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Phosphorylation of a gelatin-binding protein from L6 myoblasts by protein kinase C

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A gelatin-binding glycoprotein from L6 rat myoblasts, designated gp46, was shown to be phosphorylated in vivo. This phosphorylation was increased slightly (18%) by phorbol ester treatment of L6 suggesting protein kinase C involvement. Purified gp46 could be phosphorylated in vitro with protein kinase C, but not by the catalytic subunit of cAMP-dependent protein kinase. Comparison of the phosphotryptic peptide maps of in vitro and in vivo labeled gp46 suggested that in vivo phosphorylation of gp46 may be mediated by protein kinase C.

Phosphorylation; Protein kinase C; Differentiation; Collagen; (Muscle)

1. INTRODUCTION

We have previously identified a major cell surface glycoprotein of rat L6 myoblasts that appears to be involved in myoblast differentiation [1]. Recently, we have found that this glycoprotein (gp46) binds specifically to gelatin-Sepharose and may act as a myoblast collagen receptor (Cates, G.A. et al., submitted). Nagata and Yamada [2] reported that a possibly similar 47 kDa gelatin-binding protein from chick embryo fibroblasts was phosphorylated and that its phosphorylation was increased markedly following fibroblast transformation with Rous sarcoma virus (RSV). In the light of these findings, we examined gp46 for phosphorylation and found that it was also phosphorylated. More importantly, our results also indicated that phosphorylation of gp46 may be mediated by protein kinase C. This observation may have important implications for the regulation of myoblast differentiation and myoblast-collagen interactions.

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Abbreviations: PMA, phorbol myristate acetate; PMSF, phenylmethylsulfonyl fluoride; Mes, 2-(*N*-morpholino)ethanesulfonic acid

2. MATERIALS AND METHODS

2.1. *Cell lines and cell culture*

A highly myogenic subclone of the rat myoblast cell line, L6, first isolated by Yaffe [3] was cultured as in [1]. JRu5 and RSV-L6 were spontaneously transformed and RSV-transformed derivatives of L6, respectively [4].

2.2. *Immunopurification of gp46 from L6 myoblasts*

gp46 was purified using immunoaffinity chromatography as described previously (Cates, G.A. et al., submitted), except that gp46 was eluted using 5 bed volumes of 50 mM diethylamine, pH 11.9. The eluate was neutralized with 2 M Tris, pH 7.5, and dialyzed against 200 vols of 20 mM Tris, pH 7.5.

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2.3. *Purification of protein kinases*

RSV-L6 myoblasts were homogenized in 10 mM Tris, pH 7.5, 0.25 M sucrose, 5 mM EGTA, 1 mM PMSF, 2 μg/ml leupeptin and 0.1% mercaptoethanol. The homogenate was centrifuged at 40000 × *g* for 30 min and the supernatant was applied to a 10 ml DEAE-cellulose column equilibrated with buffer A (10 mM Mes, pH 6.7, 1 mM EGTA, 1 mM PMSF, 2 μg/ml leupeptin and 0.1% mercaptoethanol). The column was washed with 50 ml buffer A, then with a 100 ml linear gradient of 0–0.25 M NaCl in buffer A. Fractions containing protein kinase C activity were pooled, concentrated and applied to a 1.5 × 60 cm column of Sephacryl-S200 equilibrated with buffer A. The protein kinase C activity peak was pooled and concentrated. It had a specific activity of 9000 U/mg protein. The catalytic subunit of cAMP-dependent protein kinase was purified from beef heart according to Beavo et al. [5].

2.4. *In vitro phosphorylation of gp46*

Purified gp46 was incubated for 10 min at 30°C in a reaction mixture containing 10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (10^3 cpm/pmol), 65 μg/ml phosphatidylserine, 4 μg/ml diacylglycerol, 0.8 mM $CaCl_2$ and 25 U protein kinase C. The reaction was started by the addition of ATP and stopped by adding 4-fold concentrated SDS-PAGE buffer and heating in a boiling water bath for 3 min. EGTA was added to a final concentration of 30 mM in reactions where calcium was omitted. For reactions in which cAMP-dependent protein kinase was used, the reaction mixture contained 10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (10^3 cpm/pmol), and 40 U catalytic subunit of cAMP-dependent protein kinase.

2.5. *In vivo phosphorylation of gp46*

1 ml phosphate-free medium buffered with 25 mM Hepes, pH 7.4, and containing 1 mCi [^{32}P]orthophosphate was added to each 35 mm plate. Cells were labeled for 12 h, then washed three times with ice-cold Dulbecco's phosphate-buffered saline. 0.5 ml buffer B (10 mM sodium phosphate, pH 7.4, 1% Triton X-100, 0.15 M NaCl, 50 mM NaF, 100 μM $NaVO_3$, 2 mM EDTA, 2 μg/ml leupeptin, 0.5 mM PMSF) was added to each plate. After 5 min on ice, the cells were scraped and passed three times through a 0.5 inch, 27 gauge needle. The homogenate was centrifuged at 430000 × *g* for 15 min at 4°C in a Beckman TLA100.2 rotor. The protein concentration of the supernatants was determined as described by Peterson [6]. 100 μg Triton-solubilized extract was adjusted to 800 μl with buffer C and then added to 50 μl (packed volume) of gelatin-Sepharose. After mixing for 2 h at 4°C, the beads were washed three times with 1 ml aliquots of buffer B containing 1 M NaCl. The beads were given a final wash with 1 ml of 5 mM Hepes, pH 7.5, 2 mM EDTA and then 100 μl SDS-PAGE buffer containing 2 mM EDTA was added. The beads were heated at 65°C for 15 min, then equal amounts of the supernatant were run on an SDS gel. Following staining, the relative amount of gp46 was determined by scanning with a laser densitometer. Similarly, the relative radioactivity was determined by scanning the autoradiographs.

2.6. *Two-dimensional tryptic peptide mapping*

Gel slices containing phosphorylated gp46 were digested with trypsin and the peptides separated as described by Zweig and Singer [7].

3. RESULTS

3.1. *In vivo phosphorylation of myoblast gp46*

Following myoblast labeling with [^{32}P]orthophosphate, gp46 was isolated by means of gelatin-Sepharose and found to be phosphorylated. The relative amount of gp46 and degree of phosphorylation were determined in L6 and two transformed cell lines derived from L6 (table 1). Treatment with phorbol ester caused a slight, but reproducible, increase in the phosphorylation of gp46, particularly in the spontaneous transformant, JRu5. Similar results were obtained when gp46 was isolated using immobilized anti-gp46 antibodies (not shown). The transformed lines, JRu5 and RSV-L6, were found to contain 25–36% less gp46 than the wild-type L6 myoblasts. However, the relative ^{32}P/protein ratio was the same for all the cell lines, indicating that transformation of L6 had no effect on the phosphorylation state of gp46.

3.2. *In vitro phosphorylation of myoblast gp46*

Since phorbol ester caused a stimulation of gp46

Table 1

In vivo incorporation of [^{32}P]orthophosphate by gp46 in different cell lines

Cell type	PMA[a]	Relative protein[b]	Relative $^{32}PO_4$ incorporated[b]	Relative $^{32}PO_4$/ protein ratio
L6	–	1.00	1.00	1.00
L6	+	0.84 ± 0.02	0.99 ± 0.01	1.18
RSV-L6	–	0.64 ± 0.11	0.68 ± 0.04	1.06
RSV-L6	+	0.65 ± 0.09	0.76 ± 0.01	1.17
JRu5	–	0.75 ± 0.13	0.74 ± 0.04	0.99
JRu5	+	0.63 ± 0.15	0.82 ± 0.04	1.30

[a] 1 μM PMA added for 15 min at the end of the labeling period

[b] Values were calculated from peak areas of densitometric scans and expressed relative to L6 ± SE of the mean

phosphorylation in vivo, protein kinase C was tested for its ability to phosphorylate purified gp46 in vitro. In addition, the catalytic subunit of cAMP-dependent protein kinase was also used to test the in vitro phosphorylation of gp46. Fig.1 shows that protein kinase C in the presence of calcium and phospholipids is effective in phosphorylating gp46. In contrast to protein kinase C phosphorylation, no detectable phosphorylation of gp46 by the catalytic subunit of cAMP-dependent protein kinase was detected (fig.1).

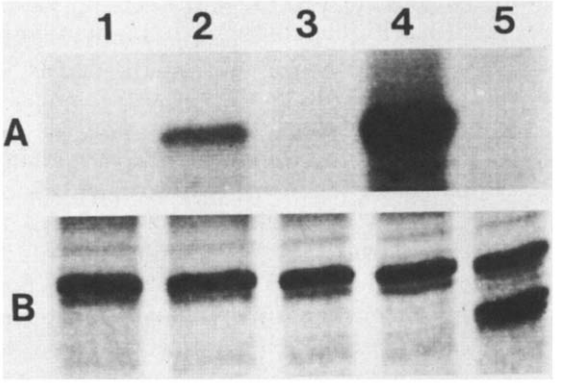

Fig.1. In vitro phosphorylation of purified gp46. Equal amounts (1 μg) of purified gp46 were incubated with protein kinase C or the catalytic subunit of cAMP-dependent protein kinase as described in section 2. (A) Autoradiograph; (B) Coomassie blue staining. Lanes: 1–4, protein kinase C added; 5, catalytic subunit of cAMP-dependent protein kinase added. Lane 1, + EGTA; 2, + phospholipids only; 3, + calcium only; 4, + phospholipids and calcium.

3.3. *Stoichiometry of in vitro phosphorylation*

Fig.2 shows the rate and amount of phosphorylation of purified gp46 by protein kinase C with time in vitro. The initial rate of gp46 phosphorylation was 0.013 pmol [^{32}P]phosphate incorporated/pmol gp46 per min. The maximum level of phosphorylation obtained was 0.32 pmol [^{32}P]phosphate/pmol gp46.

3.4. *Phosphotryptic peptide maps of gp46*

Phosphorylated gp46 obtained following in vivo labeling of L6 cells with [^{32}P]orthophosphate or in vitro labeling with protein kinase C was digested with trypsin and examined by two-dimensional peptide mapping (fig.3). Two phosphorylated peptides were observed for in vitro labeled gp46

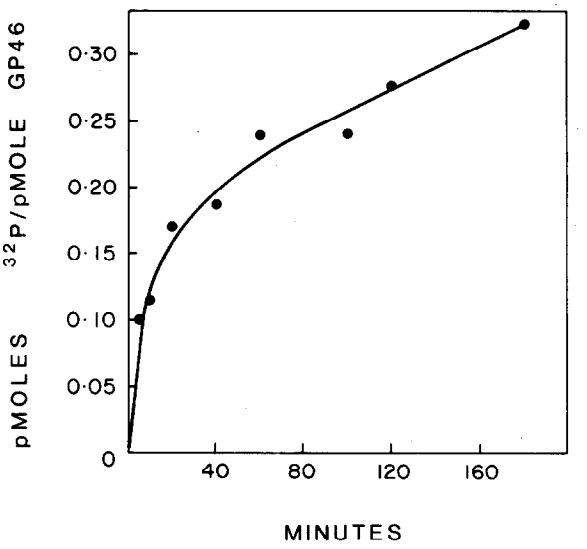

Fig.2. Stoichiometry of phosphorylation of gp46. Equal amounts of purified gp46 were phosphorylated with protein kinase C for the indicated times. Phosphorylation was stopped by the addition of SDS sample buffer and the samples were then electrophoresed on an SDS gel. [^{32}P]Phosphate incorporation was determined by cutting out and counting the gp46 band from the SDS gel.

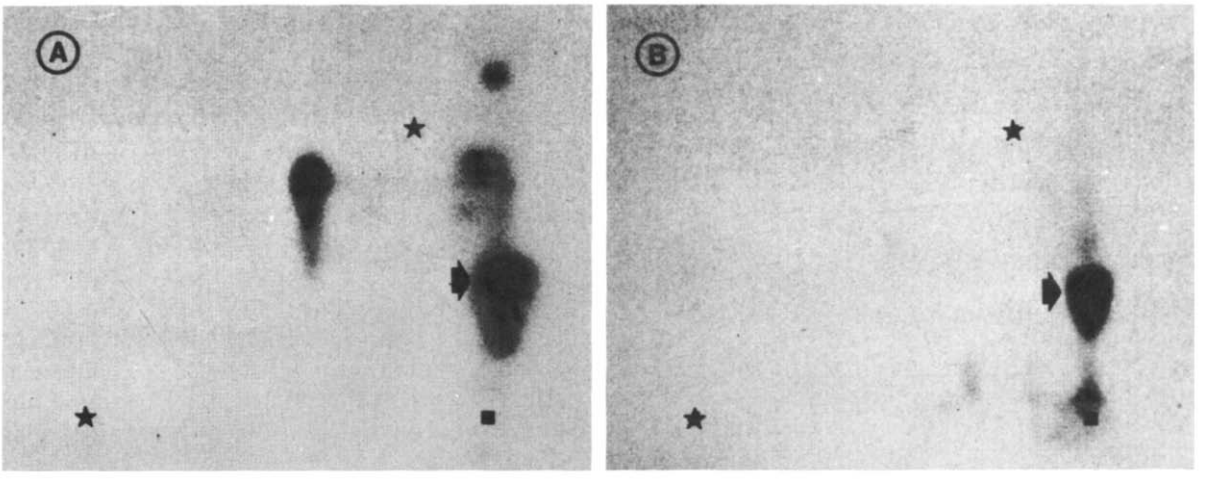

Fig.3. Phosphotryptic peptide maps of gp46. In vitro or in vivo phosphorylated gp46 bands were cut from an SDS gel and analyzed by two-dimensional tryptic peptide mapping as described in section 2. (A) Purified gp46 was phosphorylated in vitro with protein kinase C then processed for mapping; (B) untreated L6 cells were phosphorylated and gp46 purified by gelatin-Sepharose binding. This gp46 was then processed for mapping. The squares represent the respective origins and the stars represent the migration of internal reference dyes.

(fig.3A) whereas only one phosphorylated peptide was seen for the in vivo labeled protein (fig.3B). The migrations of the phosphopeptides indicated by arrows in fig.3A and B relative to the internal dye markers (stars) were very similar for both in vitro and in vivo labeled gp46, suggesting that gp46 may be constitutively phosphorylated by protein kinase C in vivo or at least that protein kinase C phosphorylates the same site in gp46 as some other constitutively activated protein kinase.

4. DISCUSSION

We have shown that a putative collagen receptor in L6 myoblasts is phosphorylated. In this respect it is similar to a 47 kDa gelatin-binding protein seen in chick embryo fibroblasts [2]. Phosphorylation of gp46 was not increased in two transformed myoblast cell lines in contrast to the large increase in phosphorylation observed for the 47 kDa gelatin-binding protein detected in RSV-transformed chick embryo fibroblasts [2]. This indicates that increased phosphorylation of collagen-binding proteins is not a general feature of RSV infection or of transformation per se.

Although we must be cautious in concluding that protein kinase C phosphorylates gp46 in vivo, our evidence does suggest this. Firstly, phorbol ester caused a slight, but reproducible, stimulation of gp46 phosphorylation. Lack of a more marked stimulation may have been due to a very rapid down-regulation of protein kinase C by phorbol ester in these cells (unpublished). Secondly, significant in vitro phosphorylation of purified gp46 by protein kinase C was shown. Thirdly, phosphotryptic peptide mapping suggested that protein kinase C phosphorylated gp46 at the same site as that found for in vitro labeled gp46. Since we have demonstrated that gp46 is involved in myoblast differentiation [1], modulation of its phosphorylation state by protein kinase C may conceivably alter or modify the course of myoblast differentiation presumably through an effect on collagen interaction. It will be of interest to determine whether changes in the phosphorylation state of gp46 modulate its binding affinity for collagen.

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REFERENCES

[1] Cates, G.A., Brickenden, A.M. and Sanwal, B.D. (1984) J. Biol. Chem. 259, 2646–2650.
[2] Nagata, K. and Yamada, K.M. (1986) J. Biol. Chem. 261, 7531–7536.
[3] Yaffe, D. (1968) Proc. Natl. Acad. Sci. USA 61, 447–483.
[4] Seth, P.K., Rogers, J., Narindrasorasak, S. and Sanwal, B.D. (1983) J. Cell. Physiol. 116, 336–344.
[5] Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1974) Methods Enzymol. 38, 299–307.
[6] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[7] Zweig, S.E. and Singer, S.J. (1979) Biochem. Biophys. Res. Commun. 88, 1147–1152.