Involvement of a cell surface protein and an ecto-protein kinase in myogenesis

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Myogenic differentiation is composed of a sequential cascade of multiple steps leading to the formation of multinucleated myotubes. The interference with any one step would abolish myogenesis. The present investigation examined the cell surface components which might be involved in myogenesis. Studies with subconfluent day 2 cultures of rat L6 myoblasts revealed that a cell surface 112 kDa protein was phosphorylated by a Ca^{2+} -, F^- - and Mg^{2+} -dependent ecto-protein kinase [Chen & Lo (1991) *Biochem. J.* 279, 467–474]. We have shown in the present investigation that adequate ATP was present on the cell surface for efficient functioning of this ecto-protein kinase. The phosphorylation of the 112 kDa protein by this ecto-protein kinase was decreased dramatically in confluent cells and in multinucleated myotubes. The following evidence suggests that both the 112 kDa protein and the ecto-protein kinase may play important roles in myogenesis. (i) The highest phosphorylation activity was observed in subconfluent cultures, i.e. before the onset of morphological differentiation. (ii) Treatment of cells with chemical reagents resulted in a corresponding decrease in the ecto-protein kinase, the 112 kDa protein, the phosphorylated 112 kDa protein (p112) and the ability to form myotubes. (iii) The level of p112 in a conditional myogenesis-defective mutant corresponded with the cells' eventual ability to differentiate. (iv) A mutant defective in the ecto-protein kinase was impaired in the phosphorylation of the 112 kDa protein and in myogenesis. (v) A mutant containing only residual levels of the 112 kDa protein was deficient in both p112 and myogenesis. (vi) Since the level of p112 was normal in another myogenesis-defective mutant, the phosphorylation of this protein was not likely to be a consequence of myogenic differentiation. The above findings suggest that the ecto-protein kinase and the 112 kDa protein may directly or indirectly be associated with the myogenic pathway. Since the levels of the ecto-protein kinase, the 112 kDa protein and p112 decreased dramatically upon the formation of myotubes, these proteins were probably not required once morphological differentiation had been initiated.

INTRODUCTION

A number of distinct sequential events are involved in the formation of multinucleated myotubes from proliferating myoblasts (Wakelam, 1985; Schneider & Olson, 1988; Florini & Magri, 1989), as follows. (i) Biochemical differentiation. This involves the induction of muscle-specific proteins such as α -cardiac and α -skeletal actin, myosin heavy and light chains, tropomyosin, troponin-T, desmin, the muscle isoenzyme of creatine kinase, acetylcholinesterase and the nicotinic acetylcholine receptor, and voltage-gated Na^+ and Ca^{2+} channels (Gunning *et al.*, 1987; Endo & Nadal-Ginard, 1987). The induction of these muscle-specific proteins can be reversed by growth stimulation (Nguyen *et al.*, 1983). (ii) Total commitment. This occurs when cells cease DNA synthesis, and irreversibly withdraw from the cell cycle. These post-mitotic mononucleated cells are irreversibly committed to subsequent myogenesis, but have not yet begun to fuse. (iii) Terminal differentiation. This involves the formation of multinucleated myotubes by fusion. In the past two decades enormous efforts have been made to elucidate the myogenesis pathway. The identification of more than 40 different myogenesis inhibitors suggests that myogenesis is composed of a sequential cascade of multiple steps leading to terminal differentiation, and interference with any one step would seem to abolish differentiation (Schneider & Olson, 1988; Endo & Nadal-Ginard, 1987). One of the problems associated with these inhibitor studies is that most inhibitors can act on more than one target site; this makes it difficult to define conclusively the components involved in myogenesis.

Another approach to determine the components involved in myogenesis is to identify the alterations found in myogenesis-defective mutants (Cates *et al.*, 1984). We have previously examined the properties of mutants defective in myogenesis. Mutant D1/S4 was isolated from rat L6 myoblasts by its resistance to cell detachment caused by anti-L6 antibodies (D'Amore & Lo, 1988). While this antibody can activate hexose transport in the parental L6 myoblasts (Lo & Duronio, 1984a,b), it has no effect of hexose transport by the mutant (D'Amore & Lo, 1988). It was subsequently found that the anti-L6 antibody interacted with a 112 kDa plasma membrane protein, and this protein was missing in mutant D1/S4. The second myogenesis-defective mutant examined was mutant F72, which was isolated from L6 myoblasts by its ability to grow in the toxic 2-deoxy-2-fluoro-D-glucose (D'Amore *et al.*, 1986b). Although not much was known about its primary genetic defect, this mutant was impaired in the high-affinity hexose transport system (HAHT), even though cytochalasin B binding studies showed that it contained the normal level of the HAHT transporter (Chen, 1989). Mutant F72 also contained the normal level of the 112 kDa protein (D'Amore & Lo, 1988). Mutant D23 was isolated from L6 myoblasts by its ability to grow in the presence of 2-deoxy-D-glucose (D'Amore *et al.*, 1986b). This mutant was defective in the HAHT transporter and also in myogenesis (Chen & Lo, 1988; Kudo & Lo, 1990). In view of their inability to fuse, these mutants may conceivably serve as useful tools in dissecting the events involved in myogenesis.

The present investigation examined the factors involved in the initiation of myogenesis. Previous mutant analyses have implic-

Abbreviations used: BrdUrd, 5-bromo-2'-deoxyuridine; p112, phosphorylated 112 kDa protein; anti-PMV, rabbit antibodies raised against plasma membrane vesicles prepared from rat L6 myoblasts; HAHT, high-affinity hexose transporter; PBS, phosphate-buffered saline (150 mM-NaCl/80 mM- Na_2HPO_4 /15 mM- KH_2PO_4 /30 mM-KCl, pH 7.4).

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ated the possible involvement of an ecto-protein kinase in myogenesis (Lognonne & Wahrman, 1986, 1988, 1990). However, it was not clear whether this ecto-protein kinase was involved in the initiation of myogenesis, as these studies were carried out with day 4 confluent cultures. We have shown in the previous paper the presence of an ecto-protein kinase in subconfluent day 2 rat L6 myoblast cultures (Chen & Lo, 1991); the natural endogenous substrate protein for this ecto-protein kinase is a cell surface 112 kDa protein. Since initiation of biochemical differentiation should occur at an early stage of the growth phase, the possibility exists that the newly discovered ecto-protein kinase may be involved in the initiation of myogenesis. Indeed, data presented in this paper suggest that the functioning of the ecto-protein kinase and the 112 kDa protein may be required for myogenesis.

MATERIALS AND METHODS

Cell lines and culture media

Yaffe's L6 rat skeletal myoblast line (Yaffe, 1968) was maintained in Alpha medium (Flow Laboratories) supplemented with 10% (v/v) horse serum (Flow Laboratories) and gentamicin (50 µg/ml; Gibco) as previously described (Chen & Lo, 1991).

Intracellular level of ATP

The intracellular ATP level was determined as previously described (D'Amore & Lo, 1986a). Cells were first washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS) and solubilized in 1 ml of 8 M-guanidine hydrochloride. Then 20 µl of this suspension was diluted 20-fold with water; 20 µl of this dilution was used for ATP determination by the luciferase-luciferin assay (Linklater *et al.*, 1985). Luminescence was measured with a Lumac Model 2010 Luminometer (Mandel). The amount of ATP in unknown samples was determined using the linear portion of a standard curve (i.e. 0.01–0.05 nmol of ATP). Protein determinations were carried out by the method of Lowry *et al.* (1951) using BSA as a standard. All determinations were carried out in triplicate and each experiment was repeated twice, with similar results.

Levels of newly synthesized ATP

Newly synthesized ATP was labelled by incubating rat myoblasts with [³²P]phosphate for 2 h. Rat L6 myoblasts were grown in normal growth medium for 2 days to a density of 4.4×10^5 cells per 60 mm plate. After washing and standing in phosphate- and serum-free medium for 30 min, cells were then labelled with 50 µCi of H₃³²PO₄/ml for 2 h at 37 °C. The labelling medium was retained, and cells were washed twice with 15 mM-Hepes buffer (pH 7.5). Each plate was then incubated with 1 ml of 15 mM-Hepes buffer (pH 7.5) with or without trypsin (3 µg/ml) for 5 to 10 min at 37 °C. At the end of the incubation period the supernatant was collected and centrifuged to remove any detached cells. The adenine nucleotides in all samples were identified and quantified by the method described by Norman *et al.* (1974). All samples (labelling medium and supernatant from various incubations) were adjusted to pH 3–4 with 17.5% HClO₄ (1 µl/100 µl sample). To each 100 µl sample, 1 µl of each of the following nucleotide carriers was added: 0.1 M-ATP, 0.1 M-ADP and 0.1 M-AMP, and then 20 µl of each sample was spotted on to a silica gel t.l.c. plate. Plates were developed in a tank containing 100 ml of a solvent system composed of 2-methylpropan-1-ol/3-methylbutan-1-ol/ethoxyethanol/ammonia/deionized water (3:2:6:3:5, by vol.). The solvent system was matured for at least 48 h before use, and added to the tank 1 h before introduction of plates (Norman *et al.*, 1974). Optimal separation of the adenine

nucleotides was achieved by running the plates in a solvent twice. The adenine nucleotides were visualized as blue spots in u.v. light. The blue spots were cut out and counted for radioactivity in scintillation fluid. The above determinations were carried out in duplicate, and the experiment was repeated twice. The average error in various determinations was less than 5%.

Phosphorylation of the cell surface 112 kDa protein

The phosphorylation of cell surface proteins was carried out as described in the previous paper (Chen & Lo, 1991).

Ecto-protein kinase activity

The phosphorylation of exogenously added horse serum proteins was also used to assess ecto-protein kinase activity. Cell surface phosphorylation assays were carried out essentially as described earlier, except that 3 µl of horse serum was included in 1.2 ml of the phosphorylation buffer. After phosphorylation, 280 µl of the supernatant from each well was added to 93 µl of 4× SDS sample buffer. After boiling in sample buffer for 5 min, 100 µl of this sample was loaded on to the SDS/polyacrylamide gel. Each lane was loaded with the same amount of protein. After drying, the gel was exposed to a Kodak X-OMAT AR film. The level of the phosphorylated 75 kDa horse serum protein was determined by densitometry. Autoradiograms used were within the linear range of the film. All phosphorylation studies were carried out in triplicate, and each experiment was repeated at least twice with similar results. Figures illustrate the results of representative experiments.

Level of the 112 kDa cell surface protein

The level of the 112 kDa cell surface protein was determined by immunoblotting studies using rabbit antibodies raised against plasma membrane vesicles prepared from rat L6 myoblasts (anti-PMV) (D'Amore & Lo, 1988). Total cell extracts (150–200 µg) from cells grown under different conditions were subjected to SDS/PAGE. Proteins were then electrophoretically transferred from the gel on to nitrocellulose membranes (0.45 µm) using a transfer buffer containing 25 mM-Tris, 192 mM-glycine (pH 8.3), 20% methanol and 0.1% SDS (Towbin *et al.*, 1979) at 55 V for 2 h. The paper was incubated with 20% fetal calf serum in Tris buffer containing 150 mM-NaCl, 10 mM-Tris/HCl (pH 8.0) and 0.05% Tween 20 for at least 5 h at 23 °C to block non-specific protein binding. After washing once with the same Tris buffer, the paper was incubated with anti-PMV or preimmune serum for 14 h at 23 °C; this was then followed by washing 3 times with the Tris buffer (5–10 min for each washing). The immune complexes were detected with ¹²⁵I-labelled Protein A. After removal of unbound ¹²⁵I-Protein A by washing three times with the Tris buffer, blots were dried and exposed to Kodak X-OMAT AR film with an intensifying screen at –86 °C. The level of the 112 kDa protein was determined by densitometry. Immunoblotting studies were carried out in duplicate and the experiment was repeated three times with similar results in all cases.

Determination of fusion index and nuclei density

Cells were plated in 6-well Falcon plates in Alpha medium. On different days after subculturing, cells were treated with 1 mM-ZnSO₄ in 20% dimethyl sulphoxide to swell the nuclei, and then fixed in 2.5% glutaraldehyde in PBS (Chen & Lo, 1989). Cells were then stained with 6% Giemsa. The fusion index was determined by calculating the ratio of the number of nuclei in myotubes to the total number of nuclei per field (Morris & Cole, 1972; Kudo & Lo, 1990). Only structures containing at least three nuclei were considered as myotubes. Usually each field contained at least 50 nuclei. Ten fields were counted for each set of determinations.

Materials

H₃³²PO₄ and [γ -³²P]ATP were purchased from ICN Biochemicals. Phloretin and 5-bromo-2'-deoxyuridine (BrdUrd) were purchased from Sigma Chemical Co. All other chemicals were obtained from commercial sources and were of the highest available purity.

RESULTS

Level of extracellular ATP excreted by rat L6 myoblasts

As indicated in the previous paper, an ecto-protein kinase was involved in the phosphorylation of a cell surface 112 kDa protein in subconfluent rat L6 myoblasts; the K_m value of this enzyme for ATP was 40 nM (Chen & Lo, 1991). Before examining the regulation and possible roles of these proteins, it is important to determine whether there is adequate ATP on the cell surface for efficient functioning of the ecto-protein kinase. Whereas extracellular ATP concentrations in purinergic nerve terminals, circulating blood and endothelial cells have been determined, no information is available on the amount of ATP excreted by cultured skeletal myoblasts (Pearson & Gordon, 1979; Born & Kratzer, 1984; White, 1985; Gordon, 1986). In the present investigation myoblasts were first labelled with [³²P]phosphate for 2 h. The concentration of [³²P]ATP present in the growth medium was found to be around 3 nM (Table 1). Based on an estimated intracellular volume of 0.22 μ l/10⁵ cells (D'Amore & Lo, 1986a), the intracellular concentration of ATP was calculated to be around 24 mM. This was similar to the previously determined values in rat myoblasts (D'Amore & Lo, 1986a) and in human myoblasts (Mesmer & Lo, 1989, 1990). The levels of [³²P]ATP, [³²P]ADP and [³²P]AMP were calculated to be 32 μ M, 63 μ M and 38 μ M respectively. It was interesting to note that the ratios of these newly synthesized nucleotides differed from those of the steady-state cellular nucleotide levels. If the newly synthesized ATP was equilibrated with the intracellular ATP pool at the end of the incubation period, the ATP concentration in the growth medium would then be 2.26 μ M.

Since extracellular ATP is subjected to hydrolysis, the amount excreted by the cells may be higher than that present in the

growth medium after 2 h of incubation. As shown in Table 1, the concentrations of [³²P]ATP excreted from the cells at 5 and 10 min after washing were 34 and 37 nM respectively. If there was equilibration with the intracellular ATP pool, the extracellular ATP concentration would then be around 26 μ M. Studies using trypsin to inactivate cell surface hydrolytic enzymes (Pearson & Gordon, 1979) suggested that there was negligible hydrolysis of the extracellular ATP at 10 min after washing (Table 1). These studies showed that even if the newly synthesized ATP could not equilibrate with the intracellular ATP pool, there was still adequate cell surface ATP for efficient operation of the ecto-protein kinase.

A comparison of the ³²P-labelled nucleotides in the extra- and intra-cellular spaces revealed that the extracellular nucleotides were excreted by specific mechanisms. Similar percentages of all three adenine nucleotides would be released into the extracellular space if these nucleotides were externalized as a result of cell lysis. We found that 67.48 $\pm$ 3.37 pmol of ATP was synthesized by 4.4 $\times$ 10⁵ cells in 2 h. About 54% of the newly synthesized ATP was excreted out of the cells at 10 min after washing, whereas only 25% and 5% of the newly synthesized ADP and AMP were excreted during the same period of time. Thus, similar to cyclic AMP (Brunton & Heasley, 1988; Fehr *et al.*, 1990), extracellular ATP was excreted selectively by the cells, and not merely via damaged cell membranes. It was interesting to note that when cultured pig aortic endothelial cells were labelled with [³H]adenosine for 1 h, up to 50% of the [³H]ATP was released from the cells (Pearson & Gordon, 1979). Moreover, the newly synthesized ATP was able to equilibrate with the intracellular ATP pool within 1 h. In summary, our findings indicate that there is a constant and adequate supply of ATP on the cell surface for efficient operation of the newly discovered ecto-protein kinase (Chen & Lo, 1991).

Effect of myogenesis and cell density on the phosphorylation of the 112 kDa protein

We have shown in the previous paper (Chen & Lo, 1991) that the cell surface phosphorylation activity can be monitored by determining the level of the phosphorylated 112 kDa protein (p112). Changes in the phosphorylation activities at different developmental stages of the cells may shed some light on the roles and regulation of the ecto-protein kinase, the 112 kDa protein and/or p112. Phosphorylation assays were carried out with cells grown to different stages of myogenesis (Fig. 1). Rat L6 myoblasts began to line up and form multinucleated myotubes on day 4 after subculturing, and fusion was essentially complete by day 7 (Fig. 1) (Chen & Lo, 1989; Kudo & Lo, 1990). The level of p112 was found to decrease dramatically with increasing time after subculturing. Using the p112 level of the day 1 culture as 100%, about 85% and 40% activity were observed in day 2 and day 3 cultures respectively (Fig. 1). Negligible phosphorylation activity was observed upon formation of multinucleated myotubes (day 5 to day 7 cultures).

In order to determine whether the decrease in p112 is due to a mere increase in cell density, cells were seeded at different densities and grown for 2 days. Myotubes were not detected in these cultures, even with protein contents as high as 1.4 μ g/ μ l. The p112 level in these myoblasts was found to be inversely proportional to the cell density (as indicated by the protein concentration) (Fig. 2). A decrease of about 50% in p112 was observed when the cell density was doubled. A similar decrease in ecto-protein kinase activity at higher cell densities was also observed with other cell types (Mastro & Rozengurt, 1976).

A comparison of the cell density and myogenesis effects revealed that the p112 level was decreased to a much greater extent upon formation of myotubes (Fig. 2). Before the ap-

Table 1. Concentrations of nucleotides excreted by rat myoblasts

Newly synthesized nucleotides were labelled by incubating rat L6 myoblasts with [³²P]phosphate for 2 h. The amounts of intracellular ATP and newly synthesized nucleotides in both intracellular and extracellular spaces were determined by the procedures described in the Materials and methods section. The intracellular volume of rat L6 myoblasts grown under our experimental conditions was 0.22 μ l/10⁵ cells (D'Amore & Lo, 1986a). Using this value, the intracellular concentrations of [³²P]ATP and ATP were calculated to be 32 μ M and 24 mM respectively. The calculation of the ATP concentration in the extracellular space was based on the assumption that the newly synthesized ATP equilibrated with the intracellular ATP pool before excretion.

	Concentration of newly synthesized nucleotides (μ M)			Estimated concentration of ATP (μ M)
	AMP	ADP	ATP	
Intracellular space	38.10	62.81	31.92	24000
Growth medium	0	0.002	0.003	2.26
Hepes buffer (5 min)	0.001	0.015	0.034	25.56
Hepes buffer (10 min)	0.002	0.020	0.037	27.82
Hepes buffer + trypsin (10 min)	0.003	0.022	0.038	28.57

Fig. 1. Effect of growth stage on phosphorylation of the 112 kDa protein

Rat L6 myoblasts were grown in 10% horse serum for different periods of time after subculturing. The fusion index and the phosphorylation of the 112 kDa protein were determined by procedures indicated in the text. The extent of cell fusion (○) was determined by calculating the ratio of the number of nuclei in myotubes to the total number of nuclei per field (Morris & Cole, 1972). The level of p112 (●) was determined by densitometry of the autoradiogram, and is expressed as a percentage of that found in the day 1 culture.

Fig. 2. Effect of cell density and myogenesis on the phosphorylation of the 112 kDa protein

In order to determine the effect of cell density, independent of myogenesis, on the phosphorylation of the 112 kDa protein, cells were seeded at different densities and grown for 2 days. Cell surface phosphorylation by these cultures was then carried out as described in the text. The level of p112 was determined by densitometry of the autoradiogram and the level of p112 in a culture with a protein content of $0.3 \mu\text{g}/\mu\text{l}$ was taken as 100%. ●, Level of p112 in day 2 cells seeded at different densities. It was important to note that myotubes could not be observed in these cultures. ○, Level of p112 in cells seeded at the same density, but grown to different stages of myogenesis. The ○ symbols at 1.07, 1.3, 1.38 and $1.4 \mu\text{g}$ of protein/ μl correspond to the level of p112 in day 4, day 5, day 6 and day 7 cultures respectively. The fusion indices of these cultures were 7%, 23%, 72% and 92%, respectively (see Fig. 1).

pearance of myotubes (cultures with protein contents from 0.3 to $0.77 \mu\text{g}/\mu\text{l}$), the decrease in p112 in cultures grown for different periods was similar to that seen in the cell density effect. However, upon formation of myotubes (day 4 to day 7 cultures, i.e. cultures with protein contents from 1.07 – $1.40 \mu\text{g}/\mu\text{l}$), the p112 level was dramatically decreased even without any great change in the protein content of the cultures (Fig. 2). For example, at a protein content of $1.4 \mu\text{g}/\mu\text{l}$, the p112 level in myotubes was 4 times lower than that in myoblasts. Thus the cell surface phosphorylation activity was affected by myogenesis.

The effect of myogenesis on the p112 level was further examined by using cultures exhibiting different rates of fusion. The concentration of horse serum in the growth medium was found to affect the rate of fusion (Table 2). When grown in normal (10%)

Table 2. Levels of p112 in rat myoblasts exhibiting different rates of myogenesis

The conditional myogenesis-defective mutant D1 and its parental L6 cells were initially grown in 10% horse serum for 1 day, and then subcultured in 1% or 10% horse serum. The levels of p112 in the day 3 cultures were then determined as described in the text. The level of p112 in L6 myoblasts grown in 10% horse serum was taken as 100%. Fusion indices were determined for day 4 and day 5 cultures as described by Kudo & Lo (1990).

Growth conditions		Fusion index (%)		p112 in day 3 culture (%)
Culture	Horse serum concentration (%)	Day 4 culture	Day 5 culture	
1. Cultures with normal rates of myogenesis				
L6	10	7	23	100
D1	1	12	27	93
2. Culture impaired in myogenesis				
D1	10	0	0	26
3. Culture with a faster rate of myogenesis				
L6	1	21	65	33

horse serum, L6 cells exhibited 7% and 23% fusion in day 4 and day 5 cultures respectively; however 21% and 65% fusion was observed with the corresponding cultures grown in 1% horse serum. In other words, myoblasts grown in 1% horse serum have a faster rate of fusion. If the p112 level is affected by myogenesis, then day 3 cultures of L6 cells grown in 1% horse serum should have a lower p112 level than those grown in 10% horse serum. Indeed, this was found to be the case; only 33% of the normal p112 level was observed in L6 culture grown in 1% horse serum (Table 2), even though the number of nuclei per plate was quite similar. A plausible implication of this observation is that ecto-protein kinase is no longer required after myogenesis has been initiated.

Is phosphorylation of the 112 kDa protein required for myogenesis?

The relationship between phosphorylation of the 112 kDa protein and myogenesis was examined using various chemical reagents. Although their primary sites of action have not been defined, cytochalasin B, BrdUrd and phloretin are potent inhibitors of myogenesis (Delain & Wahrmann, 1975; Endo & Nadal-Ginard, 1987; Chen & Lo, 1989; Kudo & Lo, 1990). Unfortunately, both cytochalasin B and BrdUrd can also act on other proteins with similar affinities (Sanwal, 1979; Pearson, 1981; Ray *et al.*, 1987; Chen & Lo, 1988; Kobler *et al.*, 1990). Thus they cannot be used as definitive tools in examining the relationship between myogenesis and cell surface phosphorylation.

We have demonstrated that phloretin, but not phlorizin, inhibits myogenesis at concentrations that did not affect most other cellular functions (Snow *et al.*, 1978; De Jonge *et al.*, 1983; Shefcyk *et al.*, 1983; Movius *et al.*, 1989; Kudo & Lo, 1990). The effects of these reagents on cell surface phosphorylation were therefore determined. As indicated in Table 3, phosphorylation of the 112 kDa protein was inhibited by 25 and $50 \mu\text{M}$ -phloretin; this was accompanied by the eventual inhibition of myogenesis, whereas $50 \mu\text{M}$ -phlorizin had only minimal effects on these two processes. Although its site of action was not clear, BrdUrd was also found to affect both phosphorylation and myogenesis. Thus the phosphorylation of the 112 kDa protein and the ability to differentiate seemed to be affected in a similar manner.

Table 3. Inhibition of the phosphorylation of the 112 kDa protein and myogenesis by chemical reagents

On day 2 after subculturing, rat L6 myoblasts were incubated with various chemical reagents. After 24 h of incubation, cells were washed and cell surface phosphorylation was carried out as described in the text. The level of the phosphorylated 112 kDa protein was quantified by densitometry of the autoradiogram. The level of p112 in the untreated cells was taken as 100%. Fusion indices of day 6 rat L6 myoblasts treated with the above-mentioned chemical reagents were determined by the procedure described by Kudo & Lo (1990). Data are means of three independent experiments, with an average error of 5%.

Treatment	Phosphorylation of the 112 kDa protein in day 3 culture (%)	Fusion index of day 6 culture (%)
Control	100	85
Phlorizin (50 μ M)	87.2	72
Phloretin (25 μ M)	40.7	36
Phloretin (50 μ M)	36.1	15
BrdUrd (7.5 μ M)	35.6	0

Fig. 3. Level of the phosphorylated 112 kDa protein in various myogenesis-defective mutants

Day 2 cultures of various myogenesis-defective mutants and their parental L6 myoblasts were used in these studies. Cell surface phosphorylation studies were carried out as described in the text. Lanes 1, 2, 3 and 4 indicate phosphorylated protein profiles in mutants D1/S4, F72 and D23 and the parental L6 cells respectively. The same amount of protein was loaded on to each lane. The molecular mass standards used were phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa).

Studies with a conditional myogenesis-defective mutant

The relationship between myogenesis and phosphorylation of the 112 kDa protein was further explored using a conditional myogenesis-defective mutant. Mutant D1, a concanavalin A-resistant mutant, was impaired in both biochemical and morphological differentiation when grown in 10% horse serum (Zeuner *et al.*, 1988; Chen & Lo, 1989) (Table 2). However, it was able to differentiate when cultured in 1% horse serum. Thus this mutant can serve as a unique tool in examining the relationship between myogenesis and phosphorylation, without the use of chemical reagents.

In the present investigation, both L6 and D1 cells were initially grown in 10% horse serum for 1 day, and then subcultured in

Fig. 4. Morphology of (a) L6, (b) F72 and (c) D1/S4 myoblasts on day 6 after subculturing

Myoblasts were plated at a density of 10⁵ cells/100 mm plate. After subculturing for 6 days, cells were treated with 1 mM-ZnSO₄ in 20% dimethyl sulphoxide to swell the nuclei and then fixed in 2.5% glutaraldehyde. Cells were then stained with 6% Giemsa. (a) Formation of multinucleated myotubes in the day 6 L6 culture. Mutants F72 and D1/S4 were unable to fuse even on day 6 after subculturing.

1% or 10% horse serum for a further 2 days. Cell surface phosphorylation studies revealed that similar p112 levels were present in both myogenesis-competent cells, i.e. L6 cells grown in 10% horse serum and D1 cells grown in 1% horse serum. However, only 26% of the normal p112 level was observed in myogenesis-impaired cells (i.e. D1 grown in 10% horse serum). Thus there is a close correlation between the p112 level in undifferentiated myoblasts and the cells' eventual ability to fuse.

A mutant defective in the 112 kDa protein is impaired in myogenesis

Since two components are involved in the observed cell surface phosphorylation, it is not clear which component is essential for myogenesis. Mutant D1/S4 was previously shown to be defective in a cell surface 112 kDa plasma membrane protein (D'Amore & Lo, 1988). Cell surface phosphorylation studies revealed that this mutant was also defective in p112 (Fig. 3). This suggested that the cell surface 112 kDa protein might be the endogenous substrate protein of the ecto-protein kinase. Since this mutant is also defective in myogenesis (Fig. 4), it may be surmised that the

Fig. 5. Phosphorylation of a 75 kDa horse serum protein by myogenesis-defective mutants

Exogenously added horse serum was used to measure ecto-protein kinase activity. The phosphorylation of horse serum proteins was determined as described in the text. Lane 1 shows the phosphoprotein profile by L6 cells in the absence of NaF. This shows that the 75 kDa protein was not phosphorylated in the absence of NaF. Lanes 2, 3, 4 and 5 show the phosphoprotein profiles by mutant D23, L6, D1/S4 and F72 respectively. The same amount of protein was loaded on to each lane. The molecular mass standards used were as in Fig. 3.

Table 4. Levels of 112 kDa protein, ecto-protein kinase and p112 in cells impaired in myogenesis and in myotubes

Rat myoblast L6 cells were used in this study. Cells were treated with BrdUrd or with phloretin as described in Table 3. Ecto-protein kinase activity was determined using horse serum as the exogenously added substrate, as described in Fig. 5. The level of the phosphorylated 75 kDa horse serum protein was determined by densitometry. The level of the 112 kDa protein was determined by immunoblotting studies using antiserum raised against plasma membrane vesicles prepared from L6 cells (D'Amore & Lo, 1988). The level of the phosphorylated 112 kDa protein was determined as described in Fig. 1. The extent of cell fusion was determined by calculating the ratio of the number of nuclei in myotubes to the total number of nuclei per field (Kudo & Lo, 1990). Data are means of three independent experiments, with an average error of 5%.

Culture	Ecto-protein kinase activity (%)	112 kDa protein (%)	Phosphorylation of the 112 kDa protein (%)	Fusion index (%)
Day 3 culture				
Control	100	100	100	0
BrdUrd (7.5 μ M)	73	53	35.6	0
Phloretin (50 μ M)	75	53	36.1	0
Day 6 culture	17	23	4.5	70

112 kDa protein is required for myogenesis, and p112 is not just a side-product of the phosphorylation reaction.

A mutant defective in the ecto-protein kinase is also impaired in myogenesis

In our search for mutants defective in the ecto-protein kinase, various rat myoblast mutants were screened for their ability to

phosphorylate the 112 kDa protein. These studies revealed that mutant F72 contained only a residual amount of p112 (Fig. 3). Unlike mutant D1/S4, mutant F72 contained the normal level of the 112 kDa protein (D'Amore & Lo, 1988). It is therefore conceivable that this mutant may be defective in the ecto-protein kinase responsible for phosphorylation of the 112 kDa protein.

Attempts were also made to assay ecto-protein kinase activity independent of the 112 kDa protein. Among various exogenously added proteins tested, a 75 kDa horse serum protein was found to be phosphorylated by L6 cells under the optimal assay condition for the ecto-protein kinase, i.e. in the presence of NaF, Mg^{2+} and Ca^{2+} (Fig. 5). Mutant D1/S4 (lane 4) and its parental L6 cells (lane 3) could phosphorylate this 75 kDa protein to a similar extent, whereas mutant F72 (lane 5) was unable to phosphorylate the protein. Thus, in agreement with data presented in Fig. 3, mutant F72 is defective in the ecto-protein kinase. It is important to note that this mutant is also defective in myogenesis (Fig. 4). Thus the absence of ecto-protein kinase seems to correlate with the cells' inability to differentiate.

Not all myogenesis-defective mutants are defective in p112

The question that arises from the above studies is whether phosphorylation of the 112 kDa protein is involved in triggering myogenesis or is a mere consequence of myogenesis. If the latter is the case, then mutants impaired in myogenesis should all have much decreased levels of p112. These possibilities were examined using another myogenesis-defective mutant, mutant D23, which is defective in myogenesis and in the HAHT component (D'Amore *et al.*, 1986b; D'Amore & Lo, 1986b; Chen & Lo, 1988; Kudo & Lo, 1990). The ability of this mutant to phosphorylate the 112 kDa protein was therefore examined. The mutant was found to retain the normal ability to phosphorylate the 112 kDa protein and the exogenously added 75 kDa horse serum protein (Figs. 3 and 5), even though it was defective in myogenesis. This finding is important in demonstrating that phosphorylation of the 112 kDa protein is not a consequence of biochemical differentiation, but rather may be one of the important steps involved in myogenesis.

Levels of the 112 kDa protein and ecto-protein kinase in myotubes and cells impaired in myogenesis

So far we have demonstrated a close correlation between p112 and myogenesis. This alteration in p112 could be due to changes in the level of the 112 kDa protein and/or the ecto-protein kinase activity. Attempts were therefore made to determine the levels of these two proteins. The level of the 112 kDa protein was determined by immunoblotting studies using antibodies raised against plasma membrane vesicles prepared from rat L6 myoblasts (D'Amore & Lo, 1988), whereas that of the ecto-protein kinase was quantified using exogenously added horse serum (Fig. 5). Levels of these two proteins were decreased by 47% and 26% respectively in the myogenesis-impaired cells (Table 4). A comparison with the level of p112 in these cells (Table 4) suggested that decreases in these two proteins could account for the changes in the p112 level. In view of the dramatic decrease in the 112 kDa protein, it was unlikely that the decrease in p112 was due to changes in the level of ATP excreted by the cells or to alterations in the amount of unlabelled phosphate already present in the 112 kDa protein.

In the case of the fused myotubes, only 17% and 23% of the control levels of ecto-protein kinase and the 112 kDa protein were detected (Table 4). Unlike the effects of the myogenesis inhibitors, both proteins were decreased to a similar extent in the myotubes. Again, these decreases could account for the much decreased level of p112 (4.5% of control) observed in the myotubes. This finding therefore shows that myogenesis results

in a dramatic decrease of both protein kinase and the cell surface 112 kDa protein.

DISCUSSION

We have shown in the previous paper (Chen & Lo, 1991) that a cell surface 112 kDa protein in rat L6 myoblasts can be phosphorylated by extracellular ATP. An ecto-protein kinase is thought to be responsible for this process. The K_m value of this enzyme for ATP was $0.04 \mu\text{M}$, and serine residues of the 112 kDa protein were phosphorylated. Whereas the location of the catalytic site of the ecto-protein kinase and the phosphorylation site of the 112 kDa protein have been determined, not much is known about the physiological roles and identity of these proteins.

In order for the ecto-protein kinase to carry out its function, sufficient extracellular ATP should be available to the enzyme. After 2 h of labelling with [^{32}P]phosphate, the amounts of newly synthesized ATP (as indicated by [^{32}P]ATP) present in the growth medium and excreted by the cells at 10 min after washing were $0.003 \mu\text{M}$ and $0.036 \mu\text{M}$ respectively (Table 1). If the newly synthesized ATP equilibrated with the intracellular ATP pool before excretion, the ATP concentrations in the growth medium and excreted by the cells would then be $2.26 \mu\text{M}$ and $26 \mu\text{M}$ respectively (Table 1). As in cultured pig aortic endothelial cells (Pearson & Gordon, 1979), about 54% of the newly synthesized ATP was excreted in the 10 min after washing. These findings indicate that there is a constant and adequate supply of cell surface ATP for efficient functioning of the ecto-protein kinase, even if the newly synthesized ATP cannot equilibrate with the general intracellular ATP pool. A comparison of the ratios of the extracellular and intracellular amounts of ATP, ADP and ADP revealed that ATP was selectively excreted from the cells, and was not released as a result of cell lysis.

The following evidence suggests that both the exogenously added 75 kDa horse serum protein and the endogenous cell surface 112 kDa protein might be phosphorylated by the same ecto-protein kinase. (i) NaF was required for the phosphorylation of these two proteins (Fig. 5; Chen & Lo, 1991). (ii) Multinucleated myotubes exhibited a much reduced ability to phosphorylate these two proteins (Table 4). (iii) Growth in cells in $7.5 \mu\text{M}$ -BrdUrd or $50 \mu\text{M}$ -phloretin lowered the cells' ability to phosphorylate these two proteins (Table 4). (iv) The ecto-protein-kinase-defective mutant F72 was unable to phosphorylate these two proteins (Figs. 3 and 5). Thus these studies showed that the exogenously added 75 kDa protein can serve as a substrate protein for the ecto-protein kinase. This provides another means for assaying the ecto-protein kinase activity, independent of the 112 kDa protein.

The phosphorylation of the cell surface 112 kDa protein by ecto-protein kinase was affected by the physiological state of the myoblasts. As for ecto-protein kinases found in other cell types (Mastro & Rozengurt, 1976), the level of the phosphorylated 112 kDa protein (p112) in mononucleated myoblasts was inversely proportional to cell density (Fig. 2). About a 50% decrease in p112 was observed when cell density was doubled. The normal cell density used in our routine assay was considerably lower than that used by previous workers (Senechal *et al.*, 1982; Lognonne & Wahrman, 1986, 1988). This indicated that the rat myoblast ecto-protein kinase examined in the present investigation was distinct from that examined by previous workers. It should be mentioned that the phosphorylation conditions and the endogenous substrate proteins phosphorylated by these two ecto-protein kinases were quite different.

An even more dramatic decrease in p112 was observed upon the formation of myotubes. At a protein content of $1.4 \mu\text{g}/\mu\text{l}$, the p112 level in the 90% fused culture was about four times lower than that of the undifferentiated myoblasts (Fig. 2). A similar relationship between myogenesis and p112 levels was observed with L6 myoblasts exhibiting different rates of fusion. L6 myoblasts grown in 1% horse serum have a much faster rate of fusion than those grown in 10% horse serum (Table 2). On day 3 after subculturing, the former exhibited only 33% of the p112 level found in the latter cells (Table 2), even though they had a similar number of nuclei per plate. Further examination revealed that the levels of the ecto-protein kinase and 112 kDa protein in the myotubes were decreased by 83% and 77% respectively (Table 4). This decrease could account for the dramatically lowered level of p112 (4.5%) observed in the myotubes. The dramatic decrease in the myotube 112 kDa protein suggested that the observed decrease in p112 was not due to changes in the level of extracellular ATP or in the amount of unlabelled phospho-amino acids in the protein.

The observed cell density effect suggests that the ecto-protein kinase, the 112 kDa protein and/or p112 may play active roles in subconfluent cells, whereas they are not as active in confluent cultures. Since biochemical differentiation occurs at an early stage of the growth phase, these proteins may be required for the initiation of myogenesis. Two different approaches were used to test this possibility. The first approach examined the effect of myogenesis inhibitors on the level of p112. Treatment of cells with phloretin or BrdUrd resulted in a decrease in p112 level and in the eventual abolition of myogenesis; however, phlorizin had only minimal effects on these two processes (Table 3). The levels of the ecto-protein kinase and the 112 kDa protein were decreased by 26% and 47% respectively in these myogenesis-impaired cells (Table 4). It should be noted that these proteins were affected to different extents. The decreases in these proteins could account for the lowered level of p112 in these cells. Thus the levels of ecto-protein kinase, the 112 kDa protein and p112 seem to reflect the cells' eventual ability to form myotubes.

The second approach relied on the use of a conditional myogenesis-defective mutant (Chen & Lo, 1989). When grown under myogenesis-competent conditions (Table 2), both mutant D1 (grown in 1% horse serum) and its parental L6 cells (grown in 10% horse serum) contained similar levels of p112 (Table 2). However, only 26% of the normal p112 level was observed when the mutant was grown under myogenesis-incompetent conditions (i.e. in 10% horse serum) (Table 2). This again demonstrates the relationship between the p112 level in undifferentiated myoblasts and the cells' eventual ability to differentiate.

The relationship between myogenesis and p112 was further explored using isogenic mutants defective in the 112 kDa protein. Even though mutant D1/S4 exhibited normal ecto-protein kinase activity (Fig. 5), only a residual amount of p112 could be detected in this mutant. Thus, in agreement with previous findings (D'Amore & Lo, 1988), mutant D1/S4 was altered in the structural or regulatory gene for the 112 kDa protein. More importantly, this mutant was also impaired in myogenesis (Fig. 4). It is therefore conceivable that myogenesis may be dependent on the presence of the 112 kDa protein.

The other mutant that exhibited very low levels of p112 was mutant F72 (Fig. 3). Immunoblotting studies showed that this mutant contained the normal amount of the 112 kDa protein (D'Amore & Lo, 1988). This mutant was also unable to phosphorylate the 75 kDa horse serum protein (Fig. 5) and the 112 kDa protein (Fig. 3) and thus it might be altered in the structural or regulatory gene for the ecto-protein kinase. The fact that this mutant is also impaired in myogenesis (Fig. 4) suggests that myogenesis may also be dependent on ecto-protein kinase.

Since mutants D1/S4 and F72 exhibited different levels of the 112 kDa protein and the ecto-protein kinase, and they were selected by completely different methods from rat L6 myoblasts (D'Amore *et al.*, 1986a; D'Amore & Lo, 1988), it was unlikely that they were defective in the same structural gene or the same regulatory element. The finding that both mutants are defective in myogenesis suggests that the genetic elements that are defective in these mutants are involved in myogenesis. Thus the 112 kDa protein and the ecto-protein kinase may directly or indirectly be associated with the myogenic pathway. We have recently shown that the expression of various myogenic factors and muscle-specific enzymes was suppressed similarly in both mutants D1/S4 and F72, thus suggesting that they are altered at the same or closely related sites (S. R. Chen & T. C. Y. Lo, unpublished work).

Another question that arises from the above studies is whether the decrease in p112 is the cause or the result of the inactivation of myogenesis. We have previously shown that mutant D23 is defective in myogenic differentiation (Kudo & Lo, 1990). The findings that this mutant was unaltered in its ability to phosphorylate both the endogenous 112 kDa protein and the exogenous 75 kDa horse serum protein (Figs. 3 and 5), and that optimal phosphorylation activity was observed with subconfluent L6 myoblasts, suggests that the phosphorylation of the 112 kDa protein is not a secondary event resulting from biochemical differentiation.

To summarize, we have shown in this study that the ecto-protein kinase is able to function efficiently, utilizing the ATP excreted from the cells. The phosphorylation of the cell surface 112 kDa protein by this ecto-protein kinase was affected by the cell density and the developmental stage of the cells. The highest level of p112 was detected in subconfluent mononucleated myoblasts. Studies with myogenesis-defective mutants showed that the expression of ecto-protein kinase and of the 112 kDa protein correlated with the ability to form myotubes. Decreasing the p112 level by myogenesis inhibitors or in myogenesis-incompetent cells resulted in the eventual abolition of myogenesis.

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