Phosphorylation of a cell surface 112 kDa protein by an ecto-protein kinase in rat L6 myoblasts

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Studies with subconfluent day 2 cultures of rat myoblasts revealed that a cell surface 112 kDa protein could be phosphorylated by extracellular ATP. Analysis of the phosphorylated 112 kDa protein suggested the involvement of a serine protein kinase. The following evidence indicated the cell surface location of this protein kinase: (i) extracellular ATP was unable to penetrate the cell membrane under our experimental conditions; (ii) the phosphorylated protein profile of intact cells differed significantly from that of broken cells; (iii) the phosphorylation of the 112 kDa protein could be abolished by pretreatment of cells with very low concentrations of trypsin; (iv) the phosphorylated 112 kDa protein could be dephosphorylated by exogenously added alkaline phosphatase; (v) the phosphorylation of the 112 kDa protein was inhibited by exogenously added proteins; and (vi) exogenously added proteins could be phosphorylated by intact cells under similar experimental conditions. The phosphorylated 112 kDa protein was detected only when the reaction was carried out in the presence of Ca^{2+} , Mg^{2+} , and F^{-} ions. Kinetic analysis revealed that the K_m value of the ecto-protein kinase for ATP was $0.04 \mu\text{M}$, and the V_{max} value for phosphorylation of the 112 kDa protein was 1.67×10^{-4} pmol/min per 10^5 cells. Data presented in the accompanying paper [Chen & Lo (1991) *Biochem. J.* 279, 475–482] show that there was a constant and adequate supply of ATP on the cell surface of rat myoblasts for efficient functioning of this protein kinase, and that mutants defective in either the ecto-protein kinase or the 112 kDa protein were also impaired in myogenic differentiation. This and other biochemical studies suggest that the ecto-protein kinase and the 112 kDa protein might play important roles in myogenic differentiation.

INTRODUCTION

Although ATP is considered largely as an intracellular energy source, extracellular ATP has been shown to play important regulatory roles in differentiation, transduction of external stimuli and cell–cell interaction (Burnstock, 1981; Gordon, 1986; Filipini *et al.*, 1990). Extracellular ATP, even when present in micromolar concentrations, can cause depolarization and changes in membrane permeability in mast cells, mononuclear and polymorphonuclear phagocytes, hepatocytes and a number of transformed cell lines (Becker & Henson, 1975; Weisman *et al.*, 1984; Chahwala & Cantley, 1984; Gomperts, 1985; Charest *et al.*, 1985; Dubyak & De Young, 1985; Steinberg & Silverstein, 1987). Extracellular ATP is thought to interact directly with specific cell surface proteins. Of the ecto-enzymes examined, ecto-protein kinases are the likely candidates for mediating some of the observed phenomena, as phosphorylation can alter the structure and function of proteins in a reversible manner (Kubler *et al.*, 1986).

Ecto-protein kinases, and their substrate proteins, have been detected in a variety of mammalian plasma membranes. They catalyse the transfer of the terminal phosphate of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to endogenous cell surface proteins, as well as to exogenously added macromolecules. Generally speaking, ecto-protein kinases can be classified according to their dependency on cyclic AMP (cAMP). cAMP-dependent ecto-protein kinases have been detected in HeLa cell, Chinese hamster ovary (CHO) cells, mouse lymphoma S49 cells and spermatozoa (Majumder, 1978; Kubler *et al.*, 1989; Dey & Majumder, 1990). Maximum activation of phosphorylation is usually achieved with $5 \mu\text{M}$ -cAMP, whereas similar activation cannot be observed with cyclic GMP. The K_m value of the spermatozoa cAMP-dependent ecto-protein kinase for ATP

is around $500 \mu\text{M}$ (Dey & Majumder, 1990). cAMP-independent ecto-protein kinases are found in transformed 3T3 cells, adipocytes, HeLa cells, neural cells, fibroblasts, macrophages and myoblasts (Mastro & Rozengurt, 1976; Remold-O'Donnell, 1978; Kang *et al.*, 1979; Kubler *et al.*, 1982; Weisman *et al.*, 1984; Ehrlich *et al.*, 1986; Lognonne & Wahrmann, 1986, 1988). They are generally detected using ATP concentrations ranging from 1 to $10 \mu\text{M}$. Some reactions are dependent on Ca^{2+} and/or Mg^{2+} ions. Where determined, the K_m values of these protein kinases for ATP are in the $1\text{--}10 \mu\text{M}$ range (Mastro & Rozengurt, 1976; Kang *et al.*, 1979). In most cases a number of endogenous cell surface proteins, of molecular mass ranging from 40 kDa to 250 kDa, are phosphorylated by these protein kinases. Most of the ecto-protein kinases examined are serine/threonine protein kinases.

A cAMP-independent ecto-protein kinase was detected in a confluent day 4 culture of rat L6 myoblast (Senechal *et al.*, 1982; Lognonne & Wahrmann, 1986, 1988, 1990). Using $5 \mu\text{M}$ -ATP as the substrate, the overall phosphorylation of cell surface proteins of fusion-competent myoblasts was activated 2–4-fold by 2 mM - CaCl_2 ; about 20 proteins, ranging from 15 kDa to 250 kDa, were phosphorylated. Among these proteins, the phosphorylation of a 48 kDa protein was activated markedly in undifferentiated myoblasts and was inhibited significantly in myotubes. The phosphorylation of this protein was hardly detected in non-fusing mutants.

The present paper describes the presence of an ecto-protein kinase present predominantly in subconfluent undifferentiated rat L6 myoblasts. A cell surface 112 kDa protein was consistently phosphorylated by this ecto-protein kinase. The accompanying paper (Chen & Lo, 1991) reveals that mutants defective in either the ecto-protein kinase or the 112 kDa protein are impaired in

Abbreviations used: cAMP, cyclic AMP; CHO cells, Chinese hamster ovary cells; p112, the phosphorylated 112 kDa protein; FCS, fetal calf serum; PMSF, phenylmethanesulphonyl fluoride.

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myogenesis, thus suggesting the possible involvement of these two proteins in myogenic differentiation.

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 (D'Amore & Lo, 1986). Transfers were routinely made every 3 days (before fusion); 0.1 % trypsin was used to detach cells from the plates. Cells were counted with a Coulter Counter. For routine phosphorylation assays, cells were seeded at 8×10^4 cells per 60 mm plate.

Phosphorylation conditions

For routine phosphorylation the following conditions were chosen. Day 2 rat myoblast cultures were washed twice with 5 ml of buffer A [15 mM-Hepes, 5 mM-MgCl₂, 10 mM-CaCl₂, 8 g of NaCl/litre, 0.5 mM-phenylmethanesulphonyl fluoride (PMSF) and 2 µg of leupeptin/ml. Cells were then incubated with 3 ml of buffer A for 30 min. After incubation, buffer A was replaced with 1.2 ml of phosphorylation buffer (buffer A with 0.03 µM-[γ-³²P]ATP and 2 mM-NaF). Phosphorylation was allowed to proceed for 8 min. The phosphorylation reaction was terminated by washing the cells once with 5 ml of buffer A containing 10 µM-ATP, and then 2 × 5 ml of buffer A. Cells were then lysed with 0.3 ml of lysis buffer (10 mM-Tris/HCl, pH 7.5, 0.15 M-NaCl, 1 % Triton X-100, 1 mM-EGTA, 2 µg of leupeptin/ml and 0.5 mM-PMSF); 0.1 ml of 4 × SDS sample buffer was then added to each sample. After boiling for 5 min., samples were loaded on to an SDS/8.5 % polyacrylamide gel. In each set of experiments the same amount of protein was loaded on to each lane. The molecular mass markers used were: phosphorylase b, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soya bean trypsin inhibitor, 20 kDa; α-lactalbumin, 14.4 kDa. Protein bands were stained with Coomassie Blue. After drying, the gel was exposed to a Kodak X-OMAT AR film. All experiments were repeated at least three times. The level of p112 (the phosphorylated 112 kDa protein) was found to be very consistent in all cases.

The kinetic properties of the ecto-protein kinase in phosphorylating the 112 kDa protein were determined using [α-³²P]ATP at concentrations ranging from 2.3 to 20 nM; the same amount of radioactivity was added to different concentrations of unlabelled ATP. The reaction was carried out at 21 °C. After separation by SDS/PAGE, the 112 kDa band was excised from the gel and digested with 250 µl of 30 % H₂O₂ at 80 °C for 4 h. The amount of radioactivity was determined by liquid scintillation counting in scintillation fluid supplemented with 0.7 % acetic acid. The levels of p112 at 2, 3, 4 and 5 min after the addition of [γ-³²P]ATP were used to calculate the rate of phosphorylation. The data presented are results of representative experiments which were repeated at least twice.

Analysis of phosphoamino acids

Phosphoamino acid analysis was carried out as described by Litchfield & Ball (1987), with some modifications. After separation by SDS/PAGE, the [³²P]-labelled 112 kDa protein was excised from the gel. This was then incubated with shaking in 3 ml of 50 mM-ammonium bicarbonate containing 0.1 % SDS and 5 % 2-mercaptoethanol for 16 h at 37 °C. The solubilized protein was precipitated in the presence of 10 % trichloroacetic acid and 12 µg of BSA/ml. The precipitated protein was washed with ice-cold 80 % ethanol and then hydrolysed in 6 M-HCl for 2 h at 110 °C. The hydrolysed sample was then lyophilized and

washed twice with distilled water. After drying, the hydrolysate was dissolved in 10 µl of pH 1.9 buffer [2.5 % formic acid (88 % stock) and 7.8 % glacial acetic acid] containing unlabelled phosphoamino acids (phosphoserine, phosphothreonine and phosphotyrosine at 0.15 mg/ml each). The sample was then applied to a 10 cm × 10 cm cellulose-coated t.l.c. plate. The sample was electrophoresed in the first dimension using the pH 1.9 buffer towards the anode at 1250 V for 25 min. After this, the sample was electrophoresed in the second dimension using pH 3.5 buffer (0.5 % pyridine and 5.0 % glacial acetic acid) at 1250 V for 15 min. The plate was then dried, stained with ninhydrin (0.2 % in acetone) and developed in a 55 °C oven for 1 h to visualize standard phosphoamino acids, and then exposed to a Kodak X-OMAT AR film to detect the ³²P-labelled phosphoamino acids. The experiment was repeated three times.

Alkaline phosphatase treatment

Cells were phosphorylated with [γ-³²P]ATP under standard conditions. After washing with buffer A, cells were treated with or without alkaline phosphatase for 1 or 2 min. After this, cells were washed once with 5 ml of buffer A, and then lysed with 0.3 ml of the lysis buffer. After boiling with SDS sample buffer for 5 min, samples were subjected to SDS/PAGE. After drying, the gel was exposed to a Kodak X-OMAT AR film. The level of p112 was then determined by densitometry.

Hydroxylamine treatment

Hydroxylamine treatment was used to determine the presence of an acyl phosphate linkage in the ³²P-labelled 112 kDa protein (Hokin *et al.*, 1965). After separation by SDS/PAGE, the ³²P-labelled 112 kDa protein was excised from the gel and incubated with shaking in 3 ml of 50 mM-ammonium bicarbonate containing 0.1 % SDS and 5 % 2-mercaptoethanol for 16 h at 37 °C. The solubilized protein was then precipitated with 10 % trichloroacetic acid containing 12 µg of BSA/ml. The precipitated protein was washed with ice-cold 80 % ethanol and dissolved in 0.1 ml of water. It was then incubated in 0.2 ml of 0.1 M-acetate buffer (pH 5.4) containing 0.8 M-hydroxylamine at room temperature for 10 min. Hydroxylamine was prepared in the cold just before use by adding 2 parts of 8 M-NaOH to 5 parts of 4 M-hydroxylamine hydrochloride. The control sample was treated with 0.64 M-NaCl instead of hydroxylamine. After the reaction, samples were subjected to SDS/PAGE. The amount of radioactivity associated with the control and treated 112 kDa protein was determined as described above.

Materials

[α-³²P]ATP and [γ-³²P]ATP were purchased from ICN Biochemicals. Hydroxylamine hydrochloride, alkaline phosphatase and trypsin were purchased from Sigma Chemical Co. All other chemicals were obtained from commercial sources and were of the highest available purity.

RESULTS

Phosphorylation of a 112 kDa protein by extracellular ATP

When a day 2 culture of rat L6 myoblasts, originally seeded at 8×10^4 cells per 60 mm plate, was incubated with 0.03 µM-[γ-³²P]ATP at 21 °C in the phosphorylation buffer, a significant amount of radioactivity was found to be associated with these cells, even after washing with unlabelled ATP (10 µM). Analysis of these cells by SDS/PAGE revealed that a 112 kDa protein was consistently labelled by the [γ-³²P]ATP (Fig. 1). The phosphorylated 48 kDa protein found in confluent rat myoblasts (Lognonne & Wahrman, 1986, 1988, 1990) could not be detected in the present study. A number of proteins ranging from 40 to 50 kDa

Fig. 1. Phosphorylation of a 112 kDa protein by extracellular ATP
The phosphorylation of cell surface proteins by extracellular ATP was carried out as described in the text. The concentration of [γ - 32 P]ATP used was 0.03 μ M. The phosphorylation reaction was carried out in the presence of 10 mM- CaCl_2 , 5 mM- MgCl_2 and 2 mM-NaF. This Figure shows the rate of phosphorylation of the 112 kDa protein. The numbers on top of each lane indicate the duration (min) of the reaction. The molecular mass markers used were: phosphorylase b, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa.

Fig. 3. Nature of the phosphorylation reaction
The phosphorylation reaction was carried out as described in the text, for a duration of 8 min. (a) Effect of preincubation of the cells in the presence of protease inhibitors. The reaction was carried out at 21 $^{\circ}\text{C}$. Lane 1, phosphorylation of the cells without preincubation in buffer A; lane 2, phosphorylation of the cells preincubated for 30 min in buffer A lacking any protease inhibitors; lane 3, phosphorylation of the cells preincubated for 30 min in buffer A containing 0.5 mM-PMSF and 2 μ g of leupeptin/ml. (b) Effect of temperature on phosphorylation. These experiments were carried out with cells preincubated for 30 min in buffer A. Lanes 1, 2 and 3 indicate reactions carried out at 4, 21 and 37 $^{\circ}\text{C}$ respectively. (c) Phosphorylation reaction using [α - 32 P]ATP (lane 1) and [γ - 32 P]ATP (lane 2).

Fig. 2. Requirement of Ca^{2+} , F^{-} and Mg^{2+} ions for phosphorylation of the 112 kDa protein
Phosphorylation studies were carried out under standard conditions, except that the concentrations of Ca^{2+} , F^{-} and Mg^{2+} were varied as indicated. In all cases the reaction was carried out at 21 $^{\circ}\text{C}$ for 8 min. (a) Effect of varying CaCl_2 concentration. Lanes 1, 2, 3 and 4 indicate reactions carried out in the presence of 0, 1, 10 and 20 mM- CaCl_2 respectively. The concentrations of MgCl_2 and NaF were maintained at 5 mM and 2 mM respectively. (b) Requirement for MgCl_2 for phosphorylation. Lanes 1, 2, 3 and 4 indicate phosphorylation carried out in the presence of 0, 1, 5 and 10 mM- MgCl_2 respectively. The concentrations of CaCl_2 and NaF were maintained at 10 mM and 2 mM respectively. (c) Effect of fluoride on the phosphorylation of the 112 kDa protein. Lanes 1, 2, 3 and 4 indicate reactions carried out in the presence of 0, 1, 2 and 3 mM-NaF respectively. The concentrations of CaCl_2 and MgCl_2 were maintained at 10 mM and 5 mM respectively. The molecular mass markers used were as in Fig. 1.

phorylated 112 kDa protein (p112) remained relatively constant within each set of experiments. Thus only the level of p112 was monitored in this investigation.

Unlike with other ecto-protein kinases, the phosphorylation of the 112 kDa protein by extracellular ATP was detectable only when the reaction was carried out in the presence of Ca^{2+} , F^{-} and Mg^{2+} (Fig. 2) (Chang *et al.*, 1974; Mastro & Rozengurt, 1976; Kubler *et al.*, 1982, 1989). The requirements for various ions were tested over a range of concentrations. The optimal concentrations of Ca^{2+} , F^{-} and Mg^{2+} required were 10 mM, 2 mM and 5 mM respectively. In three separate experiments, phosphorylation of the 112 kDa protein could not be observed in reactions carried out in 0, 1 or 20 mM- Ca^{2+} ; phosphorylation could be observed only at 10 mM- Ca^{2+} . The absence of p112 at higher Ca^{2+} and F^{-} concentrations was probably due to precipitation of CaF_2 . The stringent Ca^{2+} requirement was not observed with intracellular protein kinases. It should be noted that a Ca^{2+} concentration in the millimolar range was also required for optimal activation of the ecto-protein kinase found in confluent rat myoblasts (Lognonne & Wahrmann, 1986). It is presently not certain whether NaF is required to inhibit dephosphorylation of p112 by phosphatases or to activate the ecto-protein kinase involved. The level of p112 was increased by pretreatment of cells with protease inhibitors such as leupeptin and PMSF for 30 min (Fig. 3a). This suggested that the rate of degradation of the protein was relatively fast. The extent of phosphorylation was found to increase with temperature (Fig 3b). Subsequent experiments were carried out at 21 $^{\circ}\text{C}$, even though the p112 level was higher at 37 $^{\circ}\text{C}$; this was intended to minimize the possibility of internalization of ATP.

Nature of p112

Attempts were made to determine the nature of the association of phosphate with the 112 kDa protein. Three pieces of evidence

were also phosphorylated. However, these proteins were not consistently phosphorylated, and the extent of phosphorylation of these lower molecular mass proteins varied considerably from experiment to experiment, whereas the level of the phos-

Fig. 4. Phosphoamino acids from the phosphorylated 112 kDa protein

After separation by SDS/PAGE the ^{32}P -labelled 112 kDa protein was excised from the gel. After elution from the gel the solubilized protein was hydrolysed and the phosphoamino acids were then separated by two-dimensional electrophoresis on t.l.c. plates, as described in the Materials and methods section. After electrophoresis the plate was dried, stained with ninhydrin to visualize standard phosphoamino acids and then exposed to a Kodak X-OMAT AR film to detect the ^{32}P -labelled phosphoamino acids. The standard phosphoamino acids used were phosphoserine (PS), phosphothreonine (PT) and phosphotyrosine (PY). 0, 1 and 2 denote the origin, the direction for the first dimension and the direction for the second dimension of electrophoresis respectively.

Fig. 5. Kinetics of phosphorylation of the 112 kDa protein

The phosphorylation of cell surface proteins by extracellular ATP was carried out under standard conditions as described in the text. The reaction was carried out at 21 °C. (a) Phosphorylation of the 112 kDa protein as a function of time. The concentration of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ used was 0.03 μM . After separation by SDS/PAGE the gel was then exposed to a Kodak X-OMAT AR film, and the level of the phosphorylated 112 kDa protein was quantified by densitometry. Autoradiograms used were within the linear range of the film. (b) Kinetics of the phosphorylation reaction. The methods used for determining the kinetic properties of the ecto-protein kinase were as described in the text. The rate of phosphorylation (v) is expressed in pmol/min per 10^5 cells. $[S]$ refers to the concentrations of ATP (in μM) used. The data presented are results of representative experiments which were repeated at least twice. Results were found to be very consistent in all cases.

Fig. 6. Integrity of the rat L6 myoblast plasma membrane

The integrity of the plasma membrane of the rat L6 myoblasts was evaluated by the permeability of the cells to the fluorescent dye ethidium bromide. Rat myoblast monolayers were first washed with buffer A, and then incubated with buffer A containing 0.5 μg of ethidium bromide/ml for 10 min. The presence of ethidium bromide inside the cells was then examined using a fluorescent microscope. Panels (a) and (c) show the cell morphology (as shown by phase-contrast microscopy) of untreated myoblasts and myoblasts treated with 0.01 % Triton X-100 for 5 min respectively. Panels (b) and (d) show the staining of the nuclei by ethidium bromide in untreated myoblasts and in myoblasts permeabilized with 0.01 % Triton X-100 respectively.

indicated that p112 was not formed by tight binding of the entire ATP molecule to the 112 kDa protein. First, the 112 kDa protein could not be labelled with $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (Fig. 3c). Secondly, after phosphorylation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, the radioactivity associated with p112 could not be displaced with 10 μM non-radioactive ATP. Thirdly, the radioactivity associated with p112 remained attached to the protein even after SDS/PAGE.

Acyl phosphate was found to be a phosphorylated intermediate upon incubation of brain $(\text{Na}^+, \text{K}^+)\text{-dependent ATPase}$ with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Hokin *et al.*, 1965). Attempts were therefore made to determine the presence of acyl phosphate in p112. In fact, the following evidence precludes the presence of acyl phosphate in the p112 examined. First, before separation by SDS/PAGE, the sample was boiled in SDS sample buffer for 5 min. This treatment would destroy any acyl phosphate bonds (Hokin *et al.*, 1965; Mastro & Rozengurt, 1976). Secondly, treatment of p112 with 0.8 M-hydroxylamine, pH 5.4, for 30 min did not decrease the amount of $[\text{P}]\text{P}_i$ incorporated into p112. The hydroxylamine-treated p112 retained 95 % of the $[\text{P}]\text{P}_i$ associated with p112.

Further characterization of the phosphorylation reaction involved identification of the amino acid phosphorylated. After elution from the SDS/polyacrylamide gel, p112 was precipitated with cold trichloroacetic acid, washed, lyophilized and hydrolysed in 6 M-HCl for 2 h at 110 °C. The identity of the phosphoamino acid was then determined by two-dimensional electrophoresis on t.l.c. plates. Serine was the only amino acid phosphorylated (Fig. 4); this therefore suggested the involvement of a serine protein kinase in the phosphorylation of the 112 kDa protein.

Kinetic properties of the phosphorylation reaction

The rate of phosphorylation of the 112 kDa protein by the ecto-protein kinase remained linear for at least 8 min (Fig. 5a). Using different ATP concentrations, the levels of p112 at 2, 3, 4 and 5 min after the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were used to calculate the rate of phosphorylation (Fig. 5b). Since the reaction was

Fig. 7. Phosphoprotein profiles of intact and permeabilized cells

Cells were first permeabilized by treatment with 1% Triton X-100 for 5 min or with 10 µg of trypsin/ml for 30 min. Cell surface phosphorylation studies were then carried out under standard conditions, as described in the text. The reaction was carried out for 8 min at 21 °C. The concentration of [γ - 32 P]ATP used was 0.03 µM. This figure shows the profiles of phosphorylated proteins in intact cells (lanes 1), in cells previously lysed by 1% Triton X-100 (a, lane 2) and in cells permeabilized with 10 µg of trypsin/ml (b, lane 2).

carried out at 21 °C, the amount of ATP secreted by the cells should be negligible during this very short period of time. The apparent K_m and V_{max} values for the phosphorylation of the 112 kDa protein were 0.04 µM and 1.67×10^{-4} pmol/min per 10^5 cells respectively. In other words, 1×10^3 molecules of the 112 kDa protein per cell could be phosphorylated in 1 min under our experimental conditions. Although the kinetic properties of the previously described rat myoblast ecto-protein kinases have not been determined (Senechal *et al.*, 1982; Lognonne & Wahrman, 1986, 1988), the present enzyme has significantly higher affinity for ATP than ecto-protein kinases found in other cell types (Mastro & Rozengurt, 1976; Kang *et al.*, 1979; Dey & Majumder, 1990).

Profiles of the phosphorylated proteins

Quite a number of intracellular protein kinases have been characterized; some are activated by cAMP or cGMP, whereas others do not require specific ions for their activities (Krebs, 1986; Edelman *et al.*, 1987; Kikkawa *et al.*, 1989). Thus if [γ - 32 P]ATP can enter the cells, a large number of proteins should be phosphorylated; this profile should be distinct from that brought about by the ecto-protein kinase. Phosphorylation studies were therefore carried out with intact whole cells and with cells that had been permeabilized with 1% Triton X-100 or trypsin (10 µg/ml). When grown under our experimental conditions, rat L6 myoblasts were normally not permeable to ethidium bromide (Fig. 6b). These cells were rendered permeable to this fluorescent dye only after treatment with Triton X-100 (Fig. 6d). The phosphoprotein profiles of intact and permeabilized cells differed significantly (Fig. 7). Whereas the 112 kDa protein was specifically phosphorylated in intact cells, a large number of proteins were phosphorylated in the permeabilized cells. This indicated that the exogenously added ATP could not enter intact cells. In agreement with this finding, cAMP and cGMP (which should activate a number of intracellular protein kinases) did not alter the phosphoprotein profile generated by the exogenously added [γ - 32 P]ATP (results not shown). In other words, the exogenously added ATP could not gain access to the intracellular cyclic-nucleotide-dependent protein kinases. A comparison of the

Fig. 8. Effect of exogenously added FCS on the phosphorylation of the 112 kDa protein

Cell surface phosphorylation assays were carried out under standard conditions, except that 3 µl of heat-inactivated FCS was included in 1.2 ml of the phosphorylation buffer. (a) Effect of heat-inactivated FCS on the phosphorylation of the 112 kDa protein. Lanes 1, 2, 3 and 4 show the effect of 0%, 0.05%, 0.1% and 0.5% FCS on the 112 kDa protein phosphorylation. (b) Phosphorylation of FCS proteins upon incubation with the cells. After phosphorylation, 280 µl of the supernatant from each well was added to 93 µl of 4× SDS sample buffer. After boiling in SDS sample buffer for 5 min, 100 µl of this sample was applied to the SDS/polyacrylamide gel. Lanes 1, 2, 3 and 4 show the phosphorylation of a 55 kDa FCS protein by cells incubated with 0%, 0.05%, 0.1% and 0.5% FCS respectively.

phosphoprotein profiles at different temperatures also revealed that ATP did not enter the cells at 37 °C (Fig. 3b).

Inhibition of cell surface phosphorylation by exogenously added proteins

If the catalytic site of the protein kinase is accessible to the external environment, then exogenously added proteins may compete with the 112 kDa protein for this site, thus preventing its phosphorylation. Exogenously added heat-inactivated fetal calf serum (FCS) was found to inhibit phosphorylation of the 112 kDa protein (Fig. 8a). Since FCS could not dephosphorylate p112 when added to the phosphorylated cells (X. Y. Chen & T. C. Y. Lo, unpublished work), the heat-inactivated FCS was not likely to contain phosphatase activity. As shown in Fig. 8(b), the exogenously added FCS proteins were phosphorylated by [γ - 32 P]ATP, albeit not very efficiently, when incubated in the presence of the cells. Similarly, a 75 kDa horse serum protein could also be phosphorylated by the ecto-protein kinase (Chen & Lo, 1991). These findings indicate that the catalytic site of the protein kinase is accessible to the external environment.

Effect of exogenously added hydrolytic enzymes

Information on the location of p112 can be obtained through the use of non-penetrating modifying reagents. Mild trypsin treatment has been shown to abolish cell surface phosphorylation of 3T3 cells (Mastro & Rozengurt, 1976). In the present study, cells were treated with trypsin prior to the phosphorylation reaction (Table 1). Treatment with 0.1 µg or 0.5 µg of trypsin/ml for 30 min lowered the amount of p112 by 23% and 54% respectively. This indicated that the 112 kDa protein and/or the

Table 1. Effect of exogenously added hydrolytic enzymes on cell surface phosphorylation

In the case of trypsin treatment, cells were preincubated with different concentrations of the enzyme for 30 min before the phosphorylation reaction. After washing, cell surface phosphorylation was then carried out under standard conditions. Alkaline phosphatase treatment was carried out with cells that had been subjected to the phosphorylation reaction. After washing with buffer A, the treated cells were lysed and processed by standard procedures. After separation by SDS/PAGE, the gel was then exposed to a Kodak X-OMAT AR film and the level of p112 was determined by densitometry. The data presented are means of three separate experiments; results were very consistent in all cases.

Enzymes	Enzyme concentration ($\mu\text{g/ml}$)	Incubation period (min)	p112 detected (%)
Trypsin	—	30	100
	0.1	30	77
	0.5	30	46
Alkaline phosphatase	—	1	100
	0.1	1	69
	0.1	2	51
	1.0	1	21

Fig. 9. The 112 kDa protein and the ecto-protein kinase are not loosely bound peripheral proteins in the cell surface

Rat L6 myoblasts were first washed with different solutions for 5 min to dissociate loosely bound cell surface peripheral proteins and then the cells were phosphorylated and processed under standard conditions. Lanes 1, 2, 3 and 4 indicate cells washed with 1 M-NaCl, control cells, cells washed with 0.4 M-glycine buffer (pH 2.0) and cells washed with 0.5 M-borate buffer (pH 10.0) respectively.

Nature of the cell surface 112 kDa protein and the ecto-protein kinase

Having determined the surface location of the phosphorylation site of the 112 kDa protein, the next question concerns the nature of the ecto-protein kinase and the 112 kDa protein. If either one of these proteins is a peripheral protein, then removal of such proteins should decrease the level of p112. We have previously shown that peripheral proteins on rat myoblasts could be removed by treatment with 0.4 M-glycine buffer (pH 2.0), 0.5 M-borate buffer (pH 10) or 1 M-NaCl (Lo & Duronio, 1984b). As shown in Fig. 9, such treatments had no effect on the level of p112. This implied that the 112 kDa protein and the ecto-protein kinase are very tightly bound peripheral proteins, amphitropic proteins or integral proteins (Burn, 1988; Ferguson & Williams, 1988).

It was interesting to note that the 112 kDa protein was phosphorylated to a greater extent in cells previously permeabilized with Triton-X 100 or 10 μg of trypsin/ml (Fig. 7). This increase in p112 was accompanied by increased phosphorylation of many other proteins. This suggested that the above-mentioned 112 kDa protein might be a transmembrane protein. However, it is also possible that another protein with a similar mobility on the SDS/polyacrylamide gel might be phosphorylated on the inner membrane surface.

DISCUSSION

The present investigation reports the phosphorylation of a rat myoblast cell surface protein by extracellular ATP. Upon incubation of undifferentiated rat myoblasts with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 112 kDa protein was consistently phosphorylated under our experimental conditions (Figs. 1 and 2). The phosphorylation of this protein was detected with very low ATP concentrations (0.03 μM), and with myoblasts grown at relatively low density (around 10^5 cells per 60 mm plate).

The following findings suggest that the 112 kDa protein was phosphorylated by extracellular $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. First, phosphorylation reactions were carried out with 0.03 μM -ATP for 8 min at 21 °C. Under these conditions, the negatively charged ATP should not penetrate the plasma membrane. Secondly, the intracellular ATP concentration in rat L6 myoblasts is around 24 mM (D'Amore & Lo, 1986; Chen & Lo, 1991). If there was no compartmentation of the intracellular ATP, extracellular ATP (0.03 μM) would be unlikely to diffuse against its concentration gradient into the cells. Thirdly, p112 could not be radioactively labelled by 0.03 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ when 10 μM unlabelled ATP was included in the phosphorylation buffer (results not shown). Thus, even if $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ could enter the cytoplasm, the specific radioactivity of ATP would be so low that specific labelling should not be observed (assuming equilibration of the newly internalized ATP with the intracellular ATP pool). It should be noted that the above findings do not eliminate the possibility that ATP might be transported across the membrane by specific proteins, thus making it accessible to the intracellular protein kinases (Krebs, 1986; Taylor *et al.*, 1990). However, the phosphoprotein profiles of intact and permeabilized cells (Fig. 7), and the lack of effect of cyclic nucleotides, suggest that the exogenously added ATP was unable to enter the cells.

The next question concerns the association of $[\text{P}^{32}]\text{P}_i$ with the 112 kDa protein. For the following reasons, our data precluded the mere binding of ATP to the 112 kDa protein and the formation of an acyl phosphate linkage in p112. (i) Whereas binding of ATP to its receptors should normally be completed in less than 1 min, the rate of phosphorylation of the 112 kDa protein remained linear for at least 8 min (Fig. 5). (ii) If the whole ATP

ecto-protein kinase were exposed on the cell surface, even before the phosphorylation reaction.

If p112 is exposed on the cell surface, then it is likely to be dephosphorylated by exogenously added alkaline phosphatase. After the phosphorylation reaction, cells were treated with 0.1 or 1 μg of alkaline phosphatase/ml for 1 or 2 min. As shown in Table 1, 0.1 μg of alkaline phosphatase/ml decreased the phosphorylation of the 112 kDa protein by 31% and 49% in 1 and 2 min respectively, whereas 1 μg of alkaline phosphatase/ml dephosphorylated 79% of p112 in 1 min. This shows that the phosphoamino acids in p112 are accessible to the exogenously added alkaline phosphatase.

molecule became bound to the protein, [α - 32 P]ATP should be just as effective as [γ - 32 P]ATP in labelling the protein; however, [α - 32 P]ATP was in fact unable to label the 112 kDa protein (Fig. 3). (iii) Non-covalently bound ATP would be dissociated from the protein by SDS/PAGE, HCl hydrolysis or trichloroacetic acid precipitation. Obviously, this was not the case (Fig. 4). (iv) The amount of [32 P]P_i associated with p112 was not affected by boiling in SDS sample buffer or by hydroxylamine treatment (Hokin *et al.*, 1965). This suggested that an acyl phosphate linkage was not present in the p112 excised from the gel. The nature of the phosphorylation reaction was revealed by phospho-amino acid analysis, which indicated the possible involvement of a serine protein kinase (Fig. 4).

A direct indication of the location of the phosphorylation sites come from studies using exogenously added macromolecules. First, the phosphorylation of the 112 kDa protein could be inhibited by heat-inactivated FCS (Fig. 8). Secondly, a 55 kDa FCS protein and a 75 kDa horse serum protein could be phosphorylated by rat myoblasts upon incubation with [γ - 32 P]ATP (Fig. 8; Chen & Lo, 1991). Unlike histone or phosvitin (Chiang *et al.*, 1979; Kubler *et al.*, 1989), the serum proteins should not disrupt the cell membrane, as they are normally included in growth medium (Klip *et al.*, 1982; Lo & Duronio, 1984a; Mesmer & Lo, 1989). These studies showed that the phosphorylation reaction must occur on the cell surface. This conclusion was corroborated by experiments using exogenously added hydrolytic enzymes. First, the level of p112 was decreased by pretreatment of the cells with very low trypsin concentrations (0.1–0.5 μ g/ml) (Table 1). Since the rat myoblast plasma membrane was not disrupted at these trypsin concentrations (Lo & Duronio, 1984b; D'Amore *et al.*, 1986), only cell surface proteins should be affected. Secondly, treatment of the phosphorylated cells with alkaline phosphatase resulted in a significant decrease in 32 P-labelled p112 (Table 1). Since alkaline phosphatase cannot penetrate the membrane, the phosphorylated serine residues of the 112 kDa protein must be exposed to the external environment.

Although the above findings show that the catalytic site of the protein kinase is accessible to the external environment, it remains to be proven that the protein kinase itself normally resides on the cell surface. It is possible that this protein kinase may be a cytoplasmic enzyme released from damaged cells which binds to the surface of intact cells after its release. The following findings suggest that this is not likely to be the case. First, the level of p112 was not altered by prewashing cells with high-salt, acidic or alkaline buffers, thus suggesting that the protein kinase was not bound to the cell surface by ionic interaction (Fig. 9). Secondly, the protein kinase involved exhibited properties distinct from those of the intracellular protein kinases. Thirdly, if the protein kinases were released from damaged cells, their activities should increase with cell density, thus leading to an elevated p112 level in confluent cultures. The accompanying paper describes that the level of p112 actually decreases with increasing cell density (Chen & Lo, 1991). Fourthly, cell lysis could not be detected in day 2 cultures of rat L6 myoblasts (Fig. 6), which exhibited significant phosphorylation of the 112 kDa protein. Fifthly, a comparison of the ATP, AMP and ADP concentrations in the extra- and intra-cellular spaces precluded the occurrence of cell lysis during normal growth conditions (Chen & Lo, 1991). The above findings therefore suggest that the protein kinase involved in phosphorylation of the 112 kDa protein is not normally a cytoplasmic enzyme.

Kinetic analysis of the phosphorylation of the 112 kDa protein indicated that the K_m value for ATP was 0.04 μ M and the V_{max} value for p112 was 1.67×10^{-4} pmol/min per 10^6 cells (Fig. 5). The K_m value for ATP was significantly different from those of

the cyclic-nucleotide-independent ecto-protein kinases found in 3T3 cells, adipocytes, HeLa cells, fibroblasts and macrophages (Mastro & Rozengurt, 1976; Remold-O'Donnell, 1978; Chiang *et al.*, 1979; Kang *et al.*, 1979; Kubler *et al.*, 1982), and also from those of the cyclic-nucleotide-dependent ecto-protein kinases found in spermatozoa (Dey & Majumder, 1990). A comparison of the K_m value of the ecto-protein kinase and the cell surface ATP concentrations in rat myoblasts suggested that there was a constant and adequate supply of ATP for efficient functioning of this ecto-protein kinase (Chen & Lo, 1991).

Similar to those present in normal and transformed fibroblasts (Mastro & Rozengurt, 1976) and in HeLa cells (Kubler *et al.*, 1982), the ecto-protein kinase examined in the present study is a phosphoserine protein kinase. Whereas ten or more proteins were phosphorylated by the ecto-protein kinases present in 3T3 cells, macrophages, HeLa cells and adipocytes (Mastro & Rozengurt, 1976; Remold-O'Donnell, 1978; Kang *et al.*, 1979; Chiang *et al.*, 1979; Kubler *et al.*, 1982), only the 112 kDa protein was consistently phosphorylated by the rat myoblast ecto-protein kinase. It should be noted that much higher ATP concentrations (ranging from 0.5 to 50 μ M) were used in previous studies.

The ecto-protein kinase examined in the present study also differed from the previously reported rat myoblast ecto-protein kinase (Lognonne & Wahrmann 1986, 1988, 1990). The latter was detected in day 4–day 6 cultures (confluent monolayers) using 5 μ M-ATP, whereas the present ecto-protein kinase was detected in the sub-confluent day 2 cultures using 0.03 μ M-ATP. Whereas more than 20 proteins (ranging from 15 kDa to 250 kDa) were phosphorylated by the ecto-protein kinase from confluent cells, only the 112 kDa protein was consistently phosphorylated by the ecto-protein kinase from subconfluent cells. The latter protein kinase was dependent on F⁻ either for activation or for inhibition of phosphatases, whereas the former did not require F⁻ for its activity. On the other hand, both rat myoblast ecto-protein kinases were cyclic-nucleotide-independent phosphoserine protein kinases, and required Mg²⁺ and Ca²⁺ for the activities.

To summarize, a cell surface phosphoserine protein kinase has been found in subconfluent rat L6 myoblasts. This enzyme is involved in the phosphorylation of a cell surface 112 kDa protein. The following paper shows that mutants defective in either one of these two proteins are also defective in myogenic differentiation (Chen & Lo, 1991).

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