Phorbol Ester, Calcium Ionophore, or Serum Added to Quiescent Rat Embryo Fibroblast Cells All Result in the Elevated Phosphorylation of Two 28,000-Dalton Mammalian Stress Proteins*

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Rat embryo fibroblast cells grown under stress (e.g. heat shock, arsenite, or amino acid analogue treatment) show elevated levels of a number of proteins with apparent molecular masses between 28,000-110,000 daltons (i.e. stress proteins). It is shown that the smaller 28,000-dalton stress proteins, which do not contain methionine, are comprised of at least four isoforms, all of which appear related as determined by one-dimensional peptide mapping. [32P]H3PO4 labeling of normal and stressed cells demonstrates that the three of the four 28-kDa isoforms are phosphoproteins. In the course of other studies phosphorylation of two 28,000-dalton proteins was observed in quiescent rat embryo fibroblasts following the addition of either the phorbol diester, phorbol-12-myristate-13 acetate, a calcium ionophore, A23187, or simply fresh serum. It is shown here that these two 28,000-dalton proteins are in fact two of the 28 kDa mammalian stress proteins.

Analysis of mammalian cells by metabolic labeling has provided the identity of proteins which show increased rates of synthesis following physiological stress. [35S]methionine labeling of cells incubated under stress conditions (e.g., heat shock, amino acid analogue, arsenite treatment, etc.) and resolution of the labeled proteins by two-dimensional gel electrophoresis have shown the major mammalian stress proteins to all be acidic polypeptides (pl values between 5 and 6) with apparent molecular masses (referred to in our laboratory) of 72, 73, 80, 90, 100, and 110,000 daltons (1, 2, and reviewed in Ref. 3). In addition to these higher molecular mass stress proteins, it is now clear that mammalian cells in response to stress also synthesize a set of smaller proteins with molecular masses between 25,000 and 30,000 daltons which lack methionine (4-6). While considerable work in many laboratories has provided a number of details regarding various post-translational modifications of the mammalian stress proteins as well as their intracellular location in both the normal and in the stressed cell, the function of any one of these proteins remains as yet unknown.

The previous studies of Kim et al. (7) and those presented here demonstrate that the small non-methionine containing mammalian stress proteins are comprised of at least four highly related isoforms with apparent molecular masses of 28,000 daltons and designated here as 28-kDa A, B, C, and D. Metabolic labeling with [32P]H3PO4 reveals that two of the 28-kDa isoforms are major phosphoprotein constituents in both normal and stressed cells. In the course of other studies in our laboratory examining the effects of various tumor promoters on tissue culture cells, an increased phosphorylation, but not an increased synthesis, of two 28,000-dalton proteins following treatment of rat embryo fibroblasts with the phorbol diester, phorbol-12-myristate-13 acetate (PMA) was observed. Similarly, Feuerstein and Cooper (8, 9) had previously reported the rapid phosphorylation of two 27,000-dalton proteins (pl ~ 5.5) following treatment of a number of different human cell lines with PMA. In the present report it is shown that these small proteins being phosphorylated in response to PMA treatment are in fact two of the 28-kDa mammalian stress proteins. In addition, it is shown that increased phosphorylation, but again not increased synthesis, of these proteins occurs also as a result of either calcium ionophore treatment of cells or addition of fresh serum to quiescent cells.

MATERIALS AND METHODS

Cell Culture and Metabolic Labeling—Rat embryo fibroblasts (REF-52) (10) were grown on 35-mm plastic dishes (Falcon) in DME supplemented with 8% fetal bovine serum. Cells were placed under stress by incubation in 1) 42°C medium (heat shock), 2) 37°C medium containing 5 mM L-azetidine-2-carboxylic acid (AzC) (Sigma), or 3) 37°C medium containing 100 μM sodium arsenite. Cells were labeled with either [3H]leucine (L-[4,5-3H]leucine, 50 Ci/mmol, Amersham Corp.) or with [32P]H3PO4 ([32P]H3PO4, carrier-free, 8 mCi/ml, Amersham Corp.) in either DME lacking leucine or DME lacking phosphate (GIBCO) under the appropriate conditions for 2 h.

For the phorbol ester, calcium ionophore, and serum stimulation experiments, REF-52 cells were plated on 35-mm dishes in DME supplemented with 8% fetal bovine serum and grown at 37°C for 4 days. The cells at >90% confluency were then labeled with [32P]H3PO4 for 1 h in DME containing no phosphate or serum. The cells, in the same medium, then were exposed to either 1) 100 ng/ml PMA (PL Biochemicals), 2) 10 μM calcium ionophore A23187 (Calbiochem-Behring), or 3) fresh 5% fetal bovine serum. After 1 h the cells were harvested, prepared for, and analyzed by two-dimensional gel electrophoreses as described previously (11).

One-dimensional Peptide Maps—Individual 28-kDa polypeptides were excised from the two-dimensional gels and mapped by proteolytic digestion using the method of Cleveland et al. (12). 0.2 μg of V8 protease were used for each digestion and the peptides analyzed on a 15-cm long, 1-mm thick, 17.5% sodium dodecyl sulfate-polyacrylamide gel. [3H]Leucine and [32P]H3PO4 peptides were detected by fluorography and autoradiography, respectively.

Two-dimensional Gel Electrophoresis—Harvesting of the cell by...

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1 The abbreviations used are: PMA, phorbol-12-myristate-13 acetate; DME, Dulbecco's modified Eagle's medium; AzC, L-azetidine-2-carboxylic acid.
sates and analysis of the labeled proteins by two-dimensional gel electrophoresis was done as described previously (11). The analysis employed pH 5-7 isoelectric focusing gels followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5% gels.

RESULTS

The changeover in the pattern of protein synthesis in mammalian cells undergoing the stress response is illustrated in Fig. 1. Shown are two-dimensional gels of the [3H]leucine-labeled proteins synthesized in cells grown at 37 °C (panel A), 42 °C (panel B), 37 °C in the presence of 5 mM AzC, an amino acid analogue of proline (panel C), or 37 °C in the presence of 100 μM sodium arsenite (panel D). In general, all three of the stress treatments resulted in the increased levels of proteins with apparent molecular masses of 110 (a), 100 (b) 90 (c), 80 (d), 73 (e), and 72 kDa (f), with the 73- and 72-kDa proteins consisting of several isoforms. There are in fact a number of subtle differences with respect to the higher molecular mass proteins induced by these three different effectors of the stress response, and these differences will be discussed more completely in a forthcoming manuscript (2). In addition, proteins with M, of approximately 28,000 also are synthesized at higher levels in the stressed cells, and it is these smaller stress proteins (designated by arrowheads) which are the focus of this report. These smaller stress proteins, which do not label with [35S]methionine, are designated as the 28-kDa A, B, C, and D species. That all of these four 28-kDa species are in fact related proteins which differ in their relative isoelectric point will be presented later in this paper.

In these exposures of the 37 °C cells, it is difficult to detect any of the 28-kDa isoforms. (The expected positions of the 28-kDa A, B, C, and D proteins are indicated in panel A, Fig. 1, for reference.) However, following a much longer exposure of the film of levels of 28-kDa A, B, and C are observed (data not shown). Both heat-shock and AzC treatment resulted in a significant increase in the 28-kDa A species and to a lesser extent an increase in the 28-kDa B species (Fig. 1, panels B and C). In contrast, arsenite treatment did not affect the production of either the 28-kDa A and B species but did result in a large increased synthesis of the 28-kDa C species, and a modest increased production of 28-kDa D (Fig. 1, panel D). In some experiments an induction of the 28-kDa C isofrom following either heat-shock or AzC treatment is also apparent. Furthermore, the relative ratios of 28-kDa A, B, C, and D proteins can differ somewhat depending upon the agent used to induce the response, the severity of the response, and the total time of labeling following induction of the response. The reason(s) for this variation in the induction and amounts of the individual 28-kDa proteins with these different effectors of the response is not clear at this time.

Phosphoprotein Patterns of Normal and Stressed Cells—Fig. 2 shows the two-dimensional gel profiles of REF-52 cells labeled with [32P]H3PO4 at 37 °C (panel A), 42 °C (panel B), 37 °C in the presence of 5 mM AzC (panel C), or 37 °C in the presence of 100 μM sodium arsenite (panel D). As we and others have shown previously, the 90-kDa stress protein (designated c) is one of the major phosphoproteins in mammalian cells. Although not obvious in these light exposures of the gels, both the 80- and 100-kDa stress proteins, as have been shown previously (11), are also phosphoproteins. Superimposition of these 32P-labeled protein gels (Fig. 2) with those of the [3H]leucine-labeled gels in Fig. 1 revealed the smaller phosphorylated proteins (indicated by arrowheads in panels A-D) to be a subset of the 28-kDa stress proteins. For example, in 37 °C cells (panel A) both 28-kDa B and to a lesser extent 28-kDa C showed 32P incorporation. Following heat-shock treatment (panel B) or AzC treatment (panel C), a modest increase in the levels of phosphorylated 28-kDa B proteins, B, C, and D are indicated with arrowheads. A-D in Fig. 2) to be a subset of the 28-kDa stress proteins. For example, in 37 °C cells (panel A) both 28-kDa B and to a lesser extent 28-kDa C showed 32P incorporation. Following heat-shock treatment (panel B) or AzC treatment (panel C), a modest increase in the levels of phosphorylated 28-kDa B proteins, B, C, and D are indicated with arrowheads. A-D in Fig. 2) to be a subset of the 28-kDa stress proteins. For example, in 37 °C cells (panel A) both 28-kDa B and to a lesser extent 28-kDa C showed 32P incorporation. Following heat-shock treatment (panel B) or AzC treatment (panel C), a modest increase in the levels of phosphorylated 28-kDa B proteins, B, C, and D are indicated with arrowheads. A-D in Fig. 2) to be a subset of the 28-kDa stress proteins. For example, in 37 °C cells (panel A) both 28-kDa B and to a lesser extent 28-kDa C showed 32P incorporation. Following heat-shock treatment (panel B) or AzC treatment (panel C), a modest increase in the levels of phosphorylated 28-kDa B proteins, B, C, and D are indicated with arrowheads.
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and C was observed as compared to control cells (panel A). Arsenite treatment, however, resulted in lower levels of the phosphorylated 28-kDa B species but increased levels of the phosphorylated 28-kDa C species. In addition, phosphorylation of the 28-kDa D species was observed only in the arsenite-treated cells.

Phosphoprotein Patterns in Cells Treated with Fresh Serum, Phorbol Ester, or Calcium Ionophore—In the course of other studies in our laboratory, it was found that addition of fresh serum to quiescent cells, phorbol ester treatment, or calcium ionophore treatment of cells all resulted in the marked phosphorylation of proteins of molecular mass of approximately 28,000 daltons and which migrated in two-dimensional gels similar to that of some of the 28-kDa stress proteins. For example, shown in Fig. 3 are the two-dimensional gel profiles of [32P]H₃PO₄-labeled proteins in quiescent 37 °C cells (panel A), quiescent cells fed fresh fetal bovine serum (panel B), cells treated with 100 ng/ml of PMA (panel C), or cells treated with 10 μM calcium ionophore A23187 (panel D). All three of these treatments resulted in an elevated phosphorylation of two 28,000-dalton proteins which are designated as the 28-kDa B and C stress proteins. Again this identification was based on superimposition of these gels with those previously shown in Figs. 1 and 2. (Further verification of the identity of these proteins will be presented below.) From the work of others it is known that addition of fresh serum to cells does not lead to any dramatic change in the specific activity of ATP pools in the cells (13). Hence, the increased [32P]H₃PO₄ associated with the 28-kDa B and C proteins most likely results from either an increased activity of a specific kinase or alternatively of a specific phosphatase followed by some kinase to maintain a constant phosphorylated state of these proteins. Although these various treatments did result in an increased phosphorylation of 28-kDa B and C, no increased synthesis of these proteins, as assayed by labeling with [3H]leucine, was observed (data not shown). It is also interesting to note that the calcium ionophore treatment of cells (panel D) resulted in lower levels of the phosphorylated 90-kDa protein (indicated by c in Fig. 3 panels). This observation is not surprising since earlier studies in our laboratory have demonstrated a decreased synthesis of 90 kDa following either glucose deprivation or treatment of cells with the calcium ionophore (11). Finally, it can also be seen in Fig. 3, panel D, that the calcium ionophore treatment resulted in a significant increase in the amount of phosphorylated vimentin (designated v).

One-dimensional Peptide Mapping—The comparison of migration on two-dimensional gels of the 28-kDa proteins whose phosphorylation increased in cells treated with either phorbol ester, calcium ionophore, or serum with those of the [3H]leucine or [32P]H₃PO₄-labeled 28-kDa proteins made in stressed cells suggested that all of these proteins were identical. To confirm this identification, some of the individual 28-kDa species were cut out from the two-dimensional gels shown in Figs. 1–3 and mapped by limited digestion with Staphylococcus aureus V8 protease. Shown in Fig. 4 are the results of these proteolytic digestions. Panel A shows the V8 maps of the [3H]leucine-labeled isoforms of the 28-kDa proteins synthesized in either the 42°C cells or the arsenite-treated cells. Judging from the molecular masses of the V8-generated peptides, it would appear that the proteins contain only a small number of aspartic and glutamic acid residues (the sites at which the protease cleaves). In addition, the apparent size of the proteolytic fragments would suggest that the enzymatic digestion did not go to completion. Similar peptide mapping results (i.e. only a few, large fragments being generated) have been obtained by Kim et al. (32) for the 25-kDa non-methionine containing stress proteins of rat myoblasts. It can be seen that the maps of 28-kDa A, B, and C appear indistinguishable from one another. In panel B are shown the maps of the [32P]-labeled 28-kDa B and C proteins from either arsenite-treated or heat-shock treated cells. Again, the maps appear identical to one another and to the [3H]leucine-labeled 28-kDa peptide maps shown in panel A. The fact that the

![Figure 3: Phosphorylation patterns of REF-52 cells treated with fresh serum, phorbol ester, or calcium ionophore.](image)

![Figure 4: One-dimensional peptide maps of [3H]leucine and [32P]H₃PO₄-labeled 28-kDa proteins.](image)
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[32P]H₂PO₄ and [3H]leucine-labeled 28-kDa peptide maps appear so similar in suggestive of the protein containing multiple phosphorylation sites. (A similar result has been observed by Kim et al. (32).) Finally, the peptide maps of the [32P]-labeled 28-kDa proteins from control, serum stimulated, or PMA-treated cells are shown in panel C. Again, the maps demonstrated that these proteins are identical to the 28-kDa stress proteins. Although not shown here, additional peptide maps of the calcium ionophore stimulated phosphorylated 28-kDa B and C proteins and the phosphorylated 28-kDa D species from arsenite-treated cells showed them to be identical to the 28-kDa stress proteins as well.

DISCUSSION

Data presented here have shown that addition of fresh serum, phorbol ester, or a calcium ionophore to quiescent cells results, in part, in the increased phosphorylation of two 28,000-dalton proteins which have been identified as members of the mammalian stress protein family. There appears to be at least four isoforms of the 28,000-dalton stress proteins, all of which lack methionine and which on the basis of peptide mapping are highly related proteins. In the case of cells placed under stress by either heat-shock or AζC treatment, the two most basic isoforms of 28 kDa, species A and B, are synthesized at rather high levels. In some experiments in which the labeling time is increased, these two treatments also resulted in a modest level of the more acidic 28-kDa C species being produced. In contrast, sodium arsenite treatment of the cells generally resulted in production primarily of the 28-kDa C species and under some cases lower levels of both 28-kDa B and D. Interestingly, in vitro translations of poly(A⁺) containing RNA isolated from 37 °C, 42 °C, and sodium arsenite-treated cells directed the synthesis primarily of the most basic, 28-kDa A species and to a lesser extent synthesis of 28-kDa B species. Presumably post-translational modifications, most likely phosphorylation, thus account for the more acidic forms (e.g. C and D) of the 28-kDa stress protein. Consistent with this idea is the [32P]H₂PO₄ in vivo labeling studies which demonstrated that the B, C, and D isoforms are indeed phosphorylated. In 37 °C cells both the 28-kDa B and C proteins are easily detected following [32P]H₂PO₄ labeling despite the fact that the proteins cannot be detected in normal exposures of the gels analyzing the [3H]leucine-labeled proteins. Following heat-shock or AζC treatment, a slight increase in the levels of phosphorylated 28-kDa B and C was observed while arsenite treatment resulted in a decreased level of phosphorylated 28-kDa B but increased amounts of 28-kDa C and D. The basis for these differences with respect to the phosphorylated isoforms of 28 kDa being observed in heat-shocked or AζC-treated cells versus arsenite-treated cells is not clear. However, the more highly oxidized form of arsenite, arsenate, is itself a phosphate analogue and thus may be affecting the differences in the 28-kDa isoforms being observed following arsenite treatment. Finally, it should be noted that there does not appear to be a net increase in the phosphate content of the various phosphorylated isoforms of 28 kDa following stress. Rather it appears that the phosphorylation state of these proteins remains constant (or even decreases) but that net synthesis of the proteins themselves is being increased during stress (i.e. there appears to be no major change in the specific activity of phosphorylation).

These results presented here examining the small stress proteins of rat fibroblasts are generally consistent with the previous studies of Kim et al. (6) examining cultured myoblasts. These authors have described at least three major 25,000-dalton stress proteins induced in rat myoblasts following exposure to sodium arsenate, sodium arsenite, or after heat-shock treatment, all of which contain no methionine and which migrate on two-dimensional gels similar to that of the 28-kDa rat fibroblast stress proteins described here.

Phosphate labeling studies have shown that two of the three rat myoblast 25-kDa proteins are phosphoproteins with the phosphate being detected exclusively on serine residues (7). In addition, S. aureus V8 digestion of the various isoforms of the 25-kDa proteins show them to be highly related polypeptides (7, 32). Moreover, the peptide fragments generated appear very similar to those described here for the 28-kDa rat fibroblast proteins. Interestingly, following induction of the stress response by sodium arsenite all three of the rat myoblast, 25-kDa proteins are found primarily in the nuclear fraction and appear to return to the cytoplasm following reversal of the stress treatment (7).

In contrast to the mammalian studies, considerable more work has been done on the smaller stress proteins induced in Drosophila cells following heat-shock treatment. Here there appears to be at least four small stress proteins which, unlike the smaller mammalian stress proteins, do contain methionine and range in size between 20,000 and 30,000 daltons (14-17). Biochemical fractionation studies have shown that these small Drosophila stress proteins are synthesized in the cytoplasm, migrate to the nucleus following heat-shock treatment (18-21), and possibly become associated with RNA (22). All of the Drosophila small stress proteins are encoded within a single gene cluster (23), and their transcription appears to be regulated during various stages of Drosophila development (24). DNA sequencing of the coding region of these genes shows them to be related to one another as well as showing homology to the mammalian lens α-crystallins (25). Avian cells also synthesize a methionine-containing 24,000-27,000-dalton protein in response to stress (26-28), and the protein has been suggested to be associated, in part, with the cytoskeleton (29). This protein appears unique to the chicken since polyclonal antibodies raised against the chicken 24-kDa stress protein did not cross-react with the smaller stress protein from other organisms (30).

An important result of the present study is the observation that two of the mammalian 28-kDa stress proteins are identical to the proteins that show a rapid increased phosphorylation in response to fresh serum, phorbol ester, or calcium ionophore. These latter agents, however, are not simply inducing a stress response in cells since they did not result in the induction of the 28-kDa stress proteins (data not shown). Other laboratories have also observed an increased phosphorylation of proteins of similar size following various treatments of mammalian cells. For example, Chambard et al. (13) reported an increased phosphorylation of a 27,000-dalton protein following addition of fresh serum or a variety of different growth factors to cell cycle arrested Chinese hamster lung fibroblasts. We think this protein may in fact be the same protein described here since we have also observed an increased phosphorylation of 28-kDa B and C in rat fibroblasts following either the addition of fresh serum (Fig. 3) or treatment of cells with various growth factors. In the case of phorbol ester treatment of cells, Feuerstein and Cooper (8, 9) have reported an increased phosphorylation of two 27-kDa proteins in human cells treated with PMA. On the basis of their reported isoelectric points for these proteins, we suggest that these two 27-kDa proteins also are likely to be identical.

3 W. J. Welch and D. Helfmaa, unpublished observations.

4 R. Franzia, manuscript in preparation.
to the two 28-kDa B and C mammalian stress proteins described here. With regards to the action of phorbol esters, it is thought that PMA treatment in vivo as well as in vitro activates, in part, a specific protein kinase referred to as protein kinase C (reviewed in Ref. 31). Considerable evidence from many laboratories has implicated a role for protein kinase C in signal transduction at the plasma membrane following treatment of cells with a variety of biological agents (e.g. thrombin, tumor promoters such as PMA, calcium ionophores, and others), many of which subsequently modulate a number of different cellular activities. A potential relationship between protein kinase C and the increased phosphorylation of the two small mammalian stress proteins is suggested since phorbol esters or calcium ionophores, agents known to activate protein kinase C, similarly stimulate the rapid and preferential phosphorylation of the two 28-kDa stress proteins. Whether in fact a protein kinase C-like mechanism is operating here in the phosphorylation of the 28-kDa proteins, either in cells placed under stress or in cells following addition of serum, phorbol ester, or calcium ionophore, is not as yet clear and will require further work. In any case, the 28-kDa stress proteins represent one of the major phosphoproteins of normal cells and appear to serve a central role in cellular responses to many diverse stimuli.

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