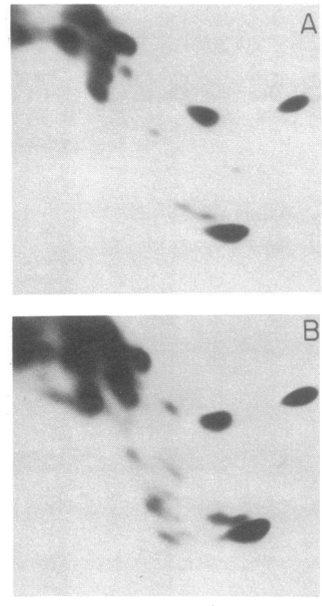

FIG. 6. Tryptic peptide maps of ^{125}I-labeled HSP 72 from 3T3 cells and of 68,000-dalton protein from HeLa cells. Polypeptides stained with Coomassie brilliant blue R-250 were cut from the dried second-dimension polyacrylamide gel and labeled with ^{125}I according to the method of Elder *et al.* (28). Tryptic peptide mapping was carried out as in Fig. 4. (*A*) HSP 72 from NIH/3T3 cells; (*B*) 68,000-dalton polypeptide from a microtubule preparation of HeLa cells.

in the cell even in the presence of excess antibody. The findings suggested that a small population of HSP 70/72 was bound to gp90 in a stable complex. Possibly, the coprecipitated molecules represent chemically modified forms or a biological subfraction of HSP 70/72. These questions relating to the quantitation of the interaction are wholly speculative, however, because the stoichiometry and kinetics of the association are not yet known.

The induction of a polypeptide of M_r about 70,000 with an isoelectric point slightly more basic than that of actin has been observed with several vertebrate and invertebrate cells subjected to heat or other conditions that disturb normal cellular metabolism (4, 12, 14). This polypeptide appears to be highly conserved. We have directly compared the heat shock-induced proteins of chicken embryo fibroblasts, murine NIH/3T3 cells, and human HeLa cells. The induced polypeptides of each cell migrated to the same position in two-dimensional gels and the ^{125}I-labeled tryptic peptide maps were similar. This homology between avian and mammalian heat shock proteins has also been reported by Wang *et al.* (14). The HeLa and avian cells also contained two isomorphic variants of the induced protein, differing in pI but not in apparent molecular weight (12, 14). With the NIH/3T3 cell, the two forms HSP 70 and HSP 72 clearly differed in electrophoretic mobility. Moreover, the fast form (HSP 70) was observed only after heat shock whereas the slower molecule (HSP 72) was present in high concentration in the absence of heat shock. Pulse–chase experiments with proteins labeled with [^{35}S]methionine prior to heat shock showed that all of the radioactivity appeared in HSP 72 and none was chased into HSP 70 (data not shown). Thus, HSP 70 did not appear to be a degradation product of HSP 72.

There is now evidence that HSP 70 is related to the cell cytoskeleton. This was first suggested to us by the substantial amounts of HSP 70/72 in the NIH/3T3 cell and the similarity of the molecule, in size, to a number of reported cytoskeleton-associated proteins. Tryptic peptide mapping confirmed this relationship. HSP 70/72 was indistinguishable from a 68,000-dalton polypeptide purified as a cytoskeleton-associated protein

of HeLa cell microtubules, generously provided by R. B. Luftig and J. A. Weatherbee (16, 17). The same finding was recently reported by Wang *et al.* (14) who also noted that the 68,000-dalton heat shock protein was indistinguishable from a component of chicken skeleton myofibrils, intermediate filament-enriched cytoskeletons prepared from a number of avian or mammalian cells grown in tissue culture, and a 68,000-dalton polypeptide that copurified with intermediate filaments from rat spinal cord neurotubules isolated from the brain.

A model suggested by these results is that the M_r 90,000 plasma membrane glycoprotein is linked to the cell cytoskeleton through an association with the heat shock-inducible proteins of M_r about 70,000. The basis for this model is the strong interaction between the molecules as evident by the coprecipitation with a monoclonal antibody. The model is supported by other known properties of gp90. The glycoprotein is an integral component of the fibroblast plasma membrane and is one of the major constituents of this structure with about 5×10^5 copies per cell (unpublished data). The glycoprotein also appears to be a common structural component of many mouse cell plasma membranes because it was found in similar concentrations among 18 different cell lines examined (18).

Many other questions concerning the association of the gp90 and HSP 70/72 remain to be examined by reconstitution studies with the purified components—for example, the stoichiometry and affinity of the interaction, the topography of gp90 in the plasma membrane, and the organization of HSP 70/72 on the cytoplasmic side of the membrane. An advanced study of such an interaction has been achieved with the human erythrocyte membrane, in which ankyrin has been identified as a link between spectrin and the cytoplasmic domain of a major integral membrane protein, band 3 (31, 32). Other associations between plasma membrane proteins and cytoskeletal components on the inner surface of the bilayer have been described; these include actin and H-2 antigens (33), actin and surface immunoglobulin (34), actin and a protein matrix on the cytoplasmic face of the plasma membrane (35), intermediate filament protein and a 170,000-dalton membrane glycoprotein (36), and fibronectin and vinculin (37).

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