CHANGES IN PLASMA MEMBRANE PHOSPHOPROTEINS DURING DIFFERENTIATION OF AN ESTABLISHED MYOGENIC CELL LINE AND A NON-FUSING α-AMANITIN RESISTANT MUTANT

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Received 21 January 1982

1. Introduction

Mononucleated myogenic cells of the L_6 line proliferate actively in culture. After several rounds of DNA replication, they reach confluency, establish contacts [1] and fuse forming multinucleated myotubes which actively synthesize muscle-specific proteins [2]. The mechanism which leads to the expression of the differentiated phenotype in myoblasts is unknown, but phenomena which take place at the external surface of the cells play a crucial role in this process [3,4]. It remains however uncertain how the plasma membrane proteins function as receptors and recognition sites during myogenesis and how myogenic cells can regulate these structures in response to a series of events. Phosphorylation may play a key role in the regulation of many functions of the eukaryotic cells [5,6]. Some of the protein kinases controlling these reactions are regulated by cyclic AMP, which modulates differentiation of avian as well as of rat myoblasts [7,8]. It was of interest, therefore, to examine the intrinsic phosphorylating systems of highly purified plasma membranes from L_6 cells at different stages of differentiation.

Our data show that such preparations contain endogenous protein kinases and that although membranes from non-differentiated as well as from differentiated cells are extensively phosphorylated, developmentally regulated changes take place in 4 phosphoproteins of app. M_r 165 000, 105 000, 60 000 and 45 000 during myogenesis. The activity of the plasma membrane protein kinases toward endogenous substrates remained unchanged during myogenesis. The enzyme(s) were found to be insensitive to stimulation by cyclic AMP and calcium. This is the first time that such a membrane-bound protein phosphorylation system has been described in muscle cells.

2. Materials and methods

2.1. *Materials*

Dulbecco's modified Eagle medium and foetal calf serum were from Gibco; adenosine 5′-[γ-^{32}P]triphosphate (spec. act. 3.26 Ci/mmol) was from Amersham; soybean trypsin inhibitor and phenylmethylsulfonylfluoride (PMSF) were from Sigma Chemical Co.; sodium lauryl sulfate (SDS) from BDH; acrylamide, *N,N′*-methylenebisacrylamide, *N,N,N′,N′*-tetramethylethylene diamine and ammonium persulfate were from BioRad Labs.; ^{14}C M_r markers for gel electrophoresis were from Amersham and Unisolve 1 from Koch-Light Labs. All other chemicals used were from Merck. All reagents were of the best available grade.

2.2. *Cell cultures*

Culture of cells of the L_6 line and of a non-fusing mutant having an altered RNA polymerase II (Ama 102) were performed as in [9].

2.3. *Plasma membrane purification*

Purified plasma membranes from radiolabelled cells of the L_6 and the Ama 102 lines were obtained according to [10]. They were shown to contain only negligible amounts of cellular contaminants by electron microscopy and by enzymatic studies [10,11]. We routinely verified the purity of the preparations. Throughout the purification procedure, 1 mg soybean trypsin inhibitor/ml and 1 mM phenylmethylsulfonyl-

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00145793/82/0000–0000/$02.75

fluoride were included in all buffer solutions to inhibit proteolysis, except when enzyme assays to check the purity of the membrane preparations were to be done. Delipidation of plasma membranes was done as in [12].

2.4. *Autophosphorylation of plasma membranes*

The standard incubation mixture contained, unless otherwise stated, glycylglycine buffer 50 mM (pH 7.4), $MgCl_2$ 10 mM, mercaptoethanol 0.1 mM, adenosine 5′-[γ-^{32}P]triphosphate 100 μM (10 μCi), 20 μg purified plasma membrane proteins and where indicated $CaCl_2$ 100 μM or ethyleneglycol bis-(β-amino-ethyl-ether) *N,N′*-tetraacetic acid (EGTA) 1 mM as well as cyclic AMP 1 μM and dibutyryl cyclic AMP 10 μM in a 40 μl total vol. The incubation was done for 12 min at 30°C. The ATP as well as the cyclic AMP and the dibutyryl cyclic AMP were added at 0, 4 and 8 min. When gels were to be run, the reaction was stopped by the addition of 40 μl sample buffer containing 1.25% SDS and 20 μg bromophenol blue/ml. The mixture was boiled for 3 min. When protein kinase activities were to be determined incubation was done for 10 min. The ATP as well as the cyclic AMP and the dibutyryl cyclic AMP were added at 0 and 5 min. The reaction was stopped by precipitation with 10% trichloroacetic acid [13].

2.5. *Labelling of the cells*

Proliferating mononucleated L_6 cells (3.5×10^3 cells/cm^2), fused cultures of this line (8–10 days after plating, 4×10^4 nuclei/cm^2, 85% of nuclei in myotubes), non-confluent Ama 102 cells (3.5×10^3 cells/cm^2), and confluent Ama 102 cells (3×10^4 cells/cm^2) were labelled at 37°C in 5 ml Dulbecco's modified Eagle medium/100 mm diam. plate containing 1×10^{-5} M leucine, 10% foetal calf serum and 0.2 μCi [^{14}C]leucine/ml. After 24 h, radioactive media were removed, and the cultures were washed several times with 10 ml ice-cold phosphate-buffered saline. The cells were scraped into ice-cold buffer 1 (0.1 M Tris–HCl (pH 7.5), 0.25 M KCl, 1 mg soybean trypsin inhibitor/ml and 1 mM of PMSF).

2.6. *SDS–polyacrylamide slab gel electrophoresis*

This performed according to [14]. Gel slabs used were 1.5 mm thick with a linear gradient of 5–15% polyacrylamide (separating gel) and a 4% stacking gel. A mixture of [^{14}C]methylated lysozyme (14 300 M_r), carbonic anhydrase (30 000 M_r), ovalbumin (46 000 M_r), bovine serum albumin (69 000 M_r), phosphorylase *b* (92 500 M_r) and myosin (220 000 M_r) were used as M_r markers on each slab. Gels were routinely run at 20 mA for 5 h. After the run the gels were fixed for 2 h in methanol:acetic acid:water (50:10:40) and left overnight in methanol:acetic acid:water (5:7:88). Gels to be autoradiographed were dried onto Whatman no. 1 filter paper using a BioRad Model 224 Slab gel dryer. Radioautography was performed by pressing the dried gel onto Kodak X-Omat AR film which was developed after an appropriate exposure period. In other experiments, the fixed gels were cut with a Mickle gel slicer into 1 mm pieces solubilised with 30% H_2O_2 overnight and counted in Unisolve 1 in an Intertechnique SL 32 scintillation counter.

Protein was determined as in [15].

3. Results

Plasma membranes were purified from non-differentiated and differentiated cells of the L_6 line which had been labelled with radioactive leucine for 24 h prior to harvesting: 25 protein peaks with app. M_r

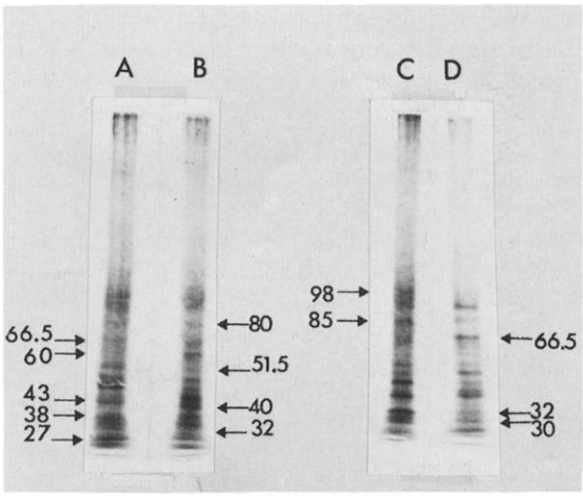

Fig.1. Autoradiogram of plasma membranes from myogenic cells of the L_6 line and Ama 102 mutants. Plasma membranes were purified from: (A) L_6 proliferating myoblasts (7×10^3 cells/cm^2); (B) L_6 myotubes (85% of nuclei included in multinucleated cells); (C) Ama 102 proliferating cells (7×10^3 cells/cm^2); or (D) Ama 102 confluent cells (3×10^4 cells/cm^2). Cells were labelled with [^{14}C]leucine (0.2 μCi/ml) for 24 h. Purified membranes were solubilised in SDS, the proteins separated on polyacrylamide gels which were fixed and treated with EN-^{3}HANCE, dried and exposed to Kodak X-Omat AR films at −80°C for 48 h. The numbers indicate the app. M_r-values ($\times 10^{-3}$).

255 000–21 500 could be identified in mononucleated cells of this line. Most of the label was incorporated into proteins migrating between 105 000–21 500 M_r. A significant increase took place at differentiation in the labelling of 7 bands of app. M_r 160 000, 80 000, 66 500, 60 000, 43 000, 40 000 and 38 000 (fig.1). The 160 000 M_r band could be identified when the radiograms were exposed for ⩾96 h (not shown). The 38 000 M_r band could not be detected in myoblasts. The incorporation of [^{14}C]leucine into the protein bands which migrated at 51 500, 32 000 and 27 000 M_r decreased at fusion (fig.1). These results confirm and extend our earlier findings where dual-label experiments were performed and the gels had been sliced [11].

The patterns of phosphorylation of purified plasma membranes from cells of the L_6 myogenic line is shown on fig.2. As is the case for membranes labelled

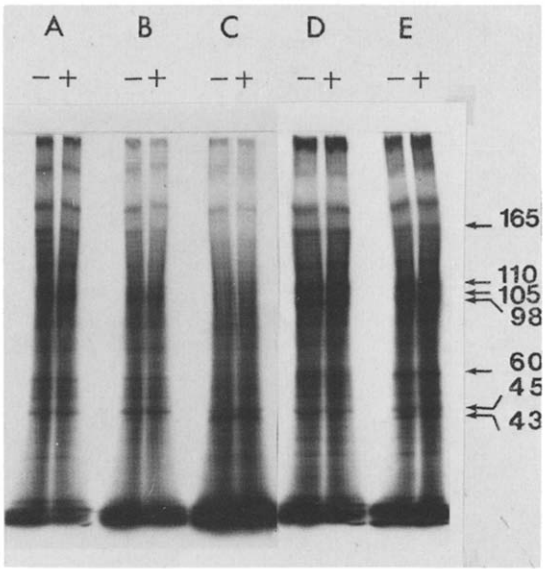

Fig.2. Autoradiogram of phosphorylated plasma membranes from myogenic cells of the L_6 line and Ama 102 mutants. Plasma membranes were purified from: (A) L_6 proliferating myoblasts (7×10^3 cells/cm^2); (B) L_6 confluent myoblasts (3×10^4 cells/cm^2); (C) L_6 myotubes (85% of nuclei included in multinucleated cells); (D) Ama 102 proliferating cells (7×10^3 cells/cm^2); or (E) Ama 102 confluent cells (3×10^4 cells/cm^2). Phosphorylation was done as in section 2 in the absence (−) or in the presence (+) of cyclic AMP. Membranes were solubilised and resolved on SDS–polyacrylamide gels. The arrows point to bands in which either changes in phosphorylation were observed during myogenesis or which could be detected only in membranes from mutant cells. The numbers indicate their app. M_r-value ($\times 10^{-3}$).

with [^{14}C]leucine 'in vitro' (see above), a minimum of 25 radioactive zones, produced under the standard incubation conditions (see section 2) could be identified by autoradiography of SDS–polyacrylamide gels of solubilised membranes from mononucleated myoblasts. A new phosphoprotein with app. M_r 43 000 appeared on plasma membranes of myotubes. On the contrary the incorporation of phosphate into 2 other bands migrating at 60 000 and 45 000 M_r decreased markedly in fused cells (fig.2). Two other phosphoproteins which migrate at 165 000 and 105 000 M_r could be identified only in autoradiograms of plasma membranes from myoblasts but not in those obtained from myotubes (fig.2). Similar results were obtained in experiments in which the gel tracks were cut into 1 mm pieces and counted. The level of phosphorylation of these proteins was 200–450 cpm over a general background of 60–90 cpm. In all our experiments, independently of the cell type studied, ~10% of total ^{32}P which could be detected on the gel migrated just beyond the tracker dye. Similar results were obtained when the phosphorylated membranes were washed extensively in the presence of 10 μM Zn^{2+}. Thus, it is unlikely that this material is free ATP. Extraction of the phosphorylated membranes with methanol/chloroform removed 80% of this radioactivity. This is a good indication that most of it represents membrane phospholipids.

Comparison with fig.1 shows that the incorporation of leucine into the bands migrating at 60 000 and 43 000 M_r increased at fusion. In contrast the amount of leucine incorporated into the 165 000, 105 000 and 45 000 M_r bands did not change during differentiation.

As shown in fig.2,3 no difference in the intensity of any of the radioactive components could be observed whether phosphorylation of L_6 cells was carried out in the presence or in the absence of cyclic AMP (10 μM) or of 10^{-5} M calcium. On the contrary, the protein kinase activity of the cytoplasmic fraction of L_6 cells as well as of Ama 102 mutants, could be stimulated by cyclic AMP (10 μM). The addition of 10^{-5} M calcium exhibited no effect (in preparation). We examined next whether quantitative changes in the activity of plasma membrane kinases take place during myogenesis. We used as a substrate endogenous purified plasma membranes. Under our assay conditions, no quantitative differences in the incorporation of ^{32}P into plasma membrane proteins could be observed regardless of the stage of differentiation of

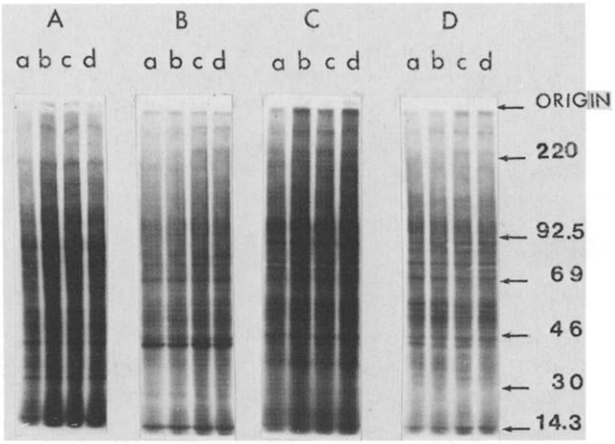

Fig.3. Influence of cyclic AMP and calcium on the phosphorylation of plasma membranes from cells of the L_6 and the Ama 102 lines. Plasma membranes were purified from: (A) L_6 proliferating myoblasts; (B) L_6 myotubes; (C) Ama 102 proliferating cells; and (D) Ama 102 confluent cells. Phosphorylation was done of: (a) EGTA; (b) EGTA and cyclic AMP; (c) calcium; and (d) calcium and cyclic AMP (section 2). The arrows indicate the migration of the protein standards. The numbers indicate their M_r-value ($\times 10^{-3}$).

the cells (table 1). The extent of ^{32}P incorporation into trichloroacetic acid precipitable material was independent of the presence of calcium (10^{-5} M) or cyclic AMP over a wide range of concentrations.

To ascertain the relationship between the observed changes and myoblast differentiation we studied the alterations in plasma membrane protein accumulation and phosphorylation which take place in cells of the Myo$^-$ non-fusing mutant line Ama 102 as the cells become confluent. As can be seen on the radiograms shown on fig.1–3 a minimum of 25 protein bands could be identified. Leucine incorporation into 4 of them (98 000, 85 000, 32 000 and 30 000 M_r) decreased at confluency while the incorporation of the amino acid increased into the 66 500 M_r band (fig.1).

As shown on fig.2, ^{32}P incorporation into 3 bands which migrated at 165 000, 60 000 and 43 000 M_r increased markedly in membranes from confluent cells. Comparison with fig.1 shows that the incorporation of leucine into these proteins remained unchanged. Similar changes were observed in membranes from L_6 cells at fusion in what concerns the 43 000 M_r band. On the contrary the 165 000 M_r peak was not phosphorylated at this stage. Two proteins with app. M_r 110 000 and 98 000 were phosphorylated only in plasma membranes from the non-fusing mutant but not in those from cells of the wild type. No difference was observed whether phosphorylation was carried out in the presence or in the absence of added cyclic AMP or calcium (radiograms, fig.3). Table 1 shows that the extent of phosphorylation in membranes from mutant cells was similar to the one obtained in the wild type. It was independent on the presence of cyclic AMP or calcium.

4. Discussion

Our results demonstrate the existence in plasma membranes from myogenic cells of an endogenous protein kinase activity. The data in [10,11] show that these membranes were essentially free of contamination by other subcellular organelles. The high salt conditions used during the purification of the membranes are known to remove most of the adsorbed peripheral proteins. Thus, the protein kinase described by us is most probably either an intrinsic membrane protein or at least tightly bound to the plasma membrane of L_6 cells.

Table 1
Activity of plasma membrane protein kinase for endogenous substrates

Additions (M)	L_6 proliferating myoblasts (7×10^3 cells/cm^2)	L_6 confluent myoblasts (3×10^4 cells/cm^2)	L_6 myotubes (85% of nuclei in myotubes)	Ama 102 proliferating cells (7×10^3 cells/cm^2)	Ama 102 confluent cells (3×10^4 cells/cm^2)
None	3.8	3.9	4.0	4.9	4.7
cAMP, 10^{-6}	3.8	3.8	4.0	5.0	4.7
cAMP, 10^{-5}	4.3	3.8	4.5	5.7	5.3
cAMP, 10^{-4}	4.0	2.7	3.5	5.6	4.5
cAMP, 10^{-3}	3.4	2.4	3.2	3.2	4.0
Ca^{2+}, 10^{-4}	3.8	3.6	3.7	4.8	4.2

Plasma membranes were purified and phosphorylation was done as in section 2. Protein kinase activity is expressed as μmol ^{32}P incorp. 10 min^{-1}. mg protein^{-1}. Similar results were obtained in 3 independent expt

We demonstrate that, during myogenesis, developmentally regulated changes took place in the phosphorylation of at least 4 proteins of app. M_r 165 000, 105 000, 60 000 and 45 000. Similar changes in the phosphorylation of a membrane protein of 43 000 M_r took place in cells of the wild type at fusion and in those of the non-fusing mutant line at confluency. It cannot be excluded that the phosphorylation of the 43 000 M_r band might be normally related to myogenesis. However, our results suggest that the observed changes alone are not sufficient to induce fusion.

Several of the developmentally regulated changes which take place in the phosphorylation of membrane proteins were not paralleled by changes in the accumulation of these molecules (section 3). This could be because either:

(i) Different protein species which comigrate on SDS-polyacrylamide gels are synthesized in myoblasts and myotubes.

(ii) Protein kinases expressed in the different cell types are different themselves. Further work using immunological procedures is being pursued.

Our data show unambiguously that the plasma membrane protein kinase from non-differentiated as well as from differentiated L_6 cells is not cyclic AMP- or calcium-dependent. This is the first time that a phosphorylating system with such characteristics has been described in membranes of myogenic cells.

The existence in the cytoplasm of L_6 myoblasts of a protein kinase which can be stimulated by the addition of cyclic AMP (in preparation, [16]) raises the question whether some of the membrane proteins could serve as substrates for these cytosolic enzymes. This becomes particularly interesting as it has been postulated that phosphorylation of membrane components by such protein kinases might be on the basis of myoblast fusion [17]. This hypothesis is under study.

In [18], several membrane proteins were phosphorylated in the presence of cyclic AMP in myoblasts but not in myotubes. The level of phosphorylation of these components was however only 40–80 cpm over a general background of 25–30 cpm. The low level of ^{32}P incorporation observed in [18] suggests that these components were not at substrate saturation. This pitfall was avoided here by the addition of a large excess of ATP.

We show that although membrane proteins from myotubes can be phosphorylated to the same extent as those from mononucleated myoblasts by endogenous protein kinases, several specific developmentally regulated changes can be identified during myogenesis. The rôle of these post-transcriptional modifications of plasma membrane proteins in muscle cell differentiation is under investigation.

Acknowledgements

This research was supported by grants from the 'Institut National de la Santé et de la Recherche Médicale', the 'Centre National de la Recherche Scientifique', the 'Union des myopathes de France' and the 'Muscular Dystrophy Association of America'. We are indebted to Professor M. L. Pearson for providing us with cells. The excellent technical help of R. Kernemp and T. Berthollet is gratefully acknowledged. We thank Miss G. Casaubon for typing this manuscript.

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