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Cell Cycle and Cyclic AMP-Dependent Phosphorylation of Plasma Membrane Proteins p14 and p24: Defects in Smooth Surface Transformed Cells

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Two proteins which are localized to the cytoplasmic surface of the plasma membrane, p14 and p24, undergo cyclic AMP-dependent phosphorylation in rapidly growing nontransformed murine embryo cells. In this cell system, growth arrest in the G_1 phase of the cell cycle induced by growth factor deprivation is associated with the reversible loss in ability to phosphorylate these substrates. By contrast, Simian virus 40 and methylcholanthrene transformed cells show both defective G_1 growth control and defects in their ability to phosphorylate p14 and p24 under all tested growth conditions. These data suggested a correlation between defects in the physophorylation of p14 and p24 and defects in the ability of transformed cells to G_1 growth arrest. The results of the current studies by contrast show that 3T3 T proadipocytes which have been transformed by the smooth surface tumorigenesis method show different characteristics. They retain the ability to G_1 growth arrest in serum-deficient medium. They show cyclic AMP-dependent phosphorylation of p14 and p24 during exponential growth. They do not, however, down regulate p14 and p24 phosphorylation in association with G_1 growth arrest. These observations suggest that neoplastic transformation is not necessarily associated with absolute defects in the ability to phosphorylate p14 and p24. Rather, the results of the current study suggest that the inability to modulate the cyclic AMP-dependent phosphorylation of plasma membrane p14 and p24 proteins during the G_1 phase of the cell cycle may be more tightly associated with neoplastic transformation.

Key words: cell cycle, cytoplasmic plasma membrane surface, control of cell proliferation, proadipocyte stem cells

The phosphorylation of specific plasma membrane proteins appears to serve a significant role in cellular growth control. We have shown that two proteins, p14 and

Abbreviations used: cAMP, 3',5'-cyclic monophosphate; SDS, sodium dodecyl sulfate; SS, smooth surface tumorigenesis.

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p24, which are localized to the cytoplasmic surface of the plasma membrane in 3T3 cells [1; Boman, Zschunke, and Scott, submitted], undergo cyclic AMP-dependent phosphorylation and that growth arrest in the G_1 phase of the cell cycle by growth factor deprivation causes down regulation in the ability of these substrates to be phosphorylated [2]. We have also reported that transformation of 3T3 cells by Simian virus 40 and methylcholanthrene abrogates normal G_1 growth control processes and results in the failure of p14 and p24 to undergo cyclic AMP-dependent phosphorylation [3]. These observations suggested that defective growth control and neoplastic transformation correlate with defects in this plasma membrane phosphorylation system.

It has also been recently reported that some transforming gene products are phosphoproteins which are localized to the cytoplasmic surface of the plasma membrane [4–8]. The avian sarcoma virus gene product $pp60^{src}$ is the best studied of these [9–12]. A variety of other factors have also been shown to stimulate the phosphorylation of plasma membrane proteins in nontransformed cells and their proliferation [13,14]. These results together provide evidence that plasma membrane phosphoproteins are involved in critical processes which regulate cell proliferation.

In this paper we present evidence which supports the conclusion that neoplastic transformation can be associated not only with absolute defects in the phosphorylation of plasma membrane proteins but also with defects in the modulation of plasma membrane protein phosphorylation during the G_1 phase of the cell cycle.

MATERIALS AND METHODS

Cell Culture

Nontransformed BALB/3T3 T proadipocytes provided by Dr. Leila Diamond were used in these studies together with clones of 3T3 T proadipocytes transformed by the smooth surface tumorigenesis (SS) method of Scott and Boone [15]. These clones are designated M3, M4, and M6.

All cells were grown in Dulbecco's modified Eagles medium containing either 10% calf serum or 10% fetal calf serum. Cells were cultured in humidified incubators at 37°C in 5–10% CO_2. No antibiotics were used in the cultures and all cells were periodically shown to be free of mycoplasma contamination by the method of Chen [17].

G_1 Growth Arrest by Serum Deprivation

To induce G_1 growth arrest as a result of growth factor deficiency, cells were first plated at low density; ie $\sim 1 \times 10^4$ cells/cm^2, in 25 cm^2 flasks or in 490 cm^2 roller bottles in DMEM containing 10% serum and allowed to attach and grow for 12–16 hr. They were then washed once in phosphate buffered saline (pH 7.4) containing 0.75 mM Ca^{++} and 0.5 mM Mg^{++} (CMPBS) and refed DMEM containing 0.5% serum. The cells were cultured in this medium for 4 days. Arrest in the G_1 phase of the cell cycle was assayed by measurement of decreased [^{3}H]thymidine incorporation into DNA and by flow microfluorimetric analysis of the DNA content in such cells. The methodology for these assays has been previously described in detail [1–3,16]. Rapidly growing cells were produced by culture in DMEM containing 10% FCS. Such cells were used during exponential growth when at a density of ~ 1–2×10^4 cells/cm^2

Plasma Membrane Isolation

Plasma membranes of 3T3 T and SS-transformed 3T3 T cell clones were isolated by the vesiculation technique previously described by Scott et al [18–20]. Briefly, cells, grown in roller bottles were washed once in CMPBS and then incubated in CMPBS containing 0.1% paraformaldehyde-2 mM dithiothreitol for 2 hr at 37°C to induce the shedding of plasma vesicles. The vesicles were sedimented by centrifugation at 30,000g at 4°C. Purified plasma membrane preparations were isolated following disruption in a Parr nitrogen bomb and sedimented at 250,000g for 1 hr at 4°C.

Plasma Membrane Phosphorylation

The phosphorylation of purified plasma membranes was perfomed in vitro as previously described [1–3]. Briefly, 50–100 μg of plasma membrane protein was phosphorylated in 165 μl of an incubation medium containing: 50 mM β-glycerol phosphate buffer (pH 6.5), 10 mM $MgCl_2$, 10 mM NaF, 2 mM theophylline, and 0.3 mM ethylene glycol bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid. The phosphorylation assay was initiated by addition of [γ-^{32}P]ATP at a final concentration of 5×10^{-5} M ($1–4 \times 10^7$ cpm/tube; specific activity, 22.5 Ci/mmole). In these assays comparisons were made of the extent of phosphorylation in various plasma membrane preparations in the presence of 5×10^{-5} M cyclic AMP. The reaction was stopped when specimens were diluted with ~4 ml 20 mM MES buffer (pH 6.5) at 4°C and sedimented at ~250,000g for 1 hr at 4°C. The results represent the final extent of phosphorylation observed following incubation for 30 min under the conditions described above. These conditions and procedures were employed because previous studies have shown that they are optimal for the detection of plasma membrane phosphoproteins in this system [1–3]. Comparable data were obtained when shorter incubation intervals were employed; the extent of labeling was, however, proportionately reduced, and autoradiographic analysis of the specimens required longer exposure times. In all these reactions phosphorylation of plasma membrane lipids was also observed. In some specimens, lipids were therefore removed by extraction with ethanol-ether (3:1, v/v) at − 20°C for 1–3 hr. In no case did the lipid extraction procedure significantly affect the appearance of p14 and/or p24 in the polyacrylamide gel analysis.

Polyacrylamide Gel Electrophoresis

Phosphorylated plasma membrane preparations were solubilized in 1% sodium dodecyl sulfate, 1 mM EDTA, 8 M urea and 50 mM dithiothreitol prepared in 100 mM phosphate buffer (pH 7.4). This suspension was heated at 90°C for 5 min to promote the dissociation of membrane proteins and then sedimented by centrifugation at 100g to remove debris. Aliquots of the sample (~50 μg) were applied to 7.5% polyacrylamide gels and electrophoresed as described [1–3]. Thereafter the gels were fixed and analyzed by autoradiography.

Materials

DMEM was purchased from GIBCO (Grand Island, NY). Serum was purchased from KC Biologicals (Kansas City, MO). [γ-^{32}P]ATP was purchased from ICN Chemical Radioisotopes Division, Irvine, CA.

RESULTS

Cell Cycle and Cyclic AMP-Dependent Phosphorylation of Plasma Membrane Proteins in Nontransformed 3T3 T Proadipocytes

Two plasma membrane proteins, p14 and p24, undergo cyclic AMP-dependent phosphorylation in exponentially growing nontransformed 3T3 T proadipocytes (Fig. 1). These cells can be growth arrested in the G_1 phase of the cell cycle by culture in serum deficient medium and associated with G_1 growth arrest a marked decrease in p14 and p24 phosphorylation occurs (Fig. 1).

Cyclic AMP-Dependent Phosphorylation of Plasma Membrane Proteins in 3T3 T Proadipocytes Transformed by the Smooth Surface Tumorigenesis Method

3T3 T proadipocytes are nontransformed mesenchymal stem cells which are related to 3T3 (clone A31) cells [15]. They are, however, distinct in that they possess the ability to differentiate into mature fat cells and are noninitiated cells with respect to the two-step model of carcinogenesis [15].

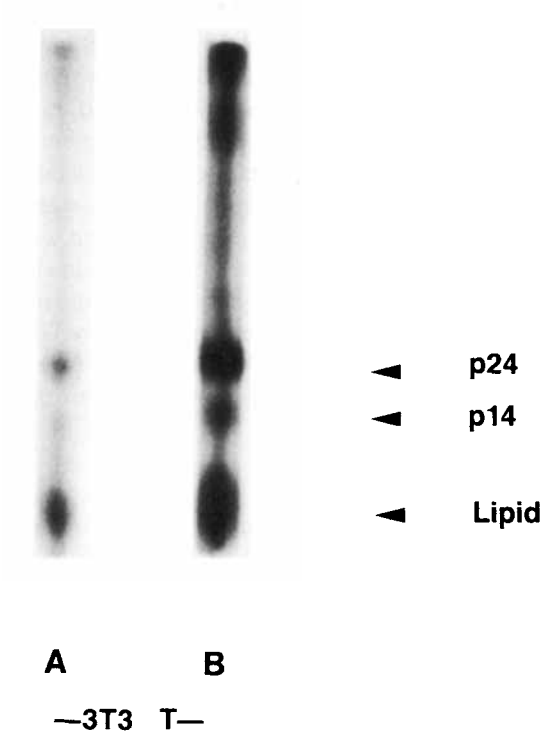

Fig. 1. Cyclic AMP-dependent phosphorylation of two plasma membrane proteins p14 and p24 in G_1 growth arrested 3T3 T proadipocytes (A) and in rapidly growing 3T3 T proadipocytes (B). Comparable quantities of plasma membrane protein were added to each gel. These specimens were extracted with ethanol–ether to remove some plasma membrane phospholipids which migrate at the electrophoresis front at the bottom of the gel.

To determine the effect of transformation of 3T3 T proadipocytes on cyclic AMP-dependent plasma membrane phosphorylation, three new clonal cell lines have been developed. They were derived from a rare tumor which formed when 3T3 T proadipocytes were implanted into a syngeneic mouse on a smooth surface plastic plate. The precise methods used to develop these clones have been previously described [16]. These new transformed 3T3 clones are designated M3, M4, and M6. It was shown that these cell lines were derived from the implanted 3T3 T cells by karyotypic analysis and it was shown that these cell lines are highly tumorigenic [16].

In this paper we have used these three transformed clonal proadipocyte cell lines to examine their cell cycle characteristics and the ability of their plasma membrane proteins to undergo cyclic AMP-dependent phosphorylation. In contrast to SV3T3 and MCA3T3 cells [3], SS-transformed proadipocytes can G_1 growth arrest growth when cultured in serum deficient medium. In particular, the data show that when SS clones M3, M4, and M6 are cultured in serum-deficient medium for 4 days DNA synthesis markedly decreases as determined by analysis of [^{3}H]thymidine incorporation into DNA and the vast majority of these cells growth arrest in the G_1 phase of the cell cycle. More specifically, after culture in serum deficient medium for 4 days the three SS-transformed 3T3 T clones show the following percentage of G_1 cells: M3, 87%; M4, 81%; M6, 86%. In results not detailed we have also found that these G_1 arrested cells can be selectively stimulated to reinitiate proliferation by the addition of either dialyzed serum or epidermal growth factor.

Figure 2 shows that in the three clones of SS-transformed 3T3 T proadipocytes cyclic AMP-dependent phosphorylation of plasma membrane proteins p14 and p24 occurs during exponential growth. Further, it shows that G_1 growth arrest induced by serum deprivation does not induce down modulation in the phosphorylation of p14 and p24 in the plasma membranes of M3 and M6 transformed proadipocyte clones. Table I summarizes these results and shows that SS-transformed proadipocyte clones M3 and M6 phosphorylate both p14 and p24 in both rapidly growth and serum arrest states. Figure 2 and Table I further show that the SS-transformed proadipocyte clone M4 shows a somewhat different response in that both p14 and p24 are phosphorylated in the rapid growth state, but following growth arrest there is a selective down modulation in the phosphorylation of p14 but not of p24.

DISCUSSION

Several transforming gene products which are localized to the cytoplasmic surface of the plasma membrane have been shown to be involved in phosphorylation reactions [4–12]. It has been suggested that these phosphoproteins are responsible at least in part for the defective growth control that is evident in transformed cells, but their prescise mechanism of action has not been established.

Our studies have focused on the cyclic AMP-dependent phosphorylation of two plasma membrane proteins in nontransformed cells, p14 and p24, and on the modulation of their phosphorylation during the G_1 phase of the cell cycle. In addition, we have focused studies on the plasma membrane of Simian virus 40 and methylcholanthrene-transformed cells and have shown that they are unable to phosphorylate p14 and p24 in the presence of cyclic AMP and also fail to modulate growth in the G_1 phase of the cell cycle when deprived of serum growth factors [3]. This suggested that defects in plasma membrane phosphorylation and defects in growth control

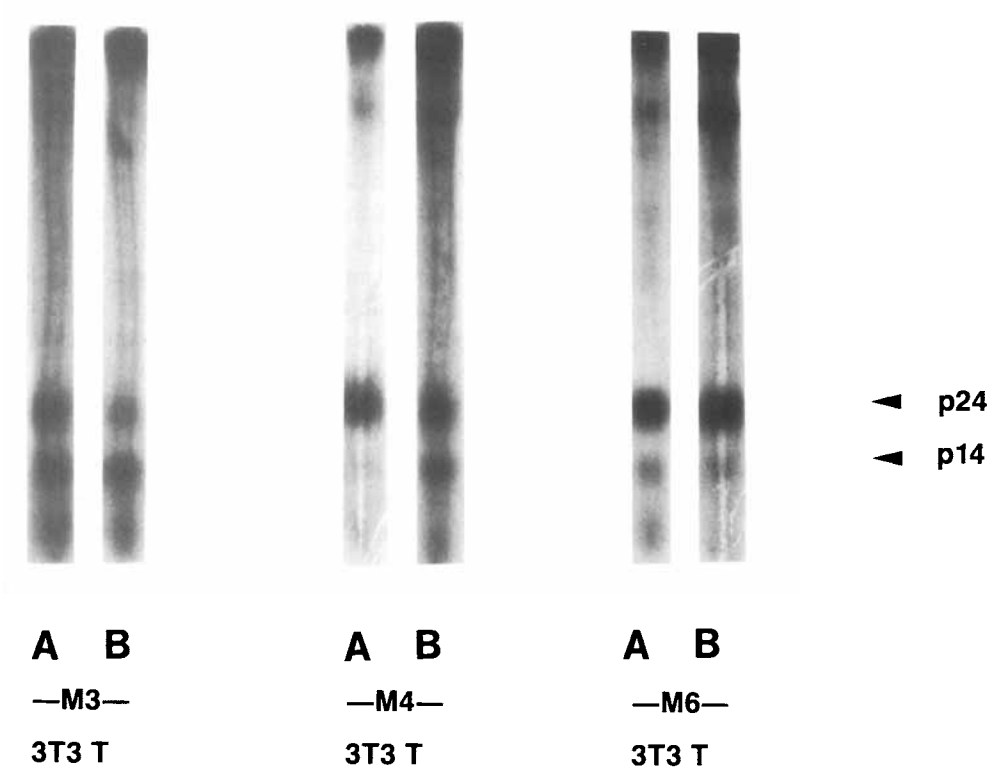

Fig. 2. Cyclic AMP-dependent phosphorylation of two plasma membrane proteins p14 and p24 in G_1 growth arrested (A) and rapidly growing (B) smooth surface transformed 3T3 T proadipocyte clones M3, M4, and M6. Comparable quantities of plasma membrane protein were added to each gel. The illustrated specimens were not ethanol-ether extracted to remove plasma membrane phospholipids.

TABLE I. Cyclic AMP-Dependent Plasma Membrane Phosphorylation in Nontransformed and Transformed Proadipocytes During Rapid Growth and After G_1 Growth Arrest

	Cyclic AMP-dependent phosphorylation			
	State B (rapid growth)		State A (serum deficiency)	
Cell line	p14	p24	p14	p24
I. Nontransformed				
3T3 T	+	+	−	−
II. SST transformed				
M3-3T3 T	+	+	+	+
M4-3T3 T	+	+	−	+
M6-3T3 T	+	+	+	+

mechanisms are associated and might provide an important mechanism of carcinogenesis.

The data presented in this paper, however, provide a new perspective. We have found that clones of smooth surface transformed 3T3 T proadipocytes can be isolated

which retain their ability to G_1 growth arrest as a result of growth factor deprivation even though they are tumorigenic. These transformed clones, in contrast to SV3T3 and MCA3T3 cells, retain their ability to phosphorylate p14 and p24 during rapid growth. They do, however, show a defect in their ability to down regulate the phosphorylation of p14 and/or p24 plasma membrane proteins in association with G_1 growth arrest. These data suggest that transformed cells do not have to show absolute defects in the cyclic AMP-dependent plasma membrane phosphorylation system. They may in fact show only defects in their ability to modulate cyclic AMP-dependent plasma membrane phosphorylation during the cell cycle. We have yet to prove that comparable reactions occur in intact cells or that they are causally related to carcinogenesis, but the available evidence supports this possibility. We concur with the hypothesis presented by others that insertion of new oncogene products in the plasma membrane or the development of defects in native plasma membrane proteins may mediate neoplastic transformation by acting on different aspects of a cascade of plasma membrane functions involving phosphorylation-dephosphorylation reactions [21,22]. We would modify this hypothesis only to suggest that the most critical aspect of this regulatory process may involve lesions in the ability of cells to modulate such reactions during the G_1 phase of the cell cycle at states where the regulation of cell proliferation is mediated.

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