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Modulation of protein synthesis in primary myogenic cells from chicken by cultivation in the serum-free, hormonally defined medium 'DMN'[1]

P. Dollenmeier[2] and H.M. Eppenberger[3]

Institute for Cell Biology, Swiss Federal Institute of Technology, CH-8093 Zürich (Switzerland),
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Summary. Chicken muscle cells secrete characteristic proteins when grown in the serum-free and hormonally defined culture medium 'DMN'. The most prominent band detected by gel electrophoresis represents a protein of mol.wt 22,000. Fibroblasts released a mol.wt 16,000 protein and fibronectin (mol.wt 220,000) into the medium. The mol.wt 22,000 protein band resolved in 2 dimensional gels into 2 spots which migrated to the same positions as small heat shock proteins as well as butyrate-inducible proteins (BIP) which can be demonstrated in whole cell extracts after butyrate treatment in the presence of serum. The synthesis and release of the mol.wt 22,000 protein is repressed by supplementing the culture medium with serum but not with chick embryo extract.

Introduction

The induction of a characteristic protein synthesis pattern as a response to environmental stress was originally detected in invertebrate cells ('heat shock response'). This characteristic cellular response can be demonstrated in a wide variety of species from plants to mammals[4–7]. Apart from elevated temperature, several toxic agents can induce an identical or a closely related response[8–10], which suggests a broad cellular stress response of which heat shock is only one of the possible initiating factors. The function of the 'stress proteins' is still unclear, but there is evidence that some of these proteins are components of the cytoskeleton and of myofibrils[11], whereas others are reported to migrate into the cell nucleus[12,13].

We report in this paper evidence that myogenic cells in a defined medium synthesize one of these stress proteins and release it into the culture medium.

Materials and methods

a) Materials: (^{35}S) methionine and En(3H)ance were from New England Nuclear, sodium dodecylsulfate (SDS) was from BDH, acrylamide from Serva and bisacrylamide from Eastman. The cell culture medium MCDB 201 was purchased from Seromed, Munich. The suppliers of the additional medium components have been listed previously[14].

b) Methods: Chicken cell cultures were prepared as described previously[14]. Sample preparation and SDS-polyacrylamide gel electrophoresis (SDS-PAGE): 30-µl aliquots of conditioned medium, labelled with 15 µCi (^{35}S)methionine/ml medium for the times indicated in the legends were boiled for 5 min in 20 µl sample buffer (in the presence of beta-mercaptoethanol) and then subjected to a 3–15% gradient SDS-polyacrylamide gel electrophoresis according to Laemmli[15]. The fixed gels were impregnated with En(3H)ance for 1 h, washed in running water for 30 min and, after drying, subjected to fluorography at −70 °C for 3–7 days. Two-dimensional gel electrophoresis and sample preparation was performed according to O'Farrell[16]. Extracts of cell cultures were essentially prepared as described by Caravatti et al.[17]. Cells were scraped from plates, sonicated and extracted for 1 h at 4 °C.

Results

Primary chick myoblasts are able to proliferate and subsequently differentiate into myotubes when cultured in the serumfree, hormonally defined medium 'DMN'. The composition of this medium has been published elsewhere[14]. Major substituting proteins in DMN were insulin, transferrin, fibronectin and fibroblast growth factor. The medium allows also proliferation of chick fibroblasts and differentiation of heart myocytes and spinal cord derived neuronal cells. These primary cells can survive for about 10 days without any medium change[14]. This is convenient for investigations of medium conditioning because it allows the chemical composition to be compared before and after cell cultivation.

We analyzed the conditioning of the DMN medium by comparing SDS-polyacrylamide gels of culture supernatants after labeling the cultures with (^{35}S)methionine. As can be seen in figure 1, the principal secretion of myogenic cells (inevitably contaminated with about 5–10% fibroblasts) into the medium is a protein of mol.wt 22,000 (22 kdalton, kDa), whereas a secondary fibroblast culture, contaminated with about 5% myogenic cells, secrets a distinct protein with a mol.wt of 16,000 (16 kDa). In addition several protein bands can be distinguished, in particular a secreted protein with a mol.wt of 220,000. The latter protein has a molecular weight identical to the fibronectin monomer, which has been recognized recently as the most prominent protein secreted by fibroblasts in serum-containing cultures[18]. By comparing the relative intensities of the 22-kDa and 16-kDa band in lanes a and b, it is clear that the 22-kDa protein is secreted mainly by muscle cells and not by the contaminating fibroblasts of the culture. Likewise, the 16-kDa and the 220-kDa proteins are secreted mainly by fibroblasts. Heart cells, however, release neither 22-kDa nor 16-kDa proteins in substantial amounts into the medium, instead additional proteins with a mol.wt higher than 220 kDa can be observed (lane c).

To investigate a possible correlation of the 22-kDa protein secretion with the state of the muscle cell differentiation (which occurs in a rather synchronized way in vitro), we analyzed the medium (fig. 2) at

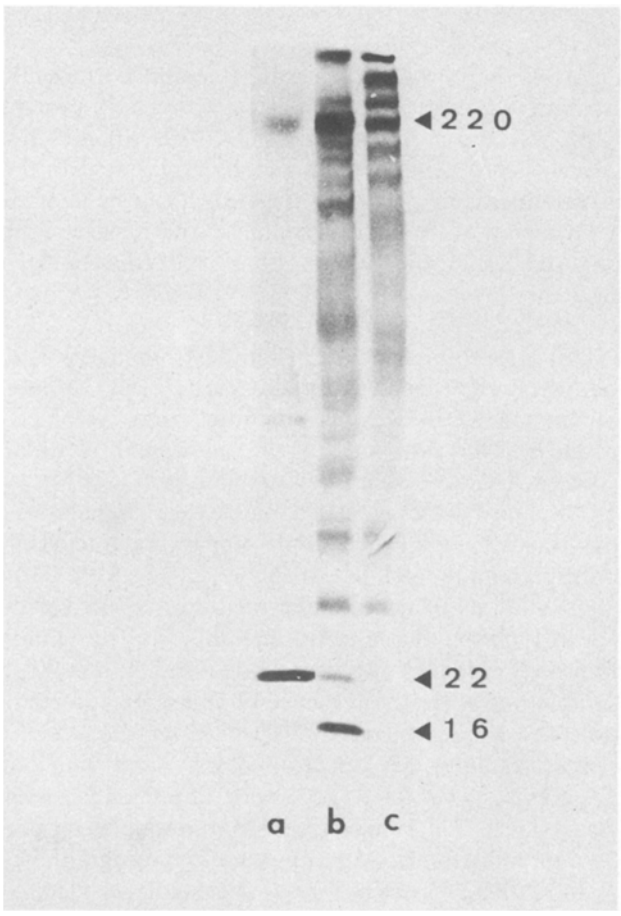

Figure 1. Fluorographed SDS-PAGE of proteins, secreted from cultured cells into conditioned medium DMN. The corresponding cultures were labelled with (^{35}S)methionine from 48 to 96 h in vitro. Medium *a* from a myogenic cell culture contaminated with fibroblasts; *b* from a fibroblast culture contaminated with myogenic cells; *c* from a heart muscle cell culture.

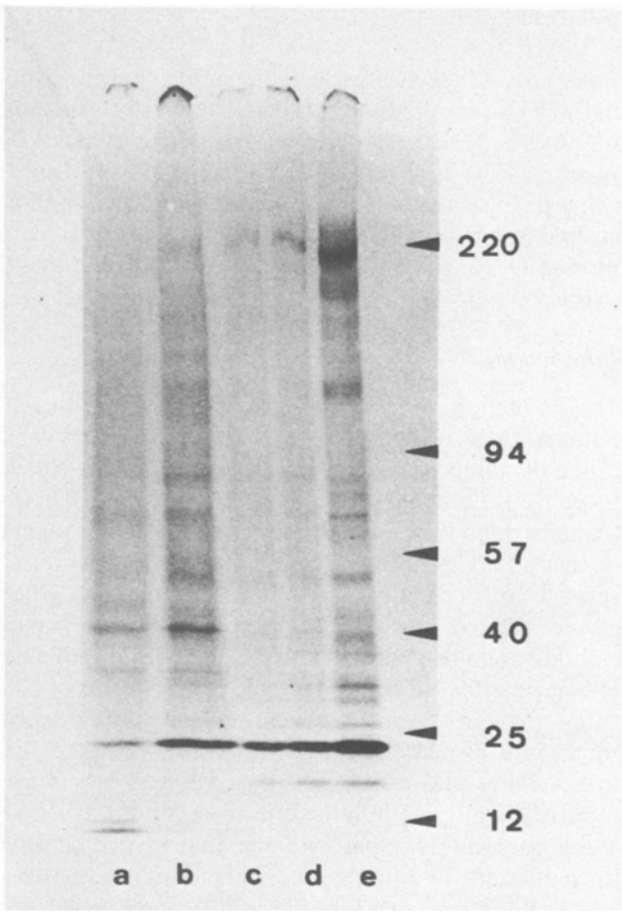

Figure 2. Fluorographed SDS-PAGE of proteins secreted from cultured cells into conditioned medium DMN. The myogenic cultures were labeled with (^{35}S)methionine for *a* 24–48 h; *b* 48–72 h; *c* 72–96 h; *d* 96–120 h; *e* 24–192 h. On the right side, the position of the mol.wt markers is indicated (kdalton).

different stages of muscle cell differentiation. We did not see any correlation between the secretion of the 22-kDa protein and the differentiation state of the myogenic cells: lane a gives the pattern derived from a culture where the majority of the cells was still in the proliferative state while in the culture of lane d almost all cells of the myogenic lineage were terminally differentiated, either postmitotic myoblasts or myotubes, but all cultures produced (although in different quantities) the 22-kDa protein. Accumulation of the 22-kDa protein in lanes c–e could also be demonstrated by Coomassie Blue staining when done under identical experimental conditions (not shown). Assuming a lower limit of detectability of 1 µg protein per band stained, the concentration of the 22-kDa protein in the conditioned medium after 96 h is higher than 30 µg/ml.

The two-dimensional gel electrophoresis of the 22-kDa protein and the 16-kDa protein was performed with (^{35}S)methionine labeled culture supernatant in the presence of unlabeled chick muscle cell extract. This allowed the identification of the 22-kDa and 16-kDa proteins on the autoradiography with respect to the positions of by Coomassie Blue stained identified muscle proteins[19]. The 22-kDa protein resolved, in the isoelectric focusing dimension, into 2 spots with an apparent pI 6.3 and 6.0 (fig. 3,a). This position correlates exactly to the position described for the butyrate-inducible proteins and to the position described for the small heat shock proteins[20]. The 16-kDa protein of the fibroblast culture medium gave a single spot with an estimated pI 6.3 (fig. 3,b). These spots could also be identified in whole cell extracts of a fibroblast contaminated muscle cell culture (fig. 3,c). Surprisingly, the spots are not detectable in 2-D gels of cell extracts from a myogenic culture grown in the standard medium containing 5% chick embryo extract and 10% horse serum (not shown). This finding was confirmed by experiments where the serumfree medium DMN was supplemented with serum or embryo extract: The analysis of the conditioned medium shows that supplementation of the DMN medium with 5% horse serum leads to a complete repression of the 22-kDa protein release (fig. 4A) and, as seen in the resolved cell extract (fig. 4B), to an almost complete repression of the 22-kDa protein synthesis, while, in contrast, supplementing the medium with up to 10% embryo extract alone was without effect on synthesis and release of the 22-kDa protein.

Discussion

The results reported here demonstrate that myogenic cells grown in the serum-free defined medium DMN as compared to serum containing medium synthesize and release a 22-kDa protein while fibroblasts grown in medium DMN release a 16-kDa protein and fibronectin. Heart cells secrete predominantly high mol.wt proteins whereas 22-kDa or 16-kDa proteins are not detected. The synthesis and release of the 22-kDa protein is inhibited by the addition of serum to the culture medium. Although it is conceivable that the 22-kDa proteins in DMN medium could arise by enhancement of a processing step from a larger protein we think it rather reflects enhanced synthesis of the 2 protein species as revealed in the 2-D-gel. If cultures were labeled in the presence of serum no labeled 22-kDa proteins can be detected. The arrows in figure 4B point to the normal positions of the 22-kDa proteins. However, the faint spots in the vicinity of the arrow heads are in a different position from the 22-kDa protein spots and are also visible in figure 3c next to the 22-kDa protein positions.

To the best of our knowledge, this is the first report of the regulatory role of serum on the synthesis of a specific protein. Although we cannot yet identify definitively the 22-kDa protein released as a 'heat shock protein', we have little doubt that it is identical with the 22-kDa butyrate-induced protein and the

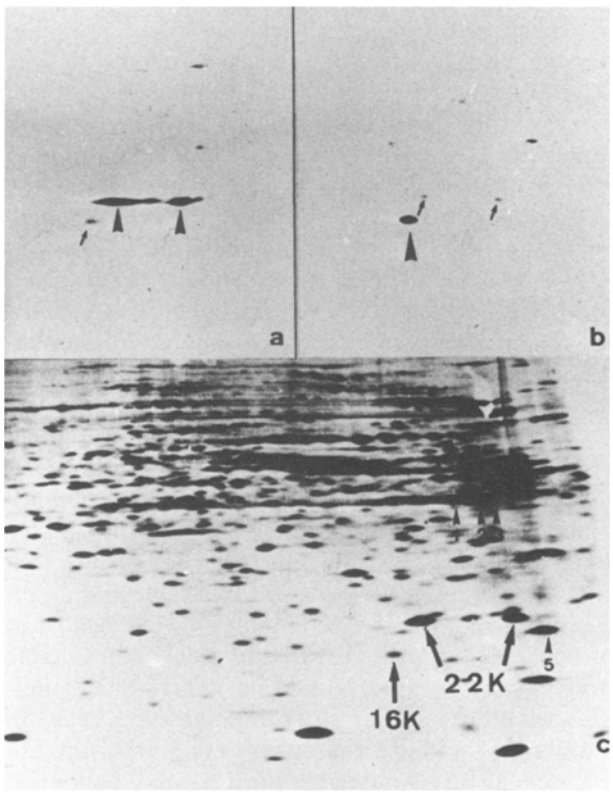

Figure 3. Fluorography of 2-dimensional gels. The cultures were labeled with (^{35}S)methionine. *a* 22-kDa protein from a myogenic culture supernatant. The culture was labeled from 120 to 174 h in vitro. The slanted arrow indicates the position of the 16-kDa protein. *b* 16-kDa protein from a fibroblast culture supernatant (contaminated with myogenic cells). The culture was labeled from 48 to 96 h after subculturing. The slanted arrows indicate the position of the 22-kDa proteins. *c* Cell extract from a myogenic culture (contaminated with fibroblasts), labelled from 120 to 174 h in vitro. The numbered spots indicate the position of 1) α-actin; 2) β-actin; 3) γ-actin; 4) desmin; 5) myosin Lc 1_f.

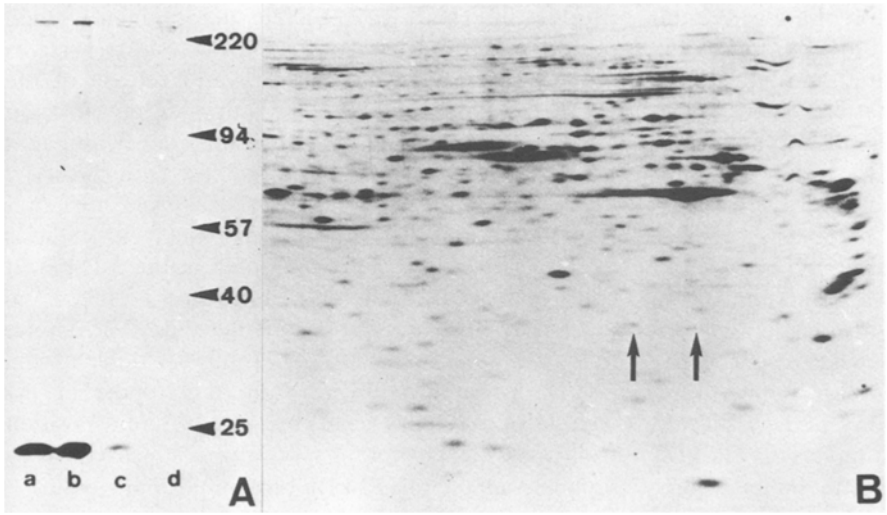

Figure 4. *A* Fluorographed SDS-PAGE of proteins released into conditioned media: The myogenic cultures were labelled from 24 to 72 h in vitro, *a* DMN medium; *b* DMN medium and 10% chick embryo extract; *c* DMN medium and 3% horse serum; *d* DMN medium and 5% horse serum. *B* Two-dimensional gel of a myogenic cell extract. The DMN medium of this culture was supplemented with 5% horse serum. The arrows indicate the position of the 22-kDa protein.

22-kDa heat shock protein as well. The evidence depends not only on the migration pattern obtained in 2-D gels but also on its atypical behavior in different electrophoretic systems. The small heat shock proteins are known to migrate (depending on the gel system used) either like a 22-kDa protein or a 27-kDa protein[8]. This is exactly the migratory behavior we also observed in our experiments: in the electrophoretic system of Laemmli the 22-kDa protein runs faster than chymotrypsinogen (25 kDa), whereas in the 2nd dimension of the 2-D gel where a different acrylamide/bisacrylamide ratio has been used, the 22-kDa protein runs slower than the light chain of myosin (also 25 kDa). The function of the 22-kDa and 16-kDa proteins secreted by skeletal muscle cells and fibroblasts respectively is still unknown. If we regard the 22-kDa protein as a cellular response to unfavorable culturing conditions ('stress') the suppression of this response by serum supplementation could indicate that a component in the serum can either fulfill a cellular requirement (e.g. a vitamin or a hormone) or can induce tolerance to the culture medium, perhaps by detoxification. Therefore the assay of the 22-kDa protein and other stress-inducible proteins in cell extracts or even in the medium could serve as a useful test in toxicology and in cell biology. For toxicological purposes, the appearance of these easily detectable proteins may be used to indicate unfavorable conditions or effects of certain compounds on cells. In addition, to optimize cell culture conditions, the absence of the stress inducible proteins may be a good criterion to decide on the performance of a serum-free or defined medium.

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2 Present address: Friedrich Miescher Institute, Ciba-Geigy Ltd, CH-4002 Basel, Switzerland.

3 To whom correspondence and reprint requests should be addressed.

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