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Changes in protein phosphorylation in wild-type and nickel-resistant cells and their involvement in morphological elongation

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Summary. Treatment of wild-type Balb/c-3T3 cells with $NiCl_2$ or N^6,2-*O*-dibutyl-adenosine 3′,5′-monophosphate (Bt_2-cAMP) resulted in a high degree and frequency of cellular elongation. Nickel-resistant Balb/c-3T3 cells (B200) treated with Bt_2-cAMP elongated at the same exposure concentration as wild-type cells. In contrast, treatment of the nickel-resistant cells with both non-cytotoxic and cytotoxic doses of $NiCl_2$ failed to induce elongation. Nickel-resistant cells had two-thirds of the total protein-phosphorylation activity of wild-type cells. Both cAMP and $NiCl_2$ enhanced phosphorylation of specific proteins in intact wild-type cells as detected by ^{32}p autoradiography of these proteins separated on two-dimensional gels. A nickel-dependent phosphorylation of specific proteins is seen following $NiCl_2$ treatment of wild-type cells but was not observed in B200 cells. In contrast, the pattern of Bt_2-cAMP-stimulated protein phosphorylation was quite similar in both wild-type and nickel-resistant cells. Although it is unclear at present how nickel ions affect the cellular protein-phosphorylation system, these results suggested that targets controlling cellular elongation are sensitive to nickel, are altered in nickel-resistant cells and appear to involve protein phosphorylation. Further characterization of these targets may help in understanding the mechanisms of nickel carcinogenesis.

Key words: Balb/c-3T3 – Nickel resistance – Cell elongation – Protein phosphorylation – Bt_2-cAMP

Introduction

Nickel compounds have been clearly implicated as human carcinogens and induce tumors in experimental animals and neoplastic transformation of cells in culture (Leonard et al. 1981; Sunderman 1984; Costa and Heck 1985). However, nickel compounds, unlike most direct-acting carcinogens, display weak mutagenic activity in most gene mutation assay systems and are not very genotoxic to mammalian cells (Sen and Costa 1986; Biggart and Costa 1986; Miyaki et al. 1979). The weak genotoxicity of nickel compounds may be due to the poor binding of Ni(II) to DNA (Lee et al. 1982). In contrast, nickel ions have relatively high binding affinities for proteins (Martell 1971). Thus, cellular proteins seem a likely target for nickel ion interaction. In attempting to understand the cellular effects of toxic and carcinogenic nickel compounds, a mouse cell line (B200) resistant to $NiCl_2$ has been derived from Balb/c-3T3 cells (Wang et al. 1988). This cell line exhibited a transformed phenotype and was tumorigenic in nude mice (Imbra et al. 1989a, b). Nickel resistance in these cells was not due to changes in the accumulation or clearance of the metal ion from cells, or due to an altered expression of metallothionein mRNA as measured by Northern blotting (Wang et al. 1988; Imbra et al. 1989a).

Recent studies suggest that two proteins (p48 and p55 with M_r 48 000 and 55 000, respectively) which bind nickel ions in wild-type Balb/c-3T3 cells exhibit an apparent defect in their ability to bind this metal in resistant cells (Wang and Costa 1989). Based upon two-dimensional SDS gel electrophoresis, there are only three major proteins in wild-type cells that retain detectable Ni binding following SDS-gel electrophoresis. Data suggest that nickel resistance may be related to the diminished binding of nickel ions to these proteins. In addition, these nickel-binding proteins exhibited charge trains when separated by two-dimensional gel electrophoresis, suggesting that they might be subject to covalent modifications such as phosphorylation (Wang and Costa 1989).

Phosphorylation and dephosphorylation of specific protein substrates are thought to be important in controlling cellular growth; altered regulation of these phosphorylation systems may play a role in neoplastic transformation (Lockwood et al. 1982, 1987; Whitfield et al. 1987; Reuse et al. 1990). Early studies showed that nickel compounds can induce morphological

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changes in Chinese hamster ovary (CHO) cells in association with the activation of cAMP-dependent protein kinase (Costa 1978) and that this effect resembled the morphological changes mediated by Bt_2-cAMP (Costa 1978). This result suggested that nickel ions may influence the cAMP-mediated signal transduction pathway to alter cell morphology. Recent studies showed that nickel sulfate can activate specific phosphorylation of nuclear proteins in peripheral blood T lymphocytes (Holst and Nordlind 1988), suggesting that nickel may affect gene expression by altering the phosphorylation of nuclear proteins. Presently, we have studied the effects of nickel chloride and N^6,2-*O*-dibutyryl-adenosine 3′,5′-monophosphate (Bt_2-cAMP) on cell morphology, cAMP-dependent protein kinase activities, and phosphorylation patterns of specific proteins from wild-type and nickel-resistant Balb/c-3T3 cells.

Materials and methods

Chemicals. Adenosine 3′,5′-monophosphate (cAMP), Bt_2-cAMP and histone type V were obtained from Sigma (St. Louis, MO). Nickel chloride was purchased from Alfa Inorganics (Danvers, MA). Adenosine [γ-^{32}P]triphosphate ([γ-^{32}P]ATP, 3000 Ci/mmol) and orthophosphoric acid ($H_3{}^{32}PO_4$) were purchased from New England Nuclear (Wilmington, DE). Dulbecco's modified Eagle's medium, newborn calf serum, and penicillin-streptomycin solution were supplied by Hazelton Research Products, Inc. (Denver, PA). All chemicals for gel electrophoresis were from Bio-Rad Laboratories (Rockville Center, NY).

Cell culture. Balb/c-3T3 mouse fibroblast obtained from the American Type Culture Collection were grown in monolayer in Dulbecco's modified Eagles medium with 10% newborn calf serum, 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Nickel-resistant B200 cells derived from Balb/c-3T3 were grown as above but in the presence of 200 μM $NiCl_2$ (Wang et al. 1988). All cultures were maintained at 37° C in a humidified atmosphere of 95% air: 5% CO_2.

Protein kinase measurements. The activity of protein kinase was determined by measuring the ability of cell lysates to phosphorylate exogenous histone V. For the assay, cells in the log growth phase were washed three times with ice-cold Puck's saline A (0.13 M NaCl, 0.05 M KCl, 5 mM glucose, 0.033 M bicarbonate buffer, pH 7.2) and scraped from the monolayer with a rubber policeman. The cells were collected by centrifugation and resuspended at a concentration of 10^6 cells/100 μl 0.01 M phosphate pH 7.0 containing 15 mM NaF and 25 mM $MgCl_2$. The suspension was homogenized with a Dounce homogenizer and the resulting homogenate was centrifuged at $20000 \times g$ for 20 min in a microcentrifuge (Wang and Costa 1989). The supernatant solution was used for the assay of protein kinase activity in the absence and presence of exogenously added and saturating cAMP (10 μM) as previously described (Costa 1978). The activity of cAMP-dependent protein kinase was estimated by expressing the activity of the enzyme assayed in the absence or presence of exogenously added and saturating cAMP (−cAMP/+cAMP). The reaction was initiated by the addition of 50 μM [γ-^{32}P]ATP and 1 μg/ml histone V to the supernatant solution (standardized by protein concentration) and allowed to proceed for 10 min at 20° C. The phosphorylated proteins were precipitated in 10% ice-cold trichloroacetic acid onto GF/C filters (Whatman) and radioactivity was measured with a Beckman LS 9800 liquid scintillation counter. Reactions were carried out in triplicate and experiments repeated at least three times.

Intact protein phosphorylation, gel electrophoresis and autoradiography. Cells were labeled with 0.5 mCi/ml of $H_3{}^{32}PO_4$ for 1 h prior to treatment with 500 μM $NiCl_2$ or 2 mM Bt_2-cAMP for an additional 6 h. Proteins were solubilized in buffer with 9 M urea, 4% NP-40, 2% mercaptoethanol and phosphoproteins were separated by two-dimensional polyacrylamide gel electrophoresis using previously described conditions (Wang and Costa 1989). Isoelectric focusing (IEF) gels, containing ampholines with a pH range of 3.5–11, were used to separate proteins in the first dimension and 12.5% SDS/PAGE was used to separate proteins according to their molecular mass in the second dimension. IEF was performed at 200 V for 18 h. The pH gradient in the first dimension was measured by slicing IEF gels into 0.5-cm pieces followed by incubating each piece with water at room temperature, and measuring the pH. In the second dimension, gels were standardized by parallel lanes with molecular mass markers: myosin (M_r 200000), β-galactosidase (116250), phosphorylase b (92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and lysozyme (14400) (Bio-Rad). The gels were photographed with Polaroid films, dried and placed in direct contact with X-ray film (X-Omat AR film) (Kodak, Rochester, NY) for autoradiography.

Results

Cells were plated at a subconfluent density of 10^6/100-mm plate and Bt_2-cAMP or $NiCl_2$ was added after 24 h of incubation. Cell morphology was monitored with a phase-contrast microscope. Figure 1 shows the elongated morphology of wild-type Balb/c-3T3 cells and nickel-resistant B200 cells after the addition of 2 mM Bt_2-cAMP. Both Balb/c-3T3 and B200 cells were slightly elongated at 6 h of treatment (less than 10% of cells), more elongation occurred at 24 h (about 60% of cells) and significant elongation was observed by 48 h (over 70% of cells). The degree of elongation in both cell lines was identical. Figure 2 shows the morphology of Balb/c-3T3 cells and B200 cells after addition of $NiCl_2$. Treatment of Balb/c-3T3 cells with 500 μM $NiCl_2$ appeared to induce morphological changes similar to those induced by Bt_2-cAMP, whereas the morphology of B200 cells treated with 500 μM $NiCl_2$ did not change.

To investigate whether the lack of response of B200 cells to elongation induced by 500 μM nickel chloride was due to reduced availability of nickel in these cells, higher doses of $NiCl_2$ were tested. Normal cellular morphology was defined as an axial ratio of $<1:3$ while an elongated morphology was defined by an axial ratio of $>1:3$. Figure 3 shows the percentage of elongated Balb/c-3T3 (a) or B200 (b) cells treated with various concentrations of either Bt_2-cAMP or $NiCl_2$. $NiCl_2$ and Bt_2-cAMP induced elongation of Balb/c-3T3 cells in both a time- and dose-dependent fashion. 500 μM $NiCl_2$ produced a similar frequency of elongated Balb/c-3T3 cells to that induced by 2 mM Bt_2-cAMP. However, nickel-resistant B200 cells have lost their ability to respond to the elongation induced by $NiCl_2$ at equally toxic (1.1 mM) or even higher doses (2.5 mM) as compared to wild-type Balb/c-3T3 cells. In contrast, Bt_2-cAMP induced similar elongation of B200 cells as observed for the wild-type cells at equivalent doses.

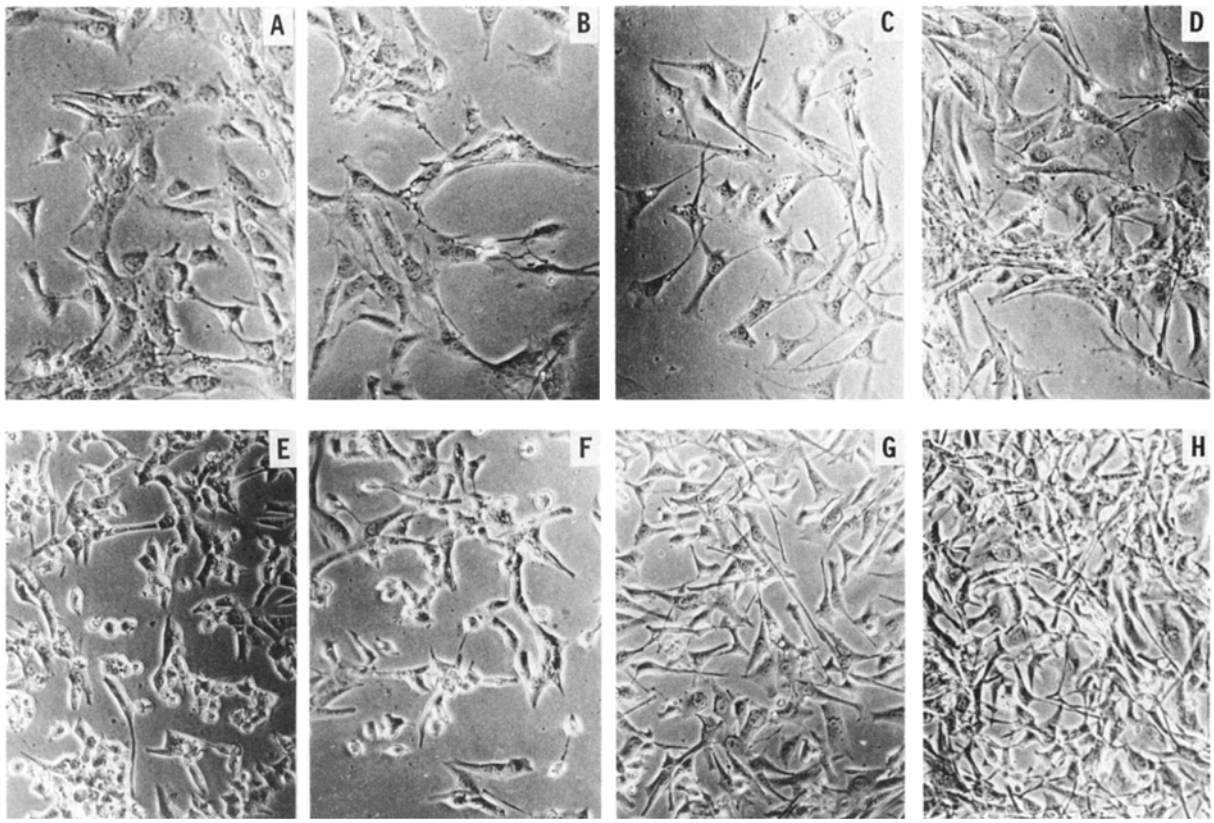

Fig. 1. Morphology of Balb/c-3T3 cells (**A–D**) and B200 cells (**E–H**) after addition of Bt_2-cAMP. Cells in the log phase of growth were exposed to 2 mM Bt_2-cAMP for 0 h (**A, E**), 6 h (**B, F**), 24 h (**C, G**) and 48 h (**D, H**). Morphology was defined as rounded, flat cell type for no alteration (axial ratio, $<1:3$) or as elongated cell type (axial ratio, $>1:3$)

Protein kinase activities in Balb/c-3T3 cells and nickel-resistant B200 cells were studied in order to investigate whether the lack of response in nickel-resistant B200 cells to nickel-chloride-induced elongation was due to an altered activity of these enzymes. Although the total protein phosphorylation activity was lower in B200 cells as compared to the Balb/c-3T3 cells, the activity ratio of cAMP-dependent protein kinase ($-$cAMP/$+$cAMP) was quite similar (Table 1).

To investigate which of the phosphoproteins in wild-type or B200 cells can be further phosphorylated upon cellular exposure to nickel chloride or Bt_2-cAMP, total cellular proteins from both cell types were labeled with $H_3{}^{32}PO_4$ and the phosphorylation pattern was analyzed by two-dimensional gel electrophoresis. Many proteins exhibited elevated phosphorylation in response to Bt_2-cAMP or $NiCl_2$ in Balb and B200 cells, but one protein with a molecular mass of 46 kDa and pI of about 5 consistently appeared to lack nickel-stimulated phosphorylation in nickel-resistant B200 cells but retained this activity in wild-type cells (Fig. 4).

Discussion

The cAMP-dependent phosphorylation pathway is a major regulator of protein phosphorylation (Costa et al. 1976). The identification and characterization of specific protein substrates in this pathway may provide evidence toward understanding the mechanism by which cAMP-dependent phosphorylation exerts its pleiotropic effects on the growth and morphology of transformed cells. There are two isozymes of cAMP-dependent protein kinase, each of which is composed of a regulatory subunit dimer (R) and two catalytic subunits (C). The type I and type II isozymes differ only in their regulatory subunits, R-I (M_r 48000) and R-II (M_r 55000) (Hofmann et al. 1975). Bt_2-cAMP can induce the elongation of cells (Porter et al. 1974) through a mechanism involving an increase in the number of microtubules and a change in their distribution (Olmsted 1986). The microtubule assembly system is modulated by microtubule-associated proteins (MAPs), one of which binds cAMP (Theurkauf and Vallee 1982). Phosphorylation or dephosphorylation of these proteins as the result of the activation of protein kinase contributes to the modulation of cellular elongation (Bloom and Lockwood 1980). There are very few agents, except for nickel compounds and cAMP-elevating agents, which can induce such a striking elongation of cultured cells. One of these agents is testosterone which is capable of potentiating the cAMP-mediated elongation (Hsie and Puck 1971).

Although $NiCl_2$ induced elongation of wild-type Balb/c-3T3 cells in a dose-dependent and time-dependent pattern, it had no effect on the elongation of nickel-

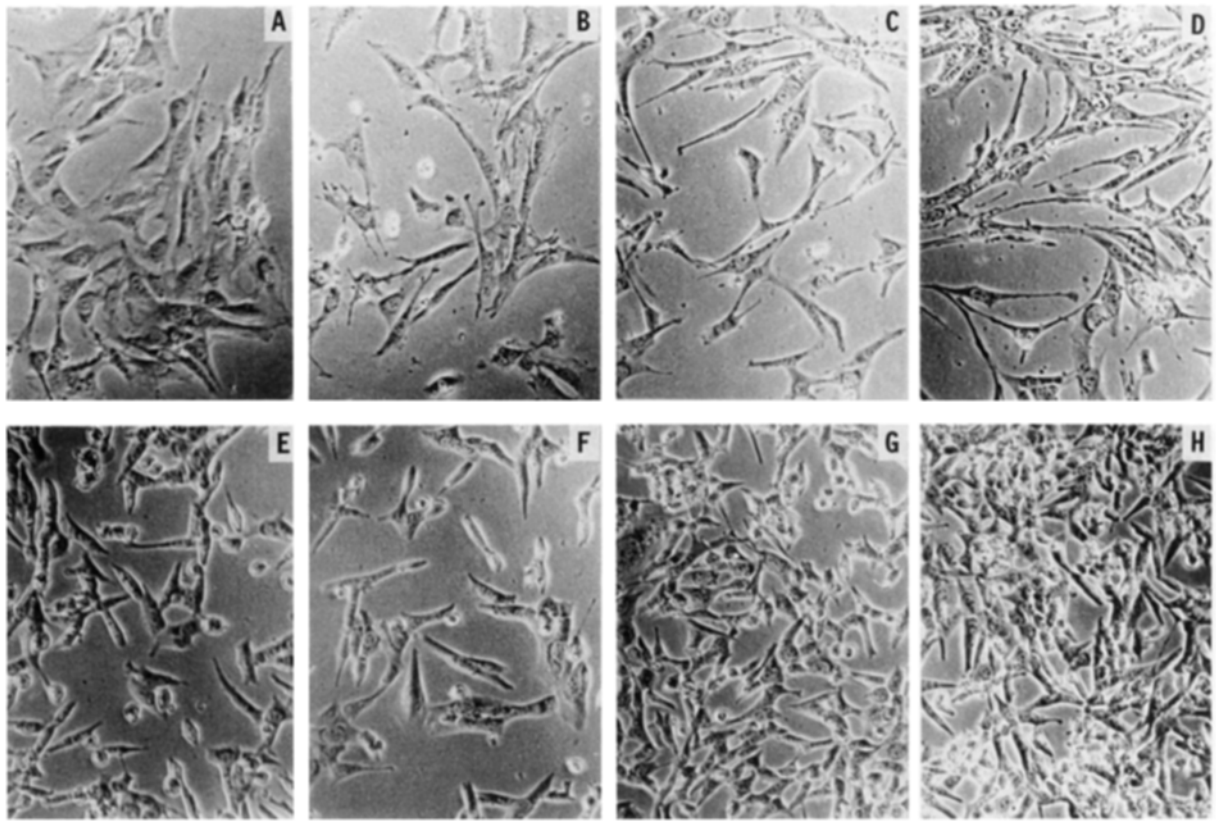

Fig. 2. Morphology of Balb/c-3T3 cells (**A–D**) and B200 cells (**E–H**) after addition of $NiCl_2$. Cells in the log phase of growth were exposed to 500 μM $NiCl_2$ for 0 h (**A, E**), 6 h (**B, F**), 24 h (**C, G**) an d 48 h (**D, H**)

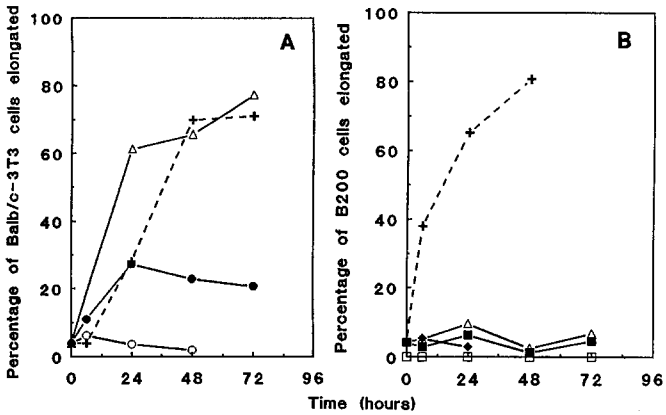

Fig. 3. Elongation of Balb/c-3T3 cells and B200 cells following addition of Bt_2-cAMP or $NiCl_2$. (**A, B**) Cells were plated at 10^6/100-mm plate and Bt_2-cAMP or $NiCl_2$ was added after a 24-h incubation. Cell morphology was monitored under phase-contrast microscope at various times and cells exhibiting elongated morphology were counted. Two plates were employed for each condition. (**A**) Balb/c-3T3 cells with no treatment (○); with 100 μM $NiCl_2$ (●); with 500 μM $NiCl_2$ (△); with 2 mM Bt_2-cAMP (+). (**B**) B200 cells with no treatment (◆); with 500 μM $NiCl_2$ (△); with 1.1 mM $NiCl_2$ (■); with 2.5 mM $NiCl_2$ (□); with 2 mM Bt_2-cAMP (+)

Table 1. Comparison of protein kinase activity in cell lysates of Balb/c-3T3 and nickel-resistant B200 cells in the absence or presence of cAMP

cAMP present	Protein kinase activity (pmol min^{-1} mg $protein^{-1}$)	
	Balb/c-3T3	B200
−cAMP	29 ± 1	19 ± 1[a]
+cAMP[c]	105 ± 28	63 ± 14[b]
Activity ratio (−cAMP/+cAMP)	0.27 ± 0.01	0.30 ± 0.03

[a] $P<0.01$, compared to parent line
[b] $P<0.1$, compared to parent line
[c] Exogenously added and saturating cAMP

resistant B200 cells even at equally toxic or at higher doses compared to wild-type cells. The LC_{50} values for 24-h exposure to $NiCl_2$ in Balb/c-3T3 cells were 100 μM, whereas 50% of survival of B200 cells required 1100 μM (Wang et al. 1988). Furthermore, although some doses of $NiCl_2$ which were used to induce elongation were relatively toxic to both wild-type and resistant cells, it is clear that elongation and cytotoxicity may be uncoupled since equally toxic doses were used in the experiments. Previous studies also showed that the cellular uptake and clearance of Ni^{2+} in both cell types were similar (unpublished results). These observations argue against the lack of biological response in resistant cells being due to a diminished uptake of Ni^{2+}. B200 cells can still be elongated upon stimulation with Bt_2-cAMP and one of the phosphorylated proteins, which

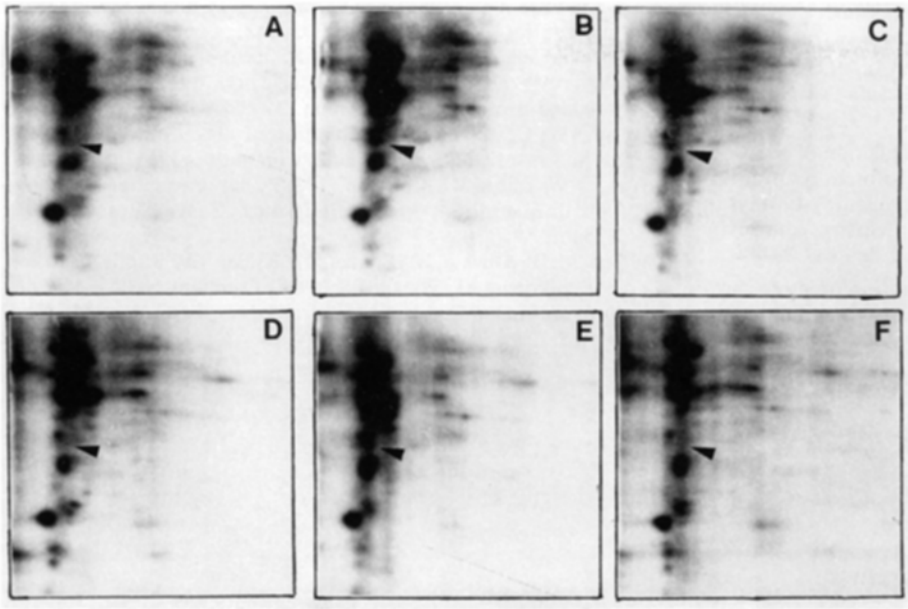

Fig. 4. Two-dimensional gel analysis of phosphoprotein from Balb/c-3T3 cells (**A–C**) and nickel-resistant B200 cells (**D–F**) in the absence (**A, D**) or presence of 2 mM Bt_2-cAMP (**B, E**) or 500 μM $NiCl_2$ (**C, F**). The acidic end of the isoelectric focusing gels is at the left. Arrows indicate the specific substrate in the cells where phosphorylation can be enhanced by Bt_2-cAMP or $NiCl_2$

exhibits a diminished nickel-dependent phosphorylation, can still be phosphorylated by Bt_2-cAMP during cellular elongation. It appears that the defect in B200 cells involves a loss of nickel-binding sites; however, since the site of the normal regulatory function(s) of nickel are unknown, it is difficult to understand their alteration during nickel resistance. It has been shown that two nickel-binding proteins (p55, p48) in wild-type Balb/c-3T3 cells lose their binding affinities to nickel ions in B200 cells (Wang and Costa 1989). The molecular masses of these nickel-binding proteins are similar to the subunits of cAMP-dependent protein kinase. It is possible that nickel ions may interact with regulatory subunits of cAMP-dependent protein kinase or other sites to elevate the cellular levels of cAMP and thus cause cell elongation.

Elongation of tissue culture cells may represent a process related to cellular differentiation. Nickel compounds are known to be potent carcinogens and cAMP is known to regulate cell growth. The ability of nickel to elongate cells in a cAMP-dependent fashion suggests that nickel may perturb the cAMP-mediated signal transduction pathway, which may in part be responsible for its carcinogenic action.

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