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PHOSPHORYLATION PATTERN OF A 25 KDALTON STRESS PROTEIN FROM RAT MYOBLASTS

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Summary: Phosphorylation of a 25 Kdalton nuclear stress protein from rat muscle was examined by two-dimensional gel electrophoresis and one-dimensional peptide mapping. These studies show that three 25 Kdalton stress proteins found in stressed rat myoblasts are actually the same protein with charge variation brought about by multiple phosphorylations. Furthermore, the predominant charge variant of 25 Kdalton protein found in cells is dependent on the intensity of the stress applied to cells.

The induction of protein synthesis by physiological or chemical stress has been described in a wide range of eukaryotes (1,2) including rat myoblasts (3). Although the response has been characterized, the mechanisms whereby stress proteins infer stress tolerance or help maintain cellular homeostasis are not known (1,2). In an attempt to better understand their mode of action, studies have been designed to determine the cellular localization (3,4,5,6) and post-translational modifications (2,5) of stress proteins.

Previous studies (3,5,6) have identified stress proteins of about 25 Kdaltons in stressed mammalian cells. In rat myoblasts and fibroblasts three 25 Kdalton stress proteins can be identified by two-dimensional gel electrophoresis. These proteins lack methionine (3), are distributed in both nuclear and cytoplasmic compartments of the cell (5) and are highly phosphorylated (5). In this work we have further characterized these three 25 Kdalton stress proteins.

MATERIALS AND METHODS

Cell Growth, Stress and Labelling: The growth and maintenance of rat myoblasts (L6 cells) has been described (7,3,5). Monolayer cultures are heat shocked by floating dishes on a 45°C water bath for 20 min (3). Cultures are stressed with arsenite or cadmium by adjusting the culture medium to 100 μM

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$NaAsO_2$ or 300 μM $CdCl_2$ for 4 hours (5). After stress treatment, 30 mm dishes are labelled for 2 hours at 37°C with 0.5 ml of leucine-free MEM (Grand Island Biological Co., Grand Island, NY) containing 5% Horse Serum and 125 μCi of [^{3}H]leucine (≥ 110 Ci/mM; New England Nuclear, Boston, MA) or 0.5 ml of DME (Grand Island Biological Co.) containing 125 μCi [^{32}P]orthophosphate (New England Nuclear). Incorporation is terminated by rinsing with Balanced Salt Solution (BSS, Grand Island Biological Co.) at 4°C followed by immediate lysis of cells in isoelectric focusing buffer (3,8).

Electrophoresis: Two-dimensional gel electrophoresis is performed as described by O'Farrell (8). Ten percent acrylamide gels are used for the second dimension (3). Gels are fixed and stained in formaldehyde (9), processed for fluorography (10) and exposed to Kodak XAR-5 film.

Cell Free Protein Synthesis: The isolation of RNA from rat myoblast cultures and *in vitro* translation in a messenger-dependent rabbit reticulocyte lysate have been described (3).

Peptide Mapping: Peptide mapping of [^{3}H]leucine and [^{32}P]orthophosphate labelled 25 Kdalton proteins is done by the procedure of Cleveland (11). Stress protein spots are cut from two-dimensional gels, digested with proteinases and fractionated in 16% SDS-polyacrylamide gels as previously described (5).

Alkaline Phosphatase Digestions: One 100 mm dish of myoblasts is stressed with $NaAsO_2$, labelled with [^{3}H]leucine and fractionated as previously described (5). Briefly, a supernatant is obtained by centrifugation of a myoblast homogenate (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$ and 1 mM Phenylmethylsulfonylfluoride) at 10,000 x g for 10 min. and is used for phosphatase digestions.

E. coli alkaline phosphatase (EC 3.1.3.1) is added to aliquots of the supernatant at 0, 100 and 300 μg/ml and allowed to incubate for 30 minutes at 37°C. Proteins are then precipitated by the addition of cold trichloroacetic acid to a final concentration of 10% (v/v) for 15 minutes at 4°C. Precipitates are collected at 5,000 x g for 10 minutes and resuspended in isoelectric focusing buffer (8) for analysis by two-dimensional gel electrophoresis.

RESULTS

Rat myoblasts grown in 100 μM sodium arsenite for four hours responded with the induction of a limited number of genes that were either quiescent or expressed at much reduced levels in non-stressed cells. Some of the more dramatic inductions are distinguished by arrows in Figure 1. Among the many inductions which occurred, the synthesis of three 25 Kdalton proteins was greatly enhanced (Figure 1, inset). When arsenite-treated myoblasts were labelled with [^{32}P]orthophosphate, the two most acidic 25 Kdalton proteins were intensely labelled (Figure 2).

When RNA was isolated from stressed myoblasts and translated in a mRNA-dependent reticulocyte lysate, however, only the two most basic 25 Kdalton stress proteins were synthesized (Figure 2). In most experiments it was noted

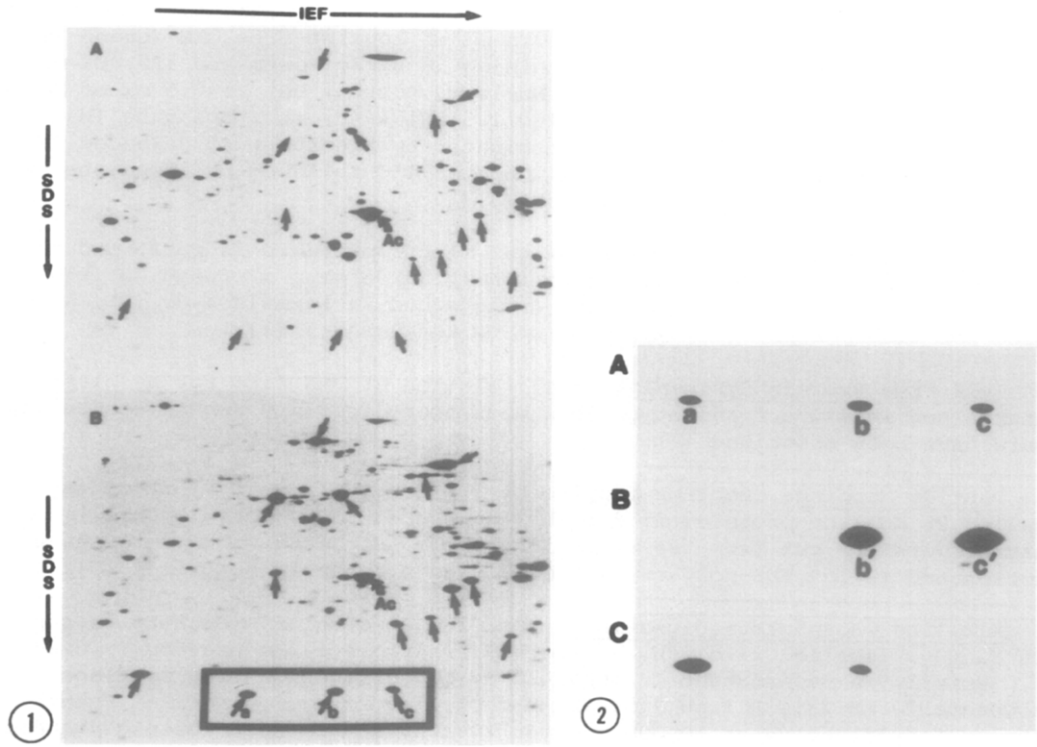


Figure 1. Induction of stress proteins in rat myoblasts. Control cells (A) and cells treated for 4 hours with 100 μM sodium arsenite (B) were grown for 1 hour in leucine-free MEM containing 5% horse serum and 25 μCi/ml [^{3}H]leucine. Cells were washed and immediately disrupted in isoelectric focusing buffer. Polypeptides synthesized during this pulse were fractionated by two-dimensional polyacrylamide gel electrophoresis and detected by fluorography (10^{6} cpm per sample). Actin (Ac) is identified by a curved arrow and serves to orient gels for comparative purposes. The major stress proteins are indicated by straight arrows in B and their absence or reduced levels of synthesis are indicated by an identical set of arrows in A. Three 25 Kdalton stress protein designated a,b,c are the subject of this study and the inset delineates the area of the two-dimensional gels which is enlarged and presented in Figures 2,4 and 5.

Figure 2. In vivo phosphorylation and in vitro translation of 25 Kdalton stress proteins. Cells stressed with sodium arsenite as in Figure 1 were incubated for 1 hour with [^{3}H]leucine as in Figure 1 (A) or for 1 hour in DME containing 125 μCi/ml [^{32}P]orthophosphate (B). RNA isolated from cells stressed as in Figure one was also translated in a cell-free, mRNA dependent rabbit reticulocyte lysate containing [^{3}H]leucine (C). In vivo (A and B) and in vitro (C) synthesized polypeptides were fractionated by two-dimensional gel electrophoresis and detected by fluorography. A portion of each two-dimensional gel corresponding to the inset in Figure one is reproduced to demonstrate the labelling pattern of three 25 Kdalton rat stress proteins (a,b,c) corresponding to those in Figure 1.

that the most basic 25 Kdalton protein was the predominant protein synthesized in cell-free translation assays. Even RNA isolated from cells in which the most acidic 25 Kdalton protein was the major in vivo stress protein (See Figure 5) directed the in vitro synthesis of the most basic 25 Kdalton protein

(data not shown). These experiments taken together suggested that the three 25 Kdalton rat stress protein might actually be a single protein.

When the three 25 Kdalton stress proteins were labelled with [^{3}H]leucine and subjected to partial proteolytic cleavage (8) with Staph aureus protease V8 (methods), the peptide fragments generated from all three proteins were identical (Figure 3). This experiment showed that these three proteins are identical with respect to primary structure and must differ only with respect to charge. Partial digestion of the [^{32}P]orthophosphate-labelled 25 Kdalton proteins shows that fragments identical in size to three of the [^{3}H]leucine-labelled fragments are phosphorylated. Increasing the protease concentration does not result in further cleavage of these fragments indicating that the 25 Kdalton protein contains multiple phosphorylation sites. We have previously shown that the phosphoamino acids are alkali labile (5) and are probably not phosphotyrosine residues. It is therefore most likely that phosphorylation occurs at multiple serine and/or threonine residues.

We treated muscle cell extracts with alkaline phosphatase to determine if the three forms of 25 Kdalton stress protein could be resolved as one charge

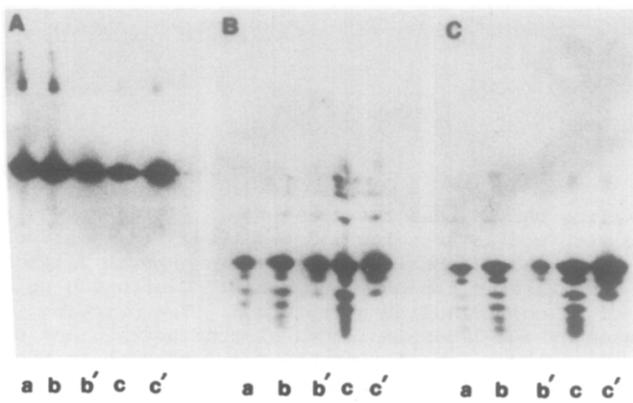
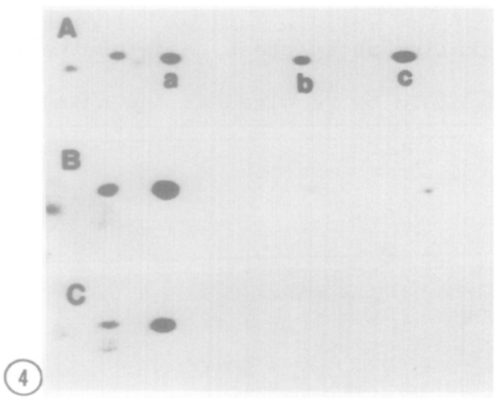


Figure 3. Cleveland peptide analysis of three 25 Kdalton proteins (a,b,c) shown in Figures 1 and 2. Three [^{3}H]leucine-labelled 25 Kdalton protein spots (a,b,c in Figure 2) and two [^{32}P]orthophosphate-labelled 25 Kdalton protein spots (b'c' in Figure 2) were cut from several two-dimensional gels. Leucine-labelled (a,b,c) and phosphate-labelled (b',c') spots were placed in the wells of a 16% SDS-polyacrylamide gel and overlaid with 0.0 µg (A) 0.1 µg (B) and 1.0 µg (C) of protease V 8 from S. aureus. Following a 30 minute digestion (5), the peptide fragments generated were fractionated in the 16% running gel and detected by fluorography.

variant if phosphate groups were removed. As extracts were incubated with increasing concentrations of E. coli alkaline phosphatase, more of the 25 Kdalton proteins appeared in the most basic form as the two acidic forms were depleted (Figure 4). Generalized degradation of proteins during phosphatase digestion was not detected indicating that this result is not an artifact of protein degradation.

Since the 25 Kdalton stress protein exists in three forms varying only by phosphorylation levels we designed experiments to determine if the ratio of phosphorylated to unphosphorylated protein was modulated in muscle cells. Figure 5 demonstrates that the level of phosphorylation of the 25 Kdalton stress protein is variable depending on the type of stress imposed on cultured cells. In our hands, heat shock is the mildest stress whereas growth in cadmium is the most intense stress to rat myoblasts. These preliminary data



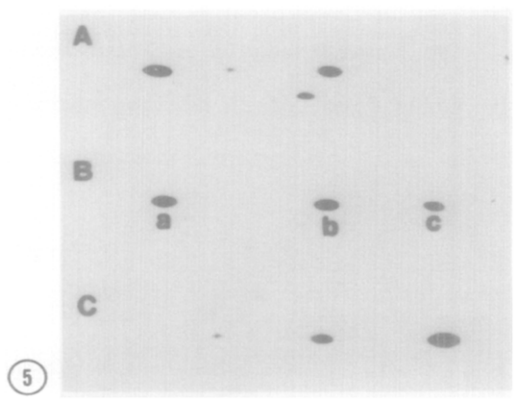


Figure 4. Alkaline phosphatase treatment of 25 Kdalton rat stress proteins. Cells stressed with sodium arsenite and labelled with [^{3}H]leucine as in Figure 1 were processed to obtain a cytoplasmic extract (methods). The extract was aliquoted and digested for 30 minutes at 37°C with 0.0 µg/ml, 100.0 µg/ml and 300 µg/ml of E. coli alkaline phosphatase. The proteins in each extract were fractionated by two-dimensional gel electrophoresis and detected by fluorography. Selected areas of the gels corresponding to the inset of Figure 1 are presented in this Figure to demonstrate the dephosphorylation of two 25 Kdalton stress proteins.

Figure 5. Stress-dependent, in vivo phosphorylation of 25 Kdalton stress protein. Monolayer cultures were stressed by heat shock (A), sodium arsenite poisoning (B) and cadmium poisoning (C) as described in methods. All cultures were labelled with [^{3}H]leucine as described and polypeptides were fractionated by two-dimensional gel electrophoresis and detected by fluorography. Selected regions of the gels corresponding to the inset of Figure 1 are presented to illustrate phosphorylation of the 25 Kdalton rat stress protein.

suggest that the phosphorylation of sp25 is proportional to stress intensity in rat myoblasts.

DISCUSSION

The experiments presented in this study demonstrate that a 25 Kdalton rat stress protein (sp25) exists in several phosphorylated forms in rat myoblasts. The level of phosphorylation is directly related to the intensity of the stress imposed on the cells. We have previously shown that sp25 is rapidly translocated to the nucleus of stressed cells (5) and preliminary results suggest that this protein is associated with the nucleoskeleton (12).

The role phosphorylation plays in the function of sp25 is yet unknown. There are several possibilities, however which are tenable. Since sp25 appears to function in the nucleus, phosphorylation could be involved in its nuclear translocation or sequestration. Alternatively, phosphorylation might influence the binding of sp25 to nuclear components such as chromatin or matrix material. Finally, phosphorylation could determine turnover kinetics of sp25 during stress recovery. At this time we have no prejudice as to which of these functions is in fact served by phosphorylation. Microlocalization studies as well as studies of phosphorylation and dephosphorylation kinetics will not only help to dissect the role of phosphorylation, but will also help explicate the role of sp25 in stressed myoblasts.

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