Biochemical Characterization of the Mammalian Stress Proteins and Identification of Two Stress Proteins as Glucose- and Ca\(^{2+}\)-Ionophore-regulated Proteins*

(Received for publication, December 16, 1982)

William J. Welch, James I. Garrels, G. Paul Thomas, Jim J.-C. Lin, and James R. Feramisco

From the Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Biochemical properties of the heat shock or stress proteins of mammalian cells have been investigated using two-dimensional gel electrophoresis and immunological techniques. Of the major mammalian stress proteins (Mr = 72,000, 73,000, and 90,000) and minor stress proteins (Mr = 80,000, 100,000, and 110,000), the 80- and 90-kDa proteins were found to be phosphoproteins in all cell types examined. The 100-kDa protein was found to incorporate phosphate in only some cell types examined. In studies of the metabolic incorporation of mannose into the stress proteins, only the 100-kDa protein was found to be a glycoprotein. Two of the stress proteins, the 80- and 100-kDa species, were found to be identical with the proteins induced in cells grown in the absence of glucose (i.e. the "glucose-regulated proteins"). These same two proteins also were induced in cells treated with calcium ionophore A23187. To begin examining the intracellular location of these multiregulated proteins, immunofluorescence microscopy studies were carried out using a monoclonal antibody against the 100-kDa stress protein. The antigen was localized primarily with the Golgi apparatus and less prominently with the plasma membrane and nucleus. Heat shock treatment resulted in an increased number of the cells exhibiting a nuclear location of 100 kDa.

Similar to what was first described in Drosophila melanogaster (1, 2), heat shock treatment of mammalian cells results in a number of coordinate changes within the cell (for review, see Refs. 3 and 4). As has been observed in various other organisms, mammalian cells placed under stress respond by altering their normal pattern of protein synthesis. Such a changeover is characterized by the dramatic increase in synthesis of a small number of polypeptides (heat shock or stress proteins) with a concomitant decrease in production of the normal array of cellular proteins. The stress proteins are not novel components of the stressed cell since most of them are expressed in cells grown under "normal" tissue culture conditions. The stress response, in general, appears to be a defensive one in nature since a brief induction of the stress proteins appears to confer a degree of protection to the cell upon subsequent stress situations (Refs. 5–7 and reviewed in Ref. 4).

To better understand the functions of the stress proteins, we have been examining the stress response in mammalian cells both at the protein and cellular levels. With regard to changes at the cellular level, we have observed that cells grown under stress appear to 1) show a more flattened and spread morphology, 2) have an increased number of intracellular actin bundles, and 3) display a rearrangement in the intermediate filament network from the normal spliced array into a tight cap near the nucleus (8). At the protein level, stressed mammalian cells synthesize at high levels proteins with apparent molecular masses of 72, 73, 80, 90, 100, and 110 kDa. The 72-, 73-, and 90-kDa proteins correspond to the major ubiquitous stress proteins described in a variety of different organisms (reviewed in Ref. 4 and see "Discussion"). In addition to the six proteins mentioned above, there are reports of smaller stress proteins in both HeLa (9, 10) and Chinese hamster fibroblasts (11). While little is known concerning the function of the stress proteins, progress has been made in the purification of several of the proteins (12, 13), in the development of specific antisera to some of the proteins (12, 14, 15), and in the determination of their subcellular location (16–20).

In the present study, utilizing two-dimensional gel electrophoresis, we have characterized some of the biochemical properties of the mammalian stress proteins including the identification of those stress proteins which are glycosylated and phosphorylated. Through a comparison of the stress proteins to the proteins induced following treatment of cells either with a calcium ionophore or with culture medium lacking glucose (i.e. the glucose-regulated proteins (21)), it was found that the latter two treatments induce the same two proteins, and that these two proteins are identical to the 80- and 100-kDa heat shock or stress proteins. Finally, a monoclonal antibody to the 100-kDa stress protein has been used to identify the intracellular localization of the antigen. These studies showed a distribution primarily coincident with the Golgi apparatus.

Experimental Procedures

Cell Culture and Radioisotope Labeling

HeLa cells, baby hamster kidney cells, gerbil fibroma cells (American Type Culture Collection CCL 146, IMR-33), Chinese hamster ovary cells, and rat-1 cells on 35-mm plastic dishes (Falcon) were grown in DME* supplemented with 10% calf serum. Upon reaching

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Recipient of National Institutes of Health Grant GM 28277.

*§ Recipient of National Institutes of Health Grant GM 26298.

† Recipient of National Institutes of Health Grant GM 27790.

¶ Recipient of National Institutes of Health Grant GM 31048.

* The abbreviations used are: DME, Dulbecco's modified Eagle's medium; ArC, l-arginine 2-carboxylic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; GRP, glucose-regulated protein.
near confluency (0.8-1.0 X 10^6 cells/dish), the cells were stressed by either the addition of fresh culture medium containing 5 mM AzC (Calbiochem) and 2% calf serum or by the addition of fresh medium prewarmed to 42 °C and containing 2% calf serum (heat shock).

Normal and AzC-treated cells were maintained at 37 °C and the heat-shocked cells were kept at 42 °C. Following the appropriate stress period (times indicated in figures) the culture medium was removed, and the cells were washed with DME lacking methionine and then were labeled for 45 min under the appropriate conditions (normal or stress) with [35S]methionine (New England Nuclear; specific activity, >800 Ci/mmol) in 0.3-0.4 ml of methionine-free DME supplemented with 2% dialyzed calf serum. After labeling, the medium was removed and the cells were washed with cold PBS. For analysis by one-dimensional polyacrylamide gel electrophoresis, the cells were solubilized by the addition of SDS-gel electrophoresis sample buffer (13) supplemented with DNase, and the samples were boiled for 2-3 min. Alternatively, for analysis by two-dimensional gel electrophoresis, the cells were harvested at 4 °C in Tris buffer containing 0.3% SDS, 1% 2-mercaptoethanol, digested briefly with DNase I and RNase A, and quickly frozen (22). The frozen samples were lyophilized and redisolved in sample buffer containing 9.95 M urea, 4% Nonidet P-40, 2% ampholites, and 100 mM dithiothreitol. Samples were stored in sample buffer at −70 °C prior to electrophoresis.

Glucose Deprivation and Calcium-Ionophore Treatments

Rat-1 cells (~1 X 10^6 cells/dish) on 35-mm plastic dishes were depleted of glucose by the addition of fresh DME containing no glucose and supplemented with 5% dialyzed calf serum. As controls, rat-1 cells were incubated in complete DME supplemented with either 5% calf serum or 5% dialyzed calf serum. Following a 21-24 h deprivation period, the cells were labeled for 45 min with [35S]methionine in methionine-free DME (complete or lacking glucose) and then prepared for two-dimensional gel electrophoresis as described above.

Rat-1 cells (~1 X 10^6 cells/dish) on 35-mm plastic dishes were treated with the calcium ionophore A23187 (Calbiochem) at a concentration of 7 X 10^-9 M in DME supplemented with 5% calf serum. Four hours later, the medium was removed and the cells were labeled with [35S]methionine in 0.3-0.4 ml of DME (methionine-free) and supplemented with both 5% dialyzed calf serum and the ionophore. Control cultures grown in DME supplemented with 5% calf serum and containing no ionophore were labeled in parallel. After a 45-min labeling period, the cells were prepared for two-dimensional gel analysis as described earlier.

\[ {^{38}}H \text{Mannose and } {^{32}}P \text{PO}_4 \text{ Labeling of Cells} \]

Normal and stressed rat-1 and HeLa cells (~1 X 10^6 cells/dish) on 35-mm plastic dishes were labeled with 300 pCi of \([^{38}H]\)mannose (Amersham; specific activity, 13.2 Ci/mmol) in 0.3-0.4 ml of DME containing glucose at a concentration of 100 µg/ml (equal to 10% of the normal concentration of glucose), 5% dialyzed calf serum, 5 mM sodium pyruvate, and nonessential amino acids. In parallel, cells were labeled with 100 pCi of \([^{38}S]\)methionine as described earlier. Following a labeling period of 3 h, the cells were prepared for two-dimensional gel analysis as described above.

SV40-transformed rat-1 cells and L6 myoblasts (~4 X 10^6 cells/dish) on 60-mm plastic dishes were labeled with 200 µCi of H_3^3-phosphate (New England Nuclear; carrier free) for 24 h in 1 ml of DME lacking phosphate and supplemented with 1% fetal calf serum. Similarly, SV40-transformed rat-1 cells and L6 myoblasts were labeled with 250 µCi of \([^{38}S]\)methionine for 2 h in 1 ml of DME lacking methionine and supplemented with 7.5% newborn calf serum. Following the labeling periods, the cells were prepared for two-dimensional gel analysis.

![Fig. 1. Polypeptide composition of normal, AzC-treated, and heat shock-treated mammalian cells.](http://www.jbc.org/)

**Fig. 1. Polypeptide composition of normal, AzC-treated, and heat shock-treated mammalian cells.**

HeLa cells, baby hamster kidney (BHK) cells, gerbil fibroma cells (GFC), Chinese hamster ovary (CHO) cells, and rat-1 cells on 35-mm plastic dishes (~1 X 10^6 cells/dish) were grown in DME supplemented with 2% calf serum at 37 °C (lane 1), at 42 °C (lane 3), or at 37 °C in the presence of 5 mM AzC (lane 2). Five hours later, the cells were washed with PBS and labeled under the appropriate conditions (i.e. normal and stressed) with [35S]methionine in methionine-free DME supplemented with 2% dialyzed calf serum. Following a 45-min labeling period, the cells were prepared for SDS-polyacrylamide gel electrophoresis (see "Experimental Procedures") and the proteins were analyzed on a 10% polyacrylamide gel. (In general, the stressed cells incorporated approximately 80-90% of the radiolabel as that observed for the normal cells.) Shown is an autoradiograph of the gel. Molecular mass markers are indicated to the left. The 110-, 100-, 90-, 80-, and 73-72-kDa proteins are indicated (in descending order) by arrowheads. Lane 1, cells grown at 37 °C. Lane 2, AzC-treated cells grown at 37 °C. Lane 3, cells grown at 42 °C (heat shocked).
Biochemical Analysis of Stress Proteins

Two-dimensional gel electrophoresis, using isoelectric focusing followed by SDS-polyacrylamide gel electrophoresis, was done as described by Garrels (22). The first dimension isoelectric focusing gels contained pH 5–7 ampholytes (LKB), and 10% acrylamide slab gels were employed in the second dimension. In general, equal amounts of trichloroacetic acid-precipitable radioactivity were applied contained pH 5–7 ampholytes (LKB), and 10% acrylamide slab gels followed by SDS-polyacrylamide gel electrophoresis, was done as described above in the presence (D) or absence (C) of 5 mM AzC. Following the labeling period, the cells were solubilized in two-dimensional gel electrophoresis sample buffer and the proteins were analyzed on pH 5–7 isoelectric focusing gels followed by electrophoresis on 10% SDS-polyacrylamide gels. (All subsequent two-dimensional gels shown were run in exactly the same manner.) Fluorographs of the gels are shown with the acidic end to the left. The stress proteins are indicated by small letters and are as follows: a, 100-kDa; b, 90-kDa; c, 80-kDa; d, 73-kDa; e, 72-kDa; and f, 73-kDa (basic). For reference, actin is indicated by a small arrow (Cappel Laboratories) was diluted 1:80 in PBS. goat anti-mouse antibody (Cappel Laboratories) was diluted 1:500 in PBS and the fluorescein-conjugated antibody was diluted 1:500 in PBS. The cells were then labeled at either 37 °C (A) or at 42 °C (B) with [35S]methionine for 45 min in methionine-free DME supplemented with 2% dialyzed calf serum. Similarly, HeLa cells on 35-mm dishes (~1.0 × 10^6 cells/dish) were grown at 37 °C in DME containing 2% calf serum (C) or in DME containing 2% calf serum and 5 mM AzC (D). Four hours later, the cells were labeled with [35S]methionine as described above in the presence (D) or absence (C) of 5 mM AzC. Following the labeling period, the cells were solubilized in two-dimensional gel electrophoresis sample buffer and the proteins were analyzed on pH 5–7 isoelectric focusing gels followed by electrophoresis on 10% SDS-polyacrylamide gels. (All subsequent two-dimensional gels shown were run in exactly the same manner.) Fluorographs of the gels are shown with the acidic end to the left. The stress proteins are indicated by small letters and are as follows: a, 100-kDa; b, 90-kDa; c, 80-kDa; d, 73-kDa; e, 72-kDa; and f, 73-kDa (basic). For reference, actin is indicated by a small arrow (Cappel Laboratories) was diluted 1:80 in PBS. goat anti-mouse antibody (Cappel Laboratories) was diluted 1:500 in PBS and the fluorescein-conjugated antibody was diluted 1:500 in PBS. Normal and heat-shocked gerbil fibroma cells on 1.2-cm round glass coverslips were prepared for indirect immunofluorescence as described elsewhere (24). The JLI5a antibody was diluted 1:500 in PBS and the fluorescein-conjugated antibody was diluted 1:500 in PBS.

**RESULTS**

Mammalian cells grown under stress respond by altering their normal pattern of protein synthesis. In Fig. 1 are shown the [35S]methionine pulse-labeled polypeptides produced in five different mammalian cell lines (HeLa, baby hamster kidney (BHK), gerbil fibroma (GFC), Chinese hamster ovary (CHO), and rat-1 (RAT) cells) following growth for 5 h at either 37 °C (lane 1), at 37 °C in the presence of 5 mM AzC, an analogue of proline (lane 2), or at 42 °C (lane 3, heat shock). In almost every case, the stressed cells synthesized at high levels proteins with approximate molecular masses of ~70, 80, 90, 100, and 110 kDa (indicated by arrowheads). (In some cases, the ~70-kDa proteins exist as doublets referred to here as the 72- and 73-kDa proteins.) It should be mentioned that others have reported stress proteins of lower molecular mass in both HeLa (9, 10) and in Chinese hamster ovary cells (11). It is not clear why we do not detect these smaller polypeptides since in the aforementioned studies (9, 11) similar cell lines, growth conditions, and metabolic labeling techniques (i.e. [35S]methionine) were used. (Hickey and Weber (10) have reported, however, that the HeLa p27 heat shock protein does not incorporate methionine to any significant extent; and indeed, in recent experiments in our laboratory using [3H]leucine for metabolic labeling, we have detected the stress-induced elevated synthesis of a 27-kDa protein.) A careful examination of the cells grown at 37 °C (lane 1) indicated that all of the stress proteins, with the exception...
Biochemical Analysis of Stress Proteins

FIG. 3. The 100-kDa stress protein is a glycoprotein. Rat-1 cells and HeLa cells on 35-mm plastic dishes (1.0 x 10⁶ cells/dish) were labeled at 37 °C with either [³⁵S]methionine in methionine-free DME containing 5% dialyzed calf serum or with [³H]mannose in DME containing only 10% the normal concentration of glucose and supplemented with 5% dialyzed calf serum. After labeling for 3 h, the cells were prepared for and analyzed by two-dimensional gel electrophoresis. Shown are fluorographs of the gels (acid end to the left). Small letters refer to: a, 100-kDa; b, 90-kDa; and c, 80-kDa. For reference, actin is indicated by a small arrow. A, [³⁵S]methionine-labeled proteins present in rat-1 cells. B, [³H]mannose-labeled proteins present in rat-1 cells. C, [³⁵S]methionine-labeled proteins present in HeLa cells. D, [³H]mannose-labeled proteins present in HeLa cells.

of the lower band at ~70,000 Da (i.e. the 72-kDa protein), are present in significant levels in the normal cells. (This is more clearly seen in Fig. 2.) Accompanying the elevated synthesis of the stress proteins is the decreased production of most other cellular proteins. The extent of such a "shut-off" of normal protein synthesis is dependent upon both the agent used to induce the response as well as the time following induction of the response within the cells are labeled. For example, AzC treatment is more effective in depressing normal protein synthesis than is heat shock treatment. AzC treatment for longer periods (e.g., 10–20 h) results in a nearly complete cessation of normal protein synthetic patterns (8, 13, 14). While the elevated synthesis of both the ~70,000- and 90,000-Da stress proteins occurs throughout the response, the enhanced production of both the 80- and 100-kDa stress proteins tends to occur early and then subsides during the latter periods of the response. For example, in rat-1 cells, a 2-h heat shock treatment (Fig. 2, A and B) resulted in the enhanced synthesis of 80-kDa while a 5-h treatment (Fig. 1) resulted in little or no 80-kDa protein synthesis. Interestingly, although the levels of the 80- and 100-kDa proteins begin to subside during the latter periods of the response, their mRNAs remain abundant in the cell in an apparently sequestered, untranslated state (8). Finally, it should be noted that the electrophoretic mobilities of each of the stress proteins synthesized in AzC-treated cells appeared marginally decreased relative to their counterparts made in either normal or heat-shock cells. This most probably reflects the incorporation of the proline analogue (AzC) into the proteins and the subsequent effects on their electrophoretic mobilities in SDS gels.

The proteins induced in cells by stress are more clearly defined when analyzed by isoelectric focusing followed by electrophoresis on SDS-polyacrylamide gels (two-dimensional gel electrophoresis). In Fig. 2 are shown the [³⁵S]methionine pulse-labeled proteins made in rat-1 cells grown at either 37 °C (A) or at 42 °C for 2 h (B) and analyzed on pH 5-7 gradients followed by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels. A similar analysis of the [³⁵S]methionine pulse-labeled proteins synthesized in HeLa cells

G. P. Thomas, unpublished observation.
Fig. 4. Phosphoprotein patterns of SV40-transformed rat-1 cells and L6 myoblasts. SV40-transformed rat-1 cells and well fused L6 myoblasts (~4 × 10⁶ cells/dish) on 60-mm plastic dishes were labeled with H₃⁢¹⁸⁵⁴P₀₄ for 24 h at 37 °C in DME lacking phosphate and supplemented with 1% fetal calf serum. Similarly, SV40-transformed rat-1 cells and partially differentiated culture of L6 muscle cells were labeled with [³⁵S]methionine for 2 h at 37 °C in DME lacking methionine and supplemented with 7.5% newborn calf serum. Following the labeling period, the cells were prepared for and analyzed by two-dimensional gel analysis. Shown are fluorographs of the gels. Small letters refer to: a, 100-kDa; b, 90-kDa; and c, 80-kDa. For reference, actin is indicated by a small arrow. A, SV40-transformed rat-1 cells labeled with [³⁵S]methionine (Fig. 3A) or with [¹⁴C]mannose (Fig. 3B) demonstrated that the 100-kDa stress protein contained mannose. B, SV40-transformed rat-1 cells labeled with H₃⁢¹⁸⁵⁴P₀₄. C, L6 muscle cells labeled with [³⁵S]methionine. D, L6 muscle cells labeled with H₁⁢¹⁸⁵⁴P₀₄.

grown either at 37 °C or at 37 °C in the presence of 5 mM AzC for 4 h is shown in Fig. 2, C and D, respectively. In both cell types, the stress proteins are indicated with respect to their apparent molecular mass and major charge isomers as the (a) 100-kDa, pI 5.0; (b) 90-kDa, pI 5.2; (c) 80-kDa, pI 5.2; (d) 73-kDa, pI 5.5; (e) 72-kDa, pI 5.6; and (f) 73/72-kDa protein, pI 6.3 (8). Examination of the stress proteins present in rat-1 cells (Fig. 2B) and those present in HeLa cells (D) reveals one major difference, that being the multiple charge variants of the ~70-kDa proteins in the human cells. A similar heterogeneity of the ~70-kDa stress proteins is also found in HeLa cells grown under heat shock conditions (14). Such charge heterogeneity of the ~70-kDa stress proteins may result from post-translational modification including methylation (54) and/or phosphorylation (42, 43; however, see below). Although not shown here, two-dimensional gel analysis of the [³⁵S]methionine-labeled proteins present in all five cell types described in Fig. 1 revealed considerable differences in both the amount and isoelectric forms of the ~70-kDa stress proteins, with the human cells displaying the largest number of ~70-kDa charge variants. The reason for the variation in the number of ~70-kDa species among different cell types is not clear at this time. Interestingly, all of the HeLa ~70-kDa isoforms appear to be highly related polypeptides as determined by one-dimensional peptide mapping (23, 13).

Glycosylation and Phosphorylation of the Stress Proteins—To examine whether any of the stress proteins are glycosylated or phosphorylated, normal and stressed cells were labeled with either [¹⁴C]mannose or with H₁⁢¹⁸⁵⁴P₀₄. Rat-1 cells and HeLa cells grown at 37 °C were labeled with [³⁵S]methionine or with [¹⁴C]mannose for a period of 3 h and the labeled proteins were analyzed as before by two-dimensional gel electrophoresis. A comparison of the gel patterns of rat-1 cells labeled with [³⁵S]methionine (Fig. 3A) or with [¹⁴C]mannose (Fig. 3B) demonstrated that the 100-kDa stress protein contained mannose. A similar result was obtained when the [³⁵S]methionine-labeling pattern of HeLa cells (Fig. 3C) was compared to the [¹⁴C]mannose-labeling pattern (Fig. 3D). (In other studies not shown, the purified 100-kDa stress protein was observed to bind to Sepharose-linked concanavalin A, a property common to glycoproteins which contain mannose.) No mannose labeling of 80-kDa or of the other stress proteins was detected. Heat shock-treated cells labeled with [¹⁴C]mannose revealed higher levels of the 100-kDa glycoprotein as compared to cells grown at 37 °C (not shown).

We next examined the phosphoprotein patterns in a variety of different cell lines. SV40-transformed rat-1 cells or L6 myoblasts grown at 37 °C and labeled with H₁⁢¹⁸⁵⁴P₀₄ revealed that the 80-, 90-, and 100-kDa stress proteins are all phosphoproteins in these two cell types (Fig. 4). Upon lower exposures of these gels, we have observed six to eight phosphorylated isoforms of the 90-kDa protein. Phosphorylation of the 80- and 90-kDa proteins also have been observed in HeLa cells, rat embryo fibroblasts (REF-52), and mouse 3T3 cells grown either at 37 or at 42 °C (not shown). In addition, it has been previously shown that the avian analogue of the mammalian 90-kDa stress protein, the 89-kDa protein, is also a phosphoprotein (55). We have not detected, however, phosphorylation of the 100-kDa stress protein in these other cell types. The reasons for the variations in the phosphorylation of the 100-kDa protein are not as yet clear.

Specific Induction of the 80- and 100-kDa Stress Proteins by Glucose Deprivation and Calcium Ionophore Treatment of...
Biochemical Analysis of Stress Proteins

FIG. 5. Glucose-regulated and Ca**-ionophore-regulated synthesis of the 80- and 100-kDa stress proteins. Rat-1 cells on 35-mm plastic dishes (~1.0 x 10^6 cells/dish) were grown at 37 °C in either DME containing 5% calf serum or in DME containing no glucose and supplemented with 5% dialyzed calf serum. Twenty-four hours later, the cells were labeled for 45 min with [35S]methionine in methionine-free DME containing (A) or lacking (B) glucose and supplemented with 5% dialyzed calf serum. Similarly, rat-1 cells were grown at 37 °C in either DME containing 5% calf serum or in the same medium supplemented with the calcium ionophore A23187 (at a concentration of 7 x 10^-4 M) for 4 h. The cells were then labeled for 45 min with [35S]methionine in the absence (C) or presence (D) of the ionophore in methionine-free DME supplemented with 5% dialyzed calf serum. Following the labeling period, the cells were prepared for two-dimensional gel analysis. Shown are fluorographs of the gels. Proteins indicated by small letters are: a, 100-kDa; b, 90-kDa; and c, 80-kDa. For reference, actin is indicated by a small arrow. A, [35S]methionine-labeled proteins synthesized in rat-1 cells grown in DME plus 5% calf serum. B, [35S]methionine-labeled proteins synthesized in rat-1 cells grown for 24 h in DME lacking glucose and containing 5% dialyzed calf serum. C, [35S]methionine-labeled proteins synthesized in rat-1 cells grown in DME plus 5% calf serum. D, [35S]methionine-labeled proteins synthesized in rat-1 cells grown for 4 h in DME containing 7 x 10^-4 M A23187 and 5% calf serum.

Cells—Numerous reports from independent laboratories have described several proteins which have synthesis rates that are regulated by the concentration of glucose in the culture medium (21, 28-30). These so-called GRPs with reported molecular masses of approximately 75,000 and 95,000 Da are synthesized at high levels following depletion of glucose from the culture medium concomitant with the decreased production of a protein with an approximate molecular mass of 90,000 Da. In addition, the GRP-95-kDa but not the GRP-75-kDa was found to be a glycoprotein (28). Hence, because of the similar properties of these proteins to that of three of the stress proteins, we examined the possible relationship of the 80-, 90-, and 100-kDa stress proteins to the GRPs. Rat-1 cells therefore were grown in the presence or absence of glucose for 24 h, labeled with [35S]methionine for 4 h, and examined by two-dimensional gel electrophoresis. As shown in Fig. 5, cells grown in the absence of glucose (B) synthesized high levels of both the 80- and 100-kDa stress proteins as compared to cells grown in complete culture medium (A). In addition, synthesis of the 90-kDa stress protein was depressed following the 24-h glucose deprivation period. Lanks et al. (56) have similarly observed the decreased synthesis of an 85-kDa (equivalent to the mammalian 90-kDa) stress protein following treatment of murine L-cells in growth medium lacking glucose (56). Essentially identical results as those described above also were obtained in HeLa cells. It would appear then, that the glucose-regulated proteins are in fact a subset of the heat shock proteins.

Recently Wu et al. (31) have shown that brief exposure of tissue culture cell lines to calcium ionophores selectively stimulates the synthesis of two proteins with molecular masses of 80,000 and 100,000 Da. Again, because of their similar molecular masses and isoelectric points, we investigated whether these two calcium-sensitive proteins were in fact the 80- and 100-kDa stress proteins. Rat-1 cells therefore were treated for 4 h with the calcium ionophore A23187 (7 x 10^-4 M) and then were labeled with [35S]methionine. In Fig. 5 are shown the two-dimensional electrophoretograms of control (C) versus ionophore-treated (D) cultures. The 80- and 100-kDa proteins induced by the ionophore were found to be identical with the 80- and 100-kDa stress proteins. Although not as dramatic as observed during glucose deprivation, calcium ionophore treatment resulted in a slight depression in 90-kDa synthesis.

Production and Characterization of Polyclonal and Monoclonal Antibodies against the 100-kDa Stress Protein—To facilitate the characterization of its subcellular location, both polyclonal and monoclonal antibodies were prepared against the 100-kDa protein. For the production of a polyclonal antibody, the 100-kDa protein was purified from HeLa cells...
Biochemical Analysis of Stress Proteins

Fig. 6. Immunoprecipitation of the 100-kDa stress protein from rat-1 cells. Rat-1 cells on 10-cm dishes (~1.2 × 10^7 cells/dish) were labeled with [35S]methionine for 24 h, the cells were solubilized by the addition of 1% Triton X-100 in PBS, and the lysate then was clarified by centrifugation. Immunoprecipitation from this lysate with the JLJ5a antibody was performed as described under “Experimental Procedures.” The cell lysate (A) and the immunoprecipitate from the cell lysate (B) were analyzed by isoelectric focusing on pH 5-7 focusing gels followed by electrophoresis on 10% SDS-polyacrylamide gels (acid end to the left). Shown are fluorographs of the gels. Small letters to indicate the proteins of interest are: a, 100-kDa; b, 90-kDa; and c, 80-kDa. For reference, actin is indicated by a small arrow in A. A, rat-1 cells labeled with [35S]methionine. B, immunoprecipitate from [35S]methionine-labeled rat-1 cells using the JLJ5a antibody.

Fig. 7. Localization of the 100-kDa stress protein in normal and heat-shocked cells by indirect immunofluorescence. Gerbil fibroma cells growing on glass coverslips at 37 or at 42°C for 4 h were fixed in 3.7% formaldehyde, extracted with acetone, and processed for immunofluorescence as described in detail elsewhere (24). The cells were first incubated with the JLJ5a antibody (diluted 1:500 in PBS) followed by fluorescein-isothiocyanate-conjugated rabbit anti-mouse IgM antibody (diluted 1:80 in PBS). The coverslips were mounted in Gelvatol (Monsanto) and photographed with a Zeiss Photomicroscope III (X63 oil, numerical aperture 1.4 lens) on Tri-X film (Kodak). A and B, phase contrast (A) and fluorescence (B) micrographs of the same field of gerbil fibroma cells grown at 37°C and stained with JLJ5a monoclonal antibody. C and D, phase contrast (C) and fluorescence (D) micrographs of gerbil fibroma cells grown at 42°C and stained with JLJ5a monoclonal antibody.
control experiments (e.g., by preabsorption of the antibody with the purified antigen and by staining with nonimmune antibodies), the staining patterns were determined to be specific for the 100-kDa protein. As exemplified in Fig. 7, A and B, the antigen was localized primarily in the perinuclear region of the cells grown at 37 °C. Although not shown here, a number of the cells (~10%) exhibited a nuclear distribution of the 100-kDa protein as well (23). The perinuclear distribution of the 100-kDa stress protein corresponds to the Golgi apparatus in D. melanogaster (23). This was shown by double-staining experiments in which the location of the 100-kDa protein, determined by immunofluorescence, was coincident with the reaction products of thiamine pyrophosphatase, a well-established enzyme marker of the Golgi apparatus (32). Furthermore, treatment of cells with either colcemid or monensin, two drugs known to disrupt and fragment the Golgi apparatus (33, 34), resulted in a rearrangement of the 100-kDa protein from its normally perinuclear location into vacuoles dispersed throughout the cytoplasm (23). It should be mentioned that polyclonal antibodies directed against the 100-kDa stress protein gave rise to staining patterns similar to those obtained using the JLJ5a monoclonal antibody. However, in addition to the perinuclear staining, some fluorescence was detected on or near the plasma membrane (data not shown).

Having found the 100-kDa protein to be present primarily in the Golgi apparatus as well as in the nucleus of some of the cells, it was of interest to determine whether its distribution changed during heat shock treatment of the cells. Hence, gerbil fibroma cells were subjected to heat treatment for 5 h at 42.5 °C and analyzed by indirect immunofluorescence. As shown in Fig. 7, C and D, the number of cells displaying nuclear staining increased. In addition, the perinuclear (or Golgi) staining became less prominent. We have found that the extent of this apparent redistribution of 100-kDa protein varies from cell line to cell line and is dependent upon the time at which the cells are examined following the heat shock treatment.

**DISCUSSION**

While the proteins induced following stress in many organisms have been described, their function and in some cases their location in the cell is still unknown. In our effort to determine the functions of the stress proteins and the presumed protective role of the response, we have investigated the stress response at both the cellular and biochemical levels. It is established from work presented here and elsewhere (4) that proteins with molecular masses of approximately 72,000, 73,000, 80,000, 90,000, 100,000, and 110,000 Da are synthesized at high rates in mammalian cells grown under stress. In addition, all of the stress proteins (with the exception of some of the minor isoforms of the 72-kDa species) are made in normal tissue culture cells, albeit at lower rates. The mammalian 72-, 73-, and 90-kDa proteins correspond to the major stress proteins described in numerous other studies dealing with heat shock in a variety of organisms. Briefly, the apparently analogous stress proteins in these various organisms include: 1) the 68,000/70,000- and 82,000-kDa proteins of *D. melanogaster* (2, 35); 2) the 73-kDa (or 70-kDa (12)) and 89-kDa proteins of chickens (36); 3) the 64- and 88-kDa proteins of myoblasts (37); 4) the 79- and 100-kDa proteins of yeast (38); 5) the 70- and 82-kDa proteins of *Dictyostelium* (39); 6) the 68- and 90-kDa proteins of *Chironomus tentans* (17); and 7) the 65- and 85-kDa proteins of higher plants (40). Interestingly, both immunological and genetic data support the idea that the ~70- and 90-kDa proteins are well conserved among the various organisms (12, 41). The 80-, 100-, and 110-kDa proteins, on the other hand, appear to be minor stress proteins and have been described only in brief or not at all in these other organisms. Herein we have described further some of the biochemical properties of the mammalian stress proteins and, in the case of the 100-kDa protein, its intracellular location.

By metabolic labeling of various mammalian tissue culture cell lines with [35S]methionine, [3]H]mannose, and H3PO4, we have determined which of the stress proteins are glycosylated and/or phosphorylated. First, of the mammalian 100-kDa protein is a glycoprotein which contains mannose. Secondly, H3PO4 labeling of a number of different cell types indicated that the 80- and 90-kDa proteins are phosphoproteins with there being six to eight phosphorylated variants of the 90-kDa protein. Incorporation of phosphate into the 100-kDa protein was found to occur in SV40-transformed rat-1 cells and in L6 myoblasts but not in normal rat embryo fibroblasts (REF-52), mouse 3T3 cells, or HeLa cells. In addition, no phosphorylation of the 100-kDa protein was found in these latter cell types when grown under stress. The reason for this variation in the phosphorylation state of the 100-kDa protein in these different cell types is not clear at this time and is under further study. While we did not detect any significant phosphorylation of any of the isoforms of the ~70-kDa stress protein in the mammalian cell types examined, Loomis et al. (42) have reported that in *D. discoideum* the ~70-kDa heat shock proteins are phosphorylated with the phosphorylated species having a short half-life of approximately 80,000 Da, which points to a subset of the stress proteins (Fig. 5). In all three cases, glucose deprivation, calcium ionophore treatment, and stress resulted in the increased synthesis of the same 80- and 100-kDa proteins. Interestingly, the same two proteins as well as the ~70- and 90-kDa stress proteins also are induced in excised tissues from whole animals (57) and in cells infected with lytic viruses (58, 59). Hence, it seems appropriate to say that the mammalian ~70-kDa stress proteins are phosphorylated transiently as in *Dictyostelium* and that we failed to detect this further. Further studies are in progress to resolve this issue.

Numerous studies have shown that glucose deprivation or exposure of cells to calcium ionophores, two treatments which could be considered as specialized forms of stress, result in the induction of proteins with molecular masses of approximately 80,000 and 100,000 Da, concomitant with a decreased synthesis of a 90,000-Da polypeptide (21, 30, 31). Because of their similar molecular mass and isoelectric charge, we investigated the relationship of these proteins to the stress proteins. These comparisons revealed that the proteins induced during either glucose deprivation or calcium ionophore treatment are indeed a subset of the stress proteins (Fig. 5). In all three cases, glucose deprivation, calcium ionophore treatment, and stress resulted in the increased synthesis of the same 80- and 100-kDa proteins. Interestingly, the same two proteins as well as the ~70- and 90-kDa stress proteins also are induced in excised tissues from whole animals (57) and in cells infected with lytic viruses (58, 59). Hence, it seems appropriate to include the depletion of glucose from the culture medium of cells, high influxes of Ca**2**+ into the cell, excision or damage to whole tissues, and viral infections to the long list of agents or conditions which elicit the stress response. Finally, while it had been previously suggested that the major, ubiquitous stress proteins, the ~70- and 90-kDa proteins, were in fact the same proteins induced by glucose deprivation (44), our results as well as those of Hightower (45) show this not to be the case. This past confusion relating to the absolute identities of both sets of proteins was most likely due to the use of one-dimensional SDS-polyacrylamide gels to make the identifications.

Because there exist considerable data concerning the prop-

---

of the proteins induced by either glucose deprivation or calcium ionophore treatment, some discussion of these previous studies is warranted. First with regard to the proteins induced by glucose deprivation, Stone et al. (46) and Isaka et al. (47) described the increased levels of two proteins with molecular masses of 75,000 and 90,000 Da (i.e. the 80- and 100-kDa proteins described here) following transformation of cells by avian sarcoma viruses. Subsequent studies by a number of independent laboratories showed, however, that the accumulation of these two proteins was not due directly to the onset of transformation; rather, their induction was due to the depletion of glucose from the culture medium of the rapidly growing transformed cells. Support for this idea comes from the observation that removal of glucose from the culture medium of normal, untransformed cells results in an increased synthesis of both proteins to levels similar to those found in the transformed cells (21, 30). Because their synthesis appeared to be regulated by the concentration of glucose in the culture medium, these proteins were referred to as GRPs, and it was suggested that they may serve some role in hexose transport (i.e. serving as transporters at the plasma membrane) (50, 51).

With regard to the proteins induced by calcium ionophores, Wu et al. (31) have described the increased synthesis of two proteins with molecular masses of 80,000 and 100,000 Da following exposure of cultured chicken pectoralis muscle cells as well as a variety of different mammalian cell lines to either ionomycin or A23187. The 80-kDa protein was described as a phosphoprotein (pI = 5.0) associated with intracellular membranes. The 100-kDa protein, was shown to have a slightly more acidic isoelectric point than the 80-kDa protein. We have described similar properties for both the 80- and 100-kDa stress proteins. (Although not presented here, we also find intracellular "vesicular" staining using a polyclonal antibody directed against the 80-kDa stress protein.5) A direct comparison of the two responses demonstrated that the ionophore-induced proteins were in fact the same as the 80- and 100-kDa stress proteins. Although it was suggested that the 100-kDa ionophore-induced protein may be the Ca** transport ATPase present in sarcoplasmic reticulum (31), this idea has yet to be tested. Because precisely the same proteins are induced by calcium ionophore, glucose deprivation, and stress, the question arises as to whether there is any connection between these various effectors. While we are continuing to investigate this question, reports have been made of a possible relationship between intracellular calcium concentrations and the activation of glucose transport (53); hence, these observations obviously deserve further attention.

Indirect immunofluorescence analysis using a monoclonal antibody against the 100-kDa stress protein showed the antigen to be present in the perinuclear region with a subpopulation of the cells exhibiting nuclear staining (Fig. 7). As was described under "Results," such perinuclear staining has been shown to correspond to the Golgi apparatus. Although not presented here, staining of cells with polyclonal serum directed against the 100-kDa protein revealed both a Golgi and what appeared to be a plasma membrane localization of the protein. This location of the 100-kDa protein is in agreement with the studies of Pouyssegur and Yamada (52) who described both cell surface and perinuclear staining using a polyclonal antibody against the glucose-regulated 95-kDa protein (or as discussed above, the 100-kDa stress protein) and is in agreement with our earlier cell fractionation studies (13). This location also agrees with the results of Zala et al. (50), which demonstrated an enrichment of both the 80- and 100-kDa proteins with intracellular membranes. In addition, McCormick et al. (60), employing indirect immunofluorescence analysis with a polyclonal antibody against the 100-kDa protein, have reported the antigen to be localized primarily in the plasma membrane and pericellular matrix. Since immunocytochemistry requires that the antigenic site(s) be available for binding, the failure of the monoclonal antibody to recognize the 100-kDa protein in the plasma membrane may result from a buried or masked antigenic site. We are in the process of generating additional hybridomas antibodies to aid in the analyzing the intracellular locations and functions of all the stress proteins.

Acknowledgments—We are indebted to J. D. Watson for his continued enthusiastic support of this work. For many stimulating discussions, we thank Robert Franz. We also thank S. Quelly, L. Cascio, B. McLaughlin Daniels, J. Emanuelle, and P. Renna for their expert technical assistance. The patience of M. Szadkowski in typing the manuscript is gratefully acknowledged.

REFERENCES

Biochemical Analysis of Stress Proteins

Biochemical characterization of the mammalian stress proteins and identification of two stress proteins as glucose- and Ca2+-ionophore-regulated proteins.

W J Welch, J I Garrels, G P Thomas, J J Lin and J R Feramisco


Access the most updated version of this article at http://www.jbc.org/content/258/11/7102

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/258/11/7102.full.html#ref-list-1