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Identification of a Ras-related Protein in Murine Erythroleukemia Cells That Is a cAMP-dependent Protein Kinase Substrate and Is Phosphorylated during Chemically Induced Differentiation*

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Murine erythroleukemia (MEL) cells deficient in cAMP-dependent protein kinase (A-kinase) activity are impaired in chemically induced differentiation (Pilz, R. B., Eigenthaler, M., and Boss, G. R. (1992) *J. Biol. Chem.* 267, 16161–16167). We identified by two-dimensional polyacrylamide gel electrophoresis two low molecular weight proteins (referred to as pp 21–1 and 21–2) that were phosphorylated when parental MEL cells, but not A-kinase-deficient MEL cells, were treated with the membrane-permeable cAMP analog 8-bromo-cAMP. We showed that pp 21–1 and 21–2: (*a*) were direct A-kinase substrates; (*b*) bound GTP; and (*c*) belonged to the *ras* superfamily of proteins. The only *ras*-related proteins that are clearly A-kinase substrates both *in vitro* and *in vivo* are Rap 1A and 1B while H- and K-Ras can be A-kinase substrates *in vitro*; we showed by immunological methods, phosphopeptide mapping, and migration on two-dimensional gels that pp 21–1 and 21–2 were not identical to one of these four proteins. We found a 3-fold increase of $^{32}PO_4$ incorporation into pp 21–2 in hexamethylene bisacetamide-treated parental MEL cells which was not secondary to an increase in pp 21–2 protein but appeared secondary to increased phosphorylation of pp 21–2 by A-kinase. Thus, pp 21–1 and 21–2 are either new *ras*-related proteins or are previously identified *ras*-related proteins not known to be A-kinase substrates, and increased phosphorylation of pp 21–2 occurs during differentiation of MEL cells.

Friend virus-infected murine erythroleukemic (MEL)[1] cells are a well-established model for studying hematopoietic cell differentiation (1). The cells proliferate as proerythroblasts and in response to a number of chemical inducers differentiate over 5–7 days into hemoglobin-producing normoblast-like cells (1). Two of the more effective inducers of MEL cell differentiation are hexamethylene bisacetamide (HMBA) and dimethyl sulfoxide (2).

In several mammalian cell systems 8-bromo-cAMP and other cAMP analogs induce cellular differentiation pointing to an important role of the cAMP-dependent protein kinase (A-kinase) (3–6). Cyclic AMP analogs do not induce differentiation of MEL cells, but there is a transient increase in the intracellular concentration of cAMP and a change in the isozyme pattern of A-kinase during chemically induced differentiation of MEL cells (7–10). To study the role of A-kinase during MEL cell differentiation, we rendered MEL cells deficient in A-kinase activity by stably transfecting them with DNA encoding either a mutant regulatory subunit of A-kinase ($R_I$mut) or the specific protein inhibitor of A-kinase (PKI, protein kinase inhibitor) (11–13). We found that the A-kinase-deficient cells were impaired in their ability to differentiate and that their degree of differentiation correlated with their residual enzyme activity. These data suggested that there were A-kinase substrates that must be phosphorylated for chemically induced differentiation to proceed.

In the present work we used two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) to identify A-kinase substrates in MEL cells. We found several low molecular weight proteins that were phosphorylated in a cAMP-dependent manner in MEL cells and showed by *in vitro* experiments using the purified catalytic (C) subunit of A-kinase that two of these proteins, referred to as pp 21–1 and 21–2, were direct A-kinase substrates. We found a 3-fold increase in $^{32}PO_4$ incorporation into pp 21–2 in HMBA-treated parental cells without an increase in the amount of pp 21–2 protein; pp 21–2 was poorly phosphorylated under all conditions in the non-differentiating A-kinase-deficient cells. We found that pp 21–1 and 21–2 belonged to the *ras* superfamily but that they were immunologically distinct from and did not comigrate on 2-D gels with Rap 1A or 1B, the only two *ras* proteins known to be A-kinase substrates both *in vitro* and *in vivo* (14–20); moreover, pp 21–2 yielded phosphopeptide maps different from that of Rap 1A and 1B.

EXPERIMENTAL PROCEDURES

Materials

MEL cells were obtained from the American Type Tissue Culture Collection (parental cells). The A-kinase-deficient cells overexpressing either $R_I$mut or PKI ($R_I$mut and PKI transfectants, respectively), and cells overexpressing the wild type regulatory subunit of A-kinase ($R_I$wt transfectants) were isolated and characterized previously (11); expression of the transfected genes was under control of the metallothionein promoter. The $R_I$mut and PKI transfectants used in the present studies

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[1] The abbreviations used are: MEL cells, Friend virus-infected murine erythroleukemic cells; HMBA, hexamethylene bisacetamide; A-kinase, cAMP-dependent protein kinase; C subunit of A-kinase, catalytic subunit of A-kinase; $R_I$wt and $R_I$mut, the wild type and a mutant form of the type I regulatory subunit of A-kinase, respectively; PKI, the specific heat-stable protein inhibitor of A-kinase; 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis; pp 21–1, 21–2 and 22, phosphoproteins of approximate molecular mass of 21, 21 and 22 kDa, respectively, denoted by the numbers 1–3, respectively; Kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly.

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(clones $R_I$mut/C3, $R_I$mut 9.10 and PKI/C1) had, in the presence of zinc, <10% of the A-kinase holoenzyme activity of parental cells (11); the $R_I$wt transfectant (clone $R_I$wt/C1) expressed a similar amount of the transfected gene in the presence of zinc as clones $R_I$mut/C3 and $R_I$mut 9.10 but had normal A-kinase activity and differentiated normally (11).

The C subunit of bovine heart A-kinase and rabbit muscle PKI were provided by Drs. S. Taylor and R. A. Maurer, respectively (21, 22). Human Rap 1A was purified from a baculovirus-based insect cell system as described previously (23). Rap 1B was purified from human platelets and was provided by Dr. T. Fischer (16); recombinant human H-Ras was provided by Dr. J. Feramisco (24).

The mouse monoclonal antibody 142–24E5 and the rat monoclonal antibody Y13–259 were raised against amino acids 96–118 and 63–73 of H-Ras, respectively, and were obtained from Quality Biotech and Oncogene Scientific. The polyclonal antibodies R61 and R195 were raised in rabbits against recombinant Rap 1A; they do not recognize H-Ras, Rap 2 or 2B, Rho A or Rab lb or 3A on Western blots (14).[2] The polyclonal antibodies R64 and SC65 were raised in rabbits against a peptide corresponding to amino acids 127–137 and 121–137 of Rap 1, respectively; R64 was provided by Dr. G. M. Bokoch (14), and SC65 was obtained from Santa Cruz Biotechnology, Inc. The streptavidin-coupled alkaline phosphatase and the biotinylated secondary antibodies were from Bio-Rad, the polyclonal goat anti-rat and anti-mouse antibodies were from Cappel, and protein A-agarose was from Calbiochem.

The pH 9–11 ampholytes were from Serva and the pH 3–10 ampholytes were from Fisher Scientific; protein standards for 2-D gels were from Bio-Rad. HMBA and forskolin were from Sigma, 8-bromo-cAMP was from Boehringer Mannheim, [γ-^{32}P]ATP, [α-^{32}P]GTP, and $^{32}PO_4$ were from DuPont NEN, and Tran^{35}S-label was from ICN.

Methods

Cell Culture—Cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum. Where indicated the $R_I$mut, PKI, and $R_I$wt transfectants were grown in the presence of 120 μM zinc for 48 h (11); at this concentration zinc had no effect on parental cells.

Protein Phosphorylation in Intact Cells—Cells were washed once in phosphate-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal bovine serum and resuspended in this medium at 1 × 10^6 cells/ml; 1 ml of the cell suspension was incubated at 37 °C for 3 h with 100 μCi of [^{32}P]orthophosphate. To selected cultures 1 mM 8-bromo-cAMP and 1 mM theophylline were added during the last 15 min of incubation; in some experiments 10 μM forskolin replaced the 8-bromo-cAMP. Cells were harvested by a brief centrifugation and extracted by shearing through a 23-gauge needle in 50 μl of 9 M urea, 4% Nonidet P-40, 2% pH 9–11 ampholytes, and 2% β-mercaptoethanol (2-D PAGE sample buffer). The extracts were cleared by centrifugation at 10,000 × *g* for 30 s.

In Vitro Protein Phosphorylation—Cells were extracted at a density of 100 × 10^6/ml by shearing through a 23-gauge needle in 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM β-glycerol phosphate, 10 μM Na_3VO_4, 4 μM microcystin, 1 μM okadaic acid, and 10 μg/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor. Five μl of the extract were incubated in a final volume of 10 μl for 10 min at 30 °C with 20 μCi of [γ^{32}-P]ATP and 2 ng of the C subunit of A-kinase. The reaction was stopped by adding 2-D PAGE sample buffer.

Analytical 2-D PAGE—The 2-D PAGE analyses were performed according to O'Farrell and Anderson (25, 26). A 50-μl aliquot of each sample from the studies of protein phosphorylation *in vitro* and in intact cells was divided in half and applied to two 1.5-mm diameter tube gels. The gels were of 6% acrylamide and contained per milliliter 80 μl of pH 3–10 ampholytes, 833 mg of urea, and 30 μl of Nonidet P-40. After prefocusing for 1 h at 200 V and focusing for 16 h at 1000 V, the gels were extruded into 2.5 ml of 125 mM Tris, pH 6.8, 10% glycerol, 2% SDS and were frozen immediately.

The cylindrical gels were thawed and applied to 12% acrylamide slab gels containing 340 mM Tris, pH 8.6, 0.1% SDS which were electrophoresed for 5 h at 100 mA and either stained with Coomassie Blue R, dried, and exposed to x-ray film or used to generate a Western blot.

A-kinase Phosphorylation of Proteins after Preparative Nondenaturing Gel Electrophoresis—Approximately 5 × 10^9 parental MEL cells were extracted in 5 ml of 125 mM Tris, pH 6.8, 0.5 mM dithiothreitol, 10% (v/v) glycerol, and the extract was centrifuged at 16,000 × *g* for 15 min at 4 °C. A preparative non-denaturing 10% acrylamide resolving gel and a 3.75% acrylamide stacking gel were prepared in a 28-mm cylindrical gel; residual persulfate was removed by pre-electrophoresis, and the cell extract was applied to the gel which was electrophoresed at 10-watt constant power for 5 h using an elution buffer of 113 mM BisTris, pH 7.0, 0.5 mM dithiothreitol, and 10% (v/v) glycerol. Appropriate fractions were concentrated and dialyzed against 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, and aliquots were incubated for 30 min at 37 °C with 50 μCi of [γ-^{32}P]ATP and 20 ng of the C subunit of A-kinase. The reaction was stopped by 2-D PAGE sample buffer, and samples were applied to analytical 2-D gels.

A-kinase Phosphorylation of Proteins on Western Blots of 2-D Gels—Cellular proteins resolved by 2-D PAGE were transferred electrophoretically from gels to polyvinylidine difluoride membranes as described previously (11). The membranes were incubated in 20 mM Tris-HCl, pH 7.4, 3% bovine serum albumin, 0.6% Tween 20, washed four times with 20 mM Tris-HCl, pH 7.4, and incubated for 1 h at room temperature in 20 mM Tris-HCl, pH 7.4, 15 μM ATP, 10 μCi of [γ-^{32}P]ATP, 10% bovine serum albumin, and 20 ng of the C subunit of A-kinase. They were then washed extensively with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% SDS, 0.6% Tween 20 and exposed to x-ray film; 2-D gel protein standards were visualized by staining the membranes with Amido Black.

Incorporation of [^{35}S]Methionine and [^{35}S]Cysteine into Cellular Proteins—Cells were incubated for 18 h at a density of 1 × 10^6 cells/ml with 50 mCi/ml Tran^{35}S-label in methionine- and cysteine-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal bovine serum. Cells were harvested and extracted as described under "Protein Phosphorylation in Intact Cells," and cell extracts were analyzed by 2-D PAGE.

Measurement of A-kinase Holoenzyme and C Subunit Activity—A-kinase holoenzyme activity was measured in cell extracts as the difference in phosphorylation of Kemptide between the presence and absence of 1 μM cAMP as described previously (11). Activity of the C subunit of A-kinase was measured similarly except cAMP was not added, and enzyme activity was determined as the difference in phosphorylation of Kemptide between the absence and presence of 10 μg/ml PKI.

Binding of [α-^{32}P]GTP to Proteins on Western Blots—Western blots of 2-D gels were incubated for 1 h at room temperature with 10 μCi of [α-^{32}P]GTP in 20 mM Tris-HCl, pH 7.4, 0.3% Tween 20, 2 μM $MgCl_2$ (27). The blots were washed and treated as described above.

Immunodetection of Proteins on Western Blots of 2-D Gels—Western blots of 2-D gels were incubated with the primary antibody (antibody 142–24E5 at a 1:2000 dilution, antibody SC65 at a 1:1000 dilution, and antibodies R61, R64, and R195 at a 1:750 dilution) followed by a biotinylated secondary antibody and streptavidin-coupled alkaline phosphatase as described previously (11).

Immunoprecipitation Using Antibodies 142–24E5, Y13–259, or SC65—Cells were incubated with [^{32}P]orthophosphate as described under "Protein Phosphorylation in Intact Cells" and were extracted in 50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM $MgCl_2$, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of leupeptin, aprotinin, and soybean trypsin inhibitor. Nuclei were removed by centrifugation and to the supernatant was added SDS and Nonidet P-40 to final concentrations of 0.05 and 0.5%, respectively. In succession, the primary antibody (1 μg), the appropriate secondary antibody, and protein A-agarose were added with gentle mixing of the samples for 1 h at 4 °C after each addition. The samples were centrifuged, and the pellet was washed six times in extract buffer containing 150 mM NaCl, 0.1% Triton X-100, and 0.005% SDS. The final pellet was resuspended in 2-D PAGE sample buffer and applied to analytical 2-D gels.

RNA Extraction and Analysis—Total cytoplasmic RNA was extracted from cells, electrophoresed on denaturing formaldehyde/agarose gels, blotted onto nitrocellulose membranes, and hybridized to radioactively labeled probes as described previously (11); the probes for Rap 1A and 1B were a 670-base pair *Eco*RI-*Bam*HI and a 600-base pair *Hin*dIII-*Bam*HI, respectively, human cDNA fragment (23).

Generation of Proteolytic Peptide Maps—Cell extracts were incubated with A-kinase and [γ-^{32}P]ATP as described above and applied to 2-D gels which were dried without fixing and exposed to x-ray film. Appropriate areas of the gel were cut out and inserted into wells of the stacking gel of a 15% one-dimensional gel; purified Rap 1A or 1B processed identically to the cell extracts were added to adjacent wells. One of several different concentrations of *Staphylococcus aureus* V8 protease were added to individual wells, and power was applied to the gel until the dye front reached the interface of the stacking and resolving gels. After a 30 min pause, power was reapplied to the gel which was processed in the usual manner and exposed to x-ray film.

Data Analysis and Scanning Densitometry—Each experiment was performed at least three times in duplicate. Enzyme activity data are

[2] L. A. Quilliam and G. M. Bokoch, unpublished observations.

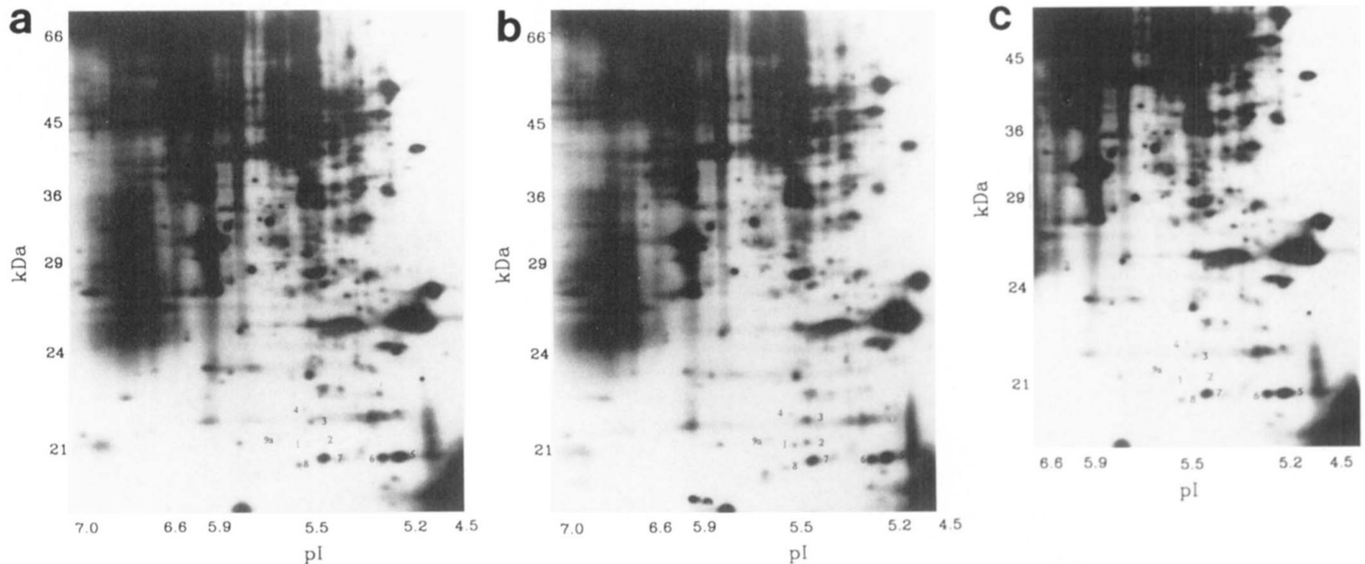

FIG. 1. **cAMP-dependent phosphorylation of proteins in MEL cells.** Cells were incubated as described under "Experimental Procedures" for 3 h with [^{32}P]orthophosphate; 1 mM 8-bromo-cAMP and 1 mM theophylline were added to some of the cultures during the last 15 min of incubation. Cell extracts were applied to 2-D gels as described under "Experimental Procedures," and the gels were exposed to x-ray film. *Panel a*, parental cells without 8-bromo-cAMP; *panel b*, parental cells with 8-bromo-cAMP; *panel c*, an A-kinase-deficient $R_I$mut transfectant (clone $R_I$mut/C3) with 8-bromo-cAMP. The numbers *1, 4, 6,* and *9a* are left of the designated protein, and the numbers *2, 3, 5, 7,* and *8* are right of the designated protein; pp 21–1, 21–2, and 22 are designated by the numbers *1–3*, respectively.

presented as the means ± S.D. Autoradiographs of gels were scanned using an LKB UltroScan XL laser densitometer in 2-D mode; the density of the proteins of interest were normalized to the density of three or more reference proteins and at least four separate autoradiographs from each condition were scanned.

RESULTS

cAMP-dependent Phosphorylation of Proteins in Intact MEL Cells—To identify proteins phosphorylated in a cAMP-dependent manner, we incubated MEL cells for 3 h with $^{32}PO_4$ and added 8-bromo-cAMP plus theophylline to some of the cultures during the last 15 min of incubation; cell extracts were analyzed on 2-D gels. Forskolin, an adenylate cyclase activator, yielded similar results as 8-bromo-cAMP.

By comparing Fig. 1*a*, parental cells incubated in the absence of 8-bromo-cAMP, to Fig. 1*b*, parental cells incubated in the presence of 1 mM 8-bromo-cAMP, cAMP-dependent phosphorylation of several low molecular weight proteins can be seen (four low molecular weight proteins showing cAMP-dependent phosphorylation are labeled *1, 2, 3,* and *9a*, and five reference proteins are labeled *4–8*). Proteins *1–3* had approximate molecular masses of 21, 21, and 22 kDa and pI values of 5.5, 5.4, and 5.4, respectively; they will be referred to as pp 21–1, 21–2 and 22, respectively. The small amount of phosphorylation of pp 21–1 and 21–2 in untreated parental MEL cells could be attributed to the activity of the free C subunit of A-kinase which was 36.5 ± 5.4 pmol/min/mg protein compared with 710 ± 64 pmol/min/mg protein for A-kinase holoenzyme activity.

Since the expression of $R_I$mut and PKI protein in the transfected cells was under control of the metallothionein promoter, we could regulate their A-kinase activity and degree of differentiation by adding zinc to the culture media (11); zinc had no effect on A-kinase activity (11), differentiation (11), or cAMP-dependent protein phosphorylation in parental cells (data not shown). In the absence of zinc, A-kinase holoenzyme activity of clone $R_I$mut/C3 was 26% of parental cells (11), the cells showed a modest degree of differentiation (11), and we observed a small amount of cAMP-dependent phosphorylation of pp 21–1, 21–2, and 22 (data not shown). However, in the presence of zinc, A-kinase holoenzyme activity of clone $R_I$mut/C3 was 6% of parental cells (11), the cells were markedly impaired in their ability to differentiate (11), and we observed no cAMP-dependent phosphorylation of pp 21–1, 21–2, and 22 (Fig. 1*c*; only cells treated with 8-bromo-cAMP are shown). Similar results were obtained with the A-kinase-deficient clones $R_I$mut/9.10 and PKI/C1.

In cells transfected with the wild type R_I subunit of A-kinase, which show the same A-kinase activity and ability to differentiate as parental cells (11), cAMP-dependent phosphorylation of pp 21–1, 21–2, and 22 was similar to that in parental cells (data not shown).

Phosphorylation of Proteins in Cell Extracts by A-kinase—Since there are known protein phosphorylation cascades (28) it seemed possible that pp 21–1, 21–2, and 22 were not direct A-kinase substrates but rather substrates of another protein kinase or phosphatase regulated by A-kinase; we, therefore, performed three separate sets of *in vitro* experiments. First, we showed that when cell extracts were incubated with [γ-^{32}P]ATP, pp 21–1 and 21–2 were detected only when the C subunit of A-kinase was added: compare Fig. 2*a*, extracts incubated in the absence of the C subunit of A-kinase, to Fig. 2*b*, extracts incubated in the presence of the C subunit of A-kinase (pp 22 was phosphorylated to a small extent in the absence of added A-kinase). Phosphorylation of pp 21–1 and 21–2 occurred within 1 min of adding the enzyme to cell extracts and was indistinguishable in extracts prepared from the $R_I$mut and PKI transfectants compared with extracts from parental cells; in all sets of extracts, phosphorylation of the three proteins was inhibited completely by adding purified PKI. Second, we fractionated MEL cell proteins on a preparative non-denaturing gel and incubated fractions containing proteins of molecular mass 20–25 kDa with [γ-^{32}P]ATP and the C subunit of A-kinase. Since most protein kinases and phosphatases are of a molecular mass higher than 25 kDa (29, 30), it seemed unlikely that a protein phosphorylation cascade occurred under these conditions. Fractions were then analyzed on 2-D gels and as shown in Fig. 3 pp 21–1 and 21–2 were clearly phosphorylated, along with several other low molecular weight proteins, while pp 22 was not. And third, we separated cellular proteins by 2-D PAGE and transferred them to a membrane which was incubated with [γ-^{32}P]ATP and the C subunit of A-kinase; pp 21–1 and 21–2

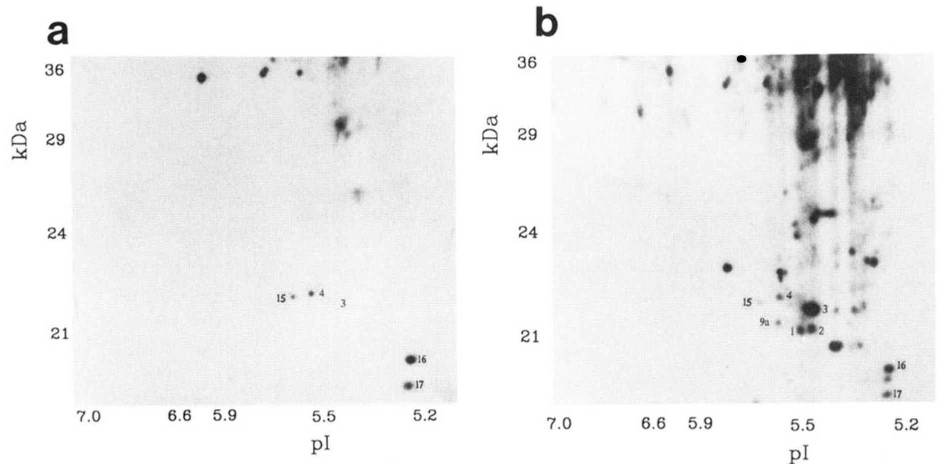

FIG. 2. **Phosphorylation of proteins in cell extracts by A-kinase.** Extracts of parental cells were incubated with [γ-^{32}P]ATP as described under "Experimental Procedures" in the absence (*panel a*) or presence (*panel b*) of the C subunit of A-kinase; the extracts were applied to 2-D gels which were exposed to x-ray film. Proteins are labeled as in Fig. 1 except the number *4* is right of the designated protein; reference proteins *15–17* were not labeled in Fig. 1.

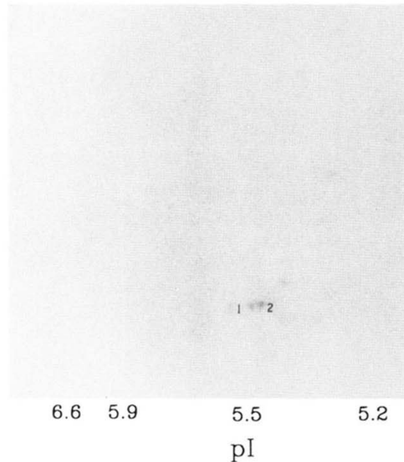

FIG. 3. **Phosphorylation of proteins by A-kinase after one-dimensional nondenaturing gel electrophoresis.** Extracts of parental cells were applied to a preparative one-dimensional nondenaturing gel as described under "Experimental Procedures." Fractions containing proteins of approximate molecular mass 20–25 kDa were incubated with [γ-^{32}P]ATP and the C subunit of A-kinase and then applied to 2-D gels as described under "Experimental Procedures." Proteins *1* and *2*, pp 21–1 and 21–2, respectively, were identified by comparing their positions to 2-D gel protein standards.

were phosphorylated on the membrane by A-kinase while pp 22 was not (data not shown). The latter two experiments indicated that pp 21–1 and 21–2 were direct A-kinase substrates and that pp 22 was either not an A-kinase substrate or that its structure was altered sufficiently under the experimental conditions that it could no longer be phosphorylated by A-kinase. The remainder of the work was devoted only to the study of pp 21–1 and 21–2.

Phosphorylation of Proteins in HMBA-treated Cells—Since pp 21–1 and 21–2 were poorly phosphorylated in A-kinase-deficient $R_I$mut and PKI transfectants and these cells show impaired differentiation in response to HMBA (11), we decided to study the effect of HMBA on these two proteins. In parental cells HMBA induced a 3-fold increase in $^{32}PO_4$ incorporation into pp 21–2 and a lesser increase of $^{32}PO_4$ incorporation into pp 21–1 (Table I and compare Fig. 4*a*, cells incubated in the presence of HMBA for 16 h, with Fig. 1*a*). When HMBA was combined with 8-bromo-cAMP, present during the last 15 min of incubation with HMBA, there was a further 10-fold increase in $^{32}PO_4$ incorporation into pp 21–2 for a total of a 30-fold increase over that observed in the basal untreated state (Table I and compare Fig. 4*b*, cells incubated in the presence of HMBA plus 8-bromo-cAMP, with Figs. 1*a* and 4*a*). The increased $^{32}PO_4$ incorporation into pp 21–2 could be seen within 8 h of adding HMBA (data not shown).

TABLE I
Effect of HMBA and 8-bromo-cAMP on $^{32}PO_4$ incorporation into PP 21–2

Parental MEL cells incubated with [^{32}P]orthophosphate for 3 h were extracted, and the proteins were separated on 2-D gels as described under "Experimental Procedures." Autoradiographs of the gels were scanned by laser densitometry, and the density of the spot corresponding to pp 21–2 was normalized to the density of three reference proteins. The HMBA-treated cells received 4 mM HMBA for 13 h prior to adding [^{32}P]orthophosphate, and the 8-bromo-cAMP-treated cells received 1 mM 8-bromo-cAMP plus 1 mM theophylline during the last 15 min of incubation with [^{32}P]orthophosphate. The values are the mean ± S.D. of four separate gels scanned for each condition; the density of the spot corresponding to pp 21–2 in untreated cells was assigned a value of 1.

	$^{32}PO_4$ incorporation into PP 21–2	
	No addition	1 mM 8-bromo-cAMP
	Relative densities	
No addition	1.0	11 ± 1.3
4 mM HMBA	3.1 ± 0.6	30 ± 1.6

In the A-kinase-deficient transfectants incubated with zinc, HMBA had no effect on $^{32}PO_4$ incorporation into pp 21–2 in the absence or presence of 8-bromo-cAMP (data not shown).

Effects of HMBA on the Amount of PP 21–2 Protein—To assess whether HMBA induced an increase in the amount of pp 21–2 protein, we performed two separate sets of experiments. First, we compared [^{35}S]methionine and [^{35}S]cysteine incorporation into pp 21–2 in untreated and HMBA-treated cells (Figs. 5, *a* and *b*, respectively; 1 mM 8-bromo-cAMP was added during the last 30 min of incubation to convert most of pp 21–2 to the phosphorylated state). And second, we quantitated pp 21–2 protein by Western blot in extracts of untreated and HMBA-treated cells (Fig. 6, *a* and *b*, respectively) using the monoclonal antibody 142–24E5 which we show later recognizes pp 21–2; the extracts were treated with [γ-^{32}P]ATP and the C subunit of A-kinase to convert most of pp 21–2 to the phosphorylated state and to assess complete transfer of the labeled protein to the membrane. Neither methionine/cysteine incorporation into pp 21–2 nor the amount of pp 21–2 detected by Western blot was significantly increased in HMBA-treated cells indicating that HMBA did not increase the amount of pp 21–2.

Activity of A-kinase C Subunit and Holoenzyme in HMBA-treated Cells—The intracellular cAMP concentration has been reported to increase severalfold within 8 h of adding HMBA to MEL cells (10); we, however, found that the activity of the free C subunit of A-kinase was the same in parental cells cultured for 1–16 h in the absence or presence of HMBA (data not shown). As previously reported (7), we found that HMBA increased A-kinase holoenzyme activity 15 and 50% at 6 and 18 h,

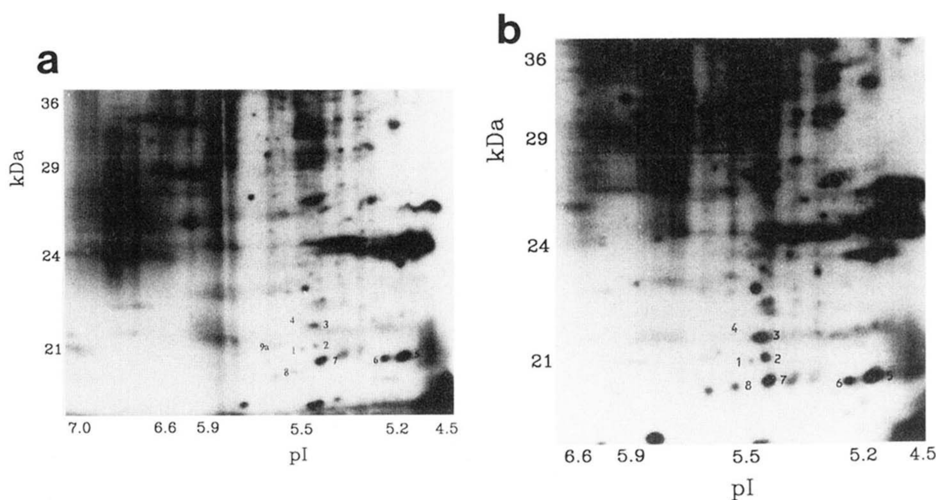

FIG. 4. **Effect of HMBA on protein phosphorylation in intact cells.** Parental cells were incubated for 16 h in the presence of 4 mM HMBA with [^{32}P]orthophosphate added during the last 3 h of incubation; to some of the cultures 1 mM 8-bromo-cAMP plus 1 mM theophylline were added during the last 15 min of incubation. *Panel a*, HMBA alone; *panel b*, HMBA plus 8-bromo-cAMP. Proteins are labeled as in Fig. 1.

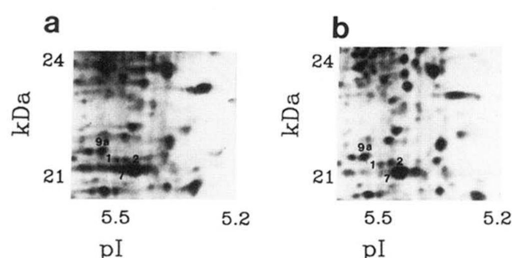

FIG. 5. **Incorporation of [^{35}S]methionine and [^{35}S]cysteine into cellular proteins.** Parental MEL cells were incubated for 12 h with [^{35}S]methionine and [^{35}S]cysteine in the absence (*panel a*) or presence (*panel b*) of 4 mM HMBA as described under "Experimental Procedures." Cell extracts were prepared and applied to 2-D gels which were exposed to x-ray film. Proteins are labeled as in Fig. 1 except the numbers *7* and *9a* are left and above, respectively, of their designated proteins.

respectively, over that in untreated cells (Fig. 7). Although the small increase in A-kinase holoenzyme activity in HMBA-treated cells appeared unlikely to explain the 3-fold increase in $^{32}PO_4$ incorporation into pp 21–2, HMBA could change the subcellular localization of A-kinase making it more accessible to pp 21–2 (31). Alternatively, HMBA could induce a post-translational modification of pp 21–2 that would make it a better A-kinase substrate.

PP 21–1 and 21–2: Binding of GTP and Reaction with Anti-Ras Antibodies—Since *ras*-related proteins have molecular masses of 20–28 kDa and play an important role in cell growth and differentiation (32, 33), we assessed whether pp 21–1 or 21–2 were *ras*-related proteins by several different methods. First, we incubated 2-D Western blots with [α-^{32}P]GTP and found that both pp 21–1 and 21–2 were GTP-binding proteins (Fig. 8).

Next, we found that the monoclonal antibody 142–24E5, which was raised against a peptide that includes the highly conserved guanine base binding domain of *ras* proteins (34), recognized pp 21–1 and 21–2 by three different methods: (i) 2-D Western immunoblotting (Fig. 6*a*); (ii) immunoprecipitation (Fig. 9*a*); and (iii) immunoaffinity chromatography (data not shown). In these experiments, cells were incubated with $^{32}PO_4$ and 8-bromo-cAMP prior to extraction; this allowed pp 21–1 and 21–2 to be identified on the immunoblots not only by their position relative to 2-D protein standards but also by comparing the immunoblot (Fig. 6*a*) with its autoradiograph (Fig. 6*c*). Antibody 142–24E5 also recognized proteins 7, 9a, and 10 on the immunoblots (Fig. 6*a*) but neither precipitated proteins 7 and 10 from cell extracts (Fig. 9*a*) nor bound these two proteins on the immunoaffinity column.

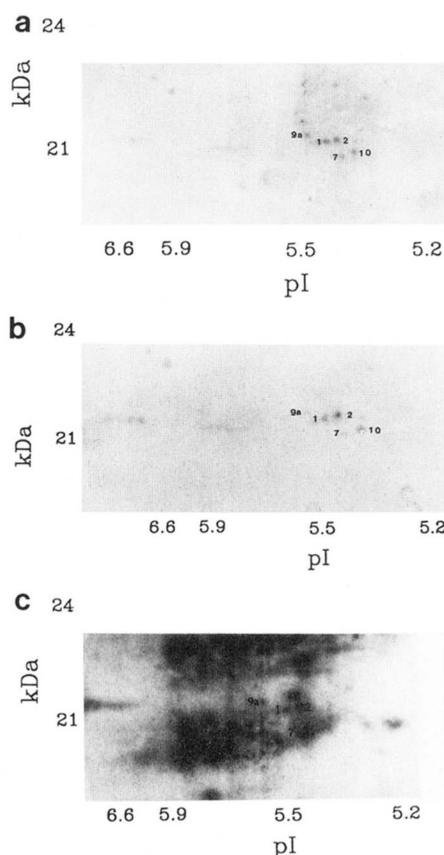

FIG. 6. **Western blot using antibody 142–24E5.** Parental cells were incubated for 16 h in the absence (*panel a*) or presence (*panel b*) of 4 mM HMBA; cell extracts were prepared, incubated with [γ-^{32}P]ATP and A-kinase, and applied to 2-D gels which were used to generate Western blots as described under "Experimental Procedures." *Panels a* and *b*, incubation of the blot with the monoclonal antibody 142–24E5 followed by an alkaline phosphatase-conjugated secondary antibody and a chromogenic substrate; *panel c*, exposure of the blot shown in *panel a* to x-ray film. Proteins *1* and *2* designate pp 21–1 and 21–2, respectively.

In a final series of experiments, we showed that the polyclonal antibodies R61 and R195, which were raised against purified Rap 1A, recognized pp 21–1 and 21–2 and protein 9a by Western immunoblotting (data not shown).

Comparison of PP 21–1 and 21–2 To Rap 1A and 1B and Expression of Rap 1A and 1B in MEL Cells—The above results indicated that pp 21–1 and 21–2 were members of the *ras* superfamily. Of all proteins in this large family only Rap 1A and 1B are clearly *in vitro* and *in vivo* A-kinase substrates; as

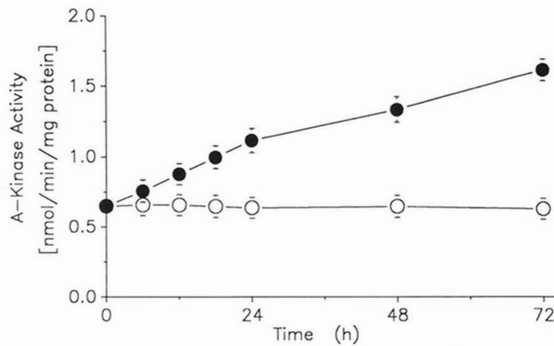

FIG. 7. **Effect of incubation time with HMBA on A-kinase activity.** Parental cells were incubated for the indicated times in the absence (*open circles*) or presence (*closed circles*) of 4 mM HMBA; A-kinase holoenzyme activity was measured as described under "Experimental Procedures." The data are the means ± S.D. (*error bars*) of three independent experiments performed in duplicate.

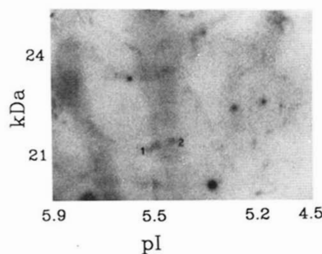

FIG. 8. **GTP-binding to phosphoproteins 21–1 and 21–2.** Extracts of parental cells were applied to 2-D gels which were blotted to polyvinylidine difluoride membranes as described under "Experimental Procedures." The membranes were incubated with [α-^{32}P]GTP and exposed to x-ray film. PP 21–1 and 21–2, proteins *1* and *2*, were identified by comparing their positions to 2-D gel protein standards stained with Amido Black on the blots.

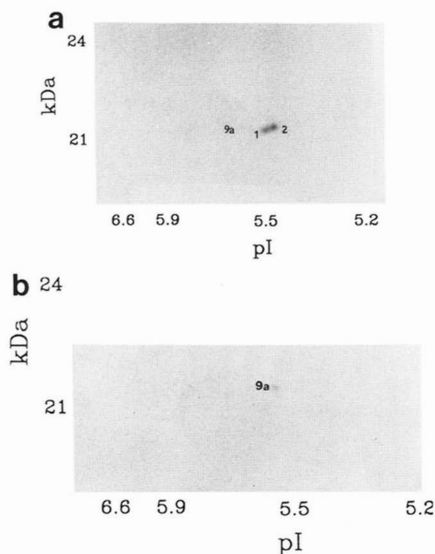

FIG. 9. **Immunoprecipitation using antibodies 142–24E5 or SC65.** Parental cells were incubated with [^{32}P]orthophosphate and 1 mM 8-bromo-cAMP as described in the legend to Fig. 1. The monoclonal antibody 142–24E5 (*panel a*) or the polyclonal antibody SC65 (*panel b*) were added to cell extracts, and immunoprecipitates were applied to 2-D gels which were exposed to x-ray film; proteins were identified using 2-D gel protein standards.

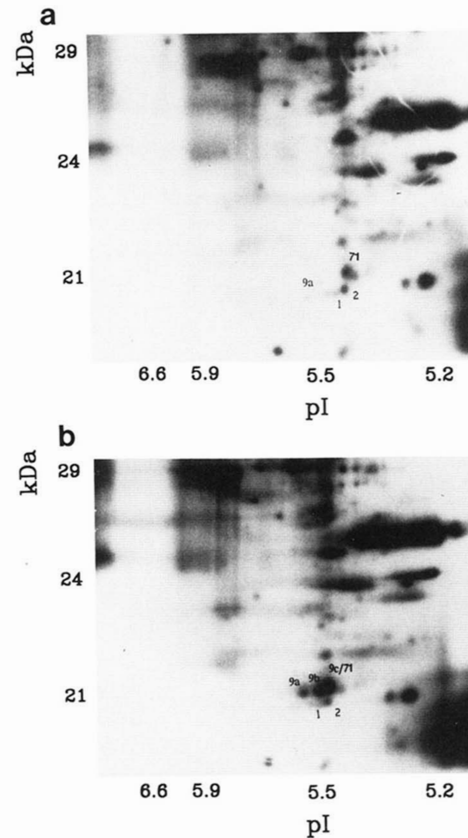

FIG. 10. **Migration of Rap 1A on 2-D gels.** Extracts of parental cells that had been incubated with [^{32}P]orthophosphate and 1 mM 8-bromo-cAMP were prepared as described in the legend to Fig. 1. Recombinant Rap 1A produced in insect cells was phosphorylated by A-kinase and [γ-^{32}P]ATP and added to half of the cell extracts. The extracts were applied to 2-D gels which were exposed to x-ray film. The acrylamide percentage was 15% in the second dimension which changed the configuration of proteins relative to that in other figures. *Panel a*, cell extract without added Rap 1A; *panel b*, cell extract with added Rap 1A. The three forms of Rap 1A are labeled as *9a*, *9b*, and *9c*. Proteins *1* and *2*, pp 21–1 and 21–2, respectively are labeled from below. Protein *71* may be protein *3* in Figs. 1 and 4.

discussed later, it is less clear whether H-Ras and K-Ras with exon 4B are A-kinase substrates *in vivo* (14–20, 35). Because antibodies R61 and R195 recognized pp 21–1 and 21–2 by immunoblotting, it seemed possible that pp 21–1 and 21–2 might be related to or identical with Rap 1A or 1B, although the latter two proteins do not bind GTP on Western blots (28, 36).[2] To assess the possible identity of pp 21–1 or 21–2 with Rap 1A or 1B, we performed three different sets of experiments. First, we determined where purified Rap 1A and 1B migrated on 2-D gels relative to pp 21–1 and 21–2. In these experiments we incubated recombinant human Rap 1A produced in insect cells or Rap 1B purified from human platelets with the C subunit of A-kinase and [γ-^{32}P]ATP and added the phosphorylated product to extracts of parental cells that had been incubated previously with [^{32}P]orthophosphate. As previously reported, we found that Rap 1A produced in insect cells yielded three separate protein-spots in 2-D gels (14); since Rap 1A is known to have only one phosphorylation site these three spots presumably represent different degrees of other post-translational modifications of the protein (14, 37). One of the forms of Rap 1A comigrated with protein 9a shown in Figs. 1, 2, and 4, and the other two forms of Rap 1A migrated with a slightly higher molecular mass than pp 21–1 and 21–2. To increase the resolution between Rap 1A and pp 21–1 and 21–2, we increased the acrylamide percentage of the second dimension gel to 15% and found that all three forms of Rap 1A were well separated from pp 21–1 and 21–2 (compare Fig. 10*a*, no added Rap 1A, to Fig. 10*b*, Rap 1A added; the three forms of Rap 1A are labeled as *9a*, *9b* and *9c*). We found that human platelet Rap 1B yielded only one spot that comigrated with protein 7 in the 2-D gel system with 12% acrylamide in the second dimension (data not shown).

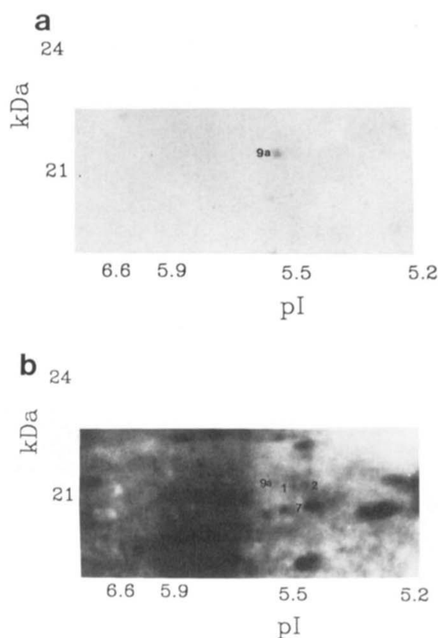

FIG. 11. **Western blot using antibody R64.** MEL cell extracts incubated with [γ-^{32}P]ATP and A-kinase were applied to 2-D gels which were used to generate a Western blot as described under "Experimental Procedures." *Panel a*, incubation of the blot with the polyclonal antibody R64, an alkaline phosphatase-conjugated secondary antibody and a chromogenic substrate; *panel b*, exposure of the blot to x-ray film.

In the second set of experiments, we used the polyclonal antibodies SC65 and R64 which were raised against Rap 1-specific peptides. We found that neither SC65 nor R64 recognized pp 21–1 or 21–2 on Western blots although they did recognize protein 9a (Fig. 11*a*; only the data with R64 are shown); from the autoradiograph of the immunoblot it was clear that pp 21–1 and 21–2 were transferred to the membrane (Fig. 11*b*). We also performed immunoprecipitation experiments on cell extracts that had been incubated with the C subunit of A-kinase and [γ-^{32}P]ATP and found that SC65 precipitated protein 9a from cell extracts but not pp 21–1 or 21–2 (Fig. 9*b*). In the final set of experiments, we compared one-dimensional phosphopeptide maps of pp 21–2 with those of purified Rap 1A and 1B. In these experiments cell extracts were incubated with [γ-^{32}P]ATP and the C subunit of A-kinase and applied to 2-D gels; the spot corresponding to pp 21–2 was cut out and the protein subjected to digestion by protease V8. We found that pp 21–2 produced a distinctly different phosphopeptide pattern than either Rap 1A or 1B (Fig. 12; only one concentration of V8 is shown but similar results were found at several other V8 protease concentrations).

Protein 9a appears to be Rap 1A based on several observations: (i) it was phosphorylated by A-kinase both *in vivo* and *in vitro* (Figs. 1 and 2); (ii) it was detected by Rap 1-specific antibodies (Figs. 9*b* and 11); and (iii) it comigrated with the most basic form of purified Rap 1A (Fig. 10). Protein 7 could represent Rap 1B since purified Rap 1B comigrated with protein 7 and antibody 142–24E5 recognized protein 7 (Fig. 6). However, protein 7 did not show *in vivo* (Fig. 1) or *in vitro* (Fig. 2) phosphorylation by A-kinase, and it was not recognized by the two Rap 1-specific antibodies. Thus, it is more likely that protein 7 does not represent Rap 1B but rather some other *ras*-related protein. Consistent with this hypothesis we could not detect Rap 1B mRNA in MEL cells by Northern blotting although we did detect Rap 1A mRNA (data not shown; both Rap 1A and 1B mRNA were detected easily in HL-60 cells).

Comparison of PP 21–1 and 21–2 to H- and K-Ras—We found that recombinant H-Ras did not comigrate on 2-D gels with pp 21–1 or 21–2 but rather migrated very close to protein 10 shown in Fig. 6*a*; small differences in migration between the recombinant protein produced in bacteria and the cellular protein could be secondary to differences in post-translational modifications. Moreover, the monoclonal antibody Y13–259 did not precipitate pp 21–1 or 21–2 from cell extracts; this antibody is specific for H-, K- and N-Ras, and we showed in preliminary experiments that it was highly efficient in immunoprecipitating H-Ras added to cell extracts.

FIG. 12. **Phosphopeptide maps of Rap 1A and 1B and pp 21–2.** MEL cell extracts incubated with [γ-^{32}P]ATP and the C subunit of A-kinase were applied to 2-D gels, and the spot corresponding to pp 21–2 was cut out and digested with V8 protease as described under "Experimental Procedures." Recombinant human Rap 1A produced in insect cells and human platelet Rap 1B were treated identically. The resulting peptides were separated by one-dimensional PAGE (*lane a*, Rap 1A; *lane b*, pp 21–2; *lane c*, Rap 1B). The three different forms of Rap 1A (proteins *9a*, *9b*, and *9c* in Fig. 10) yielded the same phosphopeptide map.

DISCUSSION

By 2-D PAGE analyses of proteins phosphorylated *in vivo*, we identified two low molecular weight proteins, pp 21–1 and 21–2, that undergo cAMP-dependent phosphorylation. We found that pp 21–1 and 21–2 were poorly phosphorylated in response to cAMP in several A-kinase-deficient cells and that the degree of phosphorylation of these two proteins correlated with the cell's A-kinase activity. We showed by three different sets of *in vitro* experiments that these two proteins were direct A-kinase substrates. We did not address the question of whether pp 21–1 and 21–2 are substrates of other protein kinases as well, but their degree of phosphorylation was very low in the basal non-A-kinase-stimulated state.

The amount of $^{32}PO_4$ incorporation into pp 21–2 increased 3-fold within the first 16 h of adding HMBA to parental MEL cells without an increase in the amount of pp 21–2 protein. This increase in $^{32}PO_4$ incorporation could occur without an increase in phosphorylation of pp 21–2 if HMBA induced dephosphorylation of pp 21–2 early after addition, thereby allowing more $^{32}PO_4$ incorporation during the later $^{32}PO_4$-labeling period; however, this mechanism is only possible if the dephosphorylation were a transient phenomenon. It seems more likely that the increase in $^{32}PO_4$ incorporation into pp 21–2 in HMBA-treated cells represented an actual increase in phosphorylation of pp 21–2. Since increased phosphorylation of pp 21–2 was not observed in the A-kinase-deficient cells treated with HMBA, it appeared that this HMBA-induced increase in pp 21–2 phosphorylation was mediated by A-kinase. There are at least two possible mechanisms to explain these data in the absence of a major increase in A-kinase activity. First, HMBA could increase the likelihood that pp 21–2 was phosphorylated by A-kinase by inducing a change in the subcellular localization of either protein; a model of A-kinase compartmentalization with creation of "target sites" for cAMP action has been proposed in other cell

types (31). And second, HMBA could induce a post-translational modification of pp 21–2 that would make it a better A-kinase substrate. Both models are consistent with the observation that the combination of HMBA and 8-bromo-cAMP yielded more phosphorylation of pp 21–2 than either agent alone (Table I and Figs. 1 and 4).

We found that pp 21–1 and 21–2 bound GTP on Western blots and by immunoblotting, immunoprecipitation, and immunoaffinity chromatography they appeared to be *ras*-related proteins, possibly related to Rap 1A or 1B. Rap 1A and 1B are clearly A-kinase substrates since *in vitro* they are phosphorylated stoichiometrically by A-kinase, and *in vivo* they undergo cAMP-dependent phosphorylation (14, 18, 36, 38). By four different criteria it appeared that pp 21–1 and 21–2 were distinct from Rap 1A and 1B: (i) pp 21–1 and 21–2 bound GTP on Western blots whereas Rap 1A and 1B do not[2] (28, 36); (ii) two antibodies raised against a specific amino acid sequence in the carboxyl terminus of Rap 1A and 1B did not recognize pp 21–1 or 21–2 on Western blots while they recognized endogenous Rap 1A; (iii) phosphopeptide maps of pp 21–2 were different from those of Rap 1A and 1B; and (iv) recombinant human Rap 1A expressed in insect cells (which are capable of all known post-translational modifications of *ras*-related proteins (23, 37, 39)) and Rap 1B purified from human platelets did not comigrate with pp 21–1 or 21–2 on 2-D gels. The mouse *rap* 1A gene has been cloned recently, and the deduced amino acid sequence is identical to that of the human protein (40). Moreover, by Northern blot analysis it appeared that there is very little if any Rap 1B expressed in MEL cells. The only other *ras* proteins that appear to be A-kinase substrates are H-Ras, which can be phosphorylated *in vitro* by A-kinase but does not show cAMP-dependent phosphorylation *in vivo* (19), and K-Ras exon 4B which is non-stoichiometrically phosphorylated by A-kinase *in vitro* (<0.1 mol PO_4/mol protein) and undergoes a small degree of cAMP-dependent phosphorylation *in vivo* when expressed at extremely high levels (19, 20). It is unlikely that pp 21–1 or 21–2 are H- or K-Ras because pp 21–1 and 21–2: (i) were good A-kinase substrates, both *in vitro* and *in vivo*; (ii) were not precipitated from cell extracts by antibody Y13–259; and (iii) were recognized by antibodies R61 and R195 on Western blots. Thus, pp 21–1 and 21–2 are either novel Ras-related proteins or are previously described members of the Ras superfamily not known to be A-kinase substrates. A search of the amino acid sequences of Ras-related proteins revealed that Rho A, Rab 8, *Ral* A and B, and G25K/CDC42Hs all have a potential A-kinase substrate consensus sequence. Since pp 21–1 and 21–2 reacted with antibodies R61 and R195, which were raised against Rap 1A, it seems unlikely that they are one of these other Ras-related proteins which are distantly related to the Rap family. Since pp 21–1 and 21–2 migrated very close to each other on 2-D gels and reacted with the same antibodies, it is possible that they are two different post-translationally modified forms of the same protein.

It should be noted that we observed several other proteins that underwent cAMP-dependent phosphorylation in parental MEL cells that were not phosphorylated in the A-kinase-deficient cells; any one of these proteins could be important to differentiation of MEL cells. Ras-related proteins are known to be involved in regulating cell growth and differentiation (32, 33) but have received little attention in MEL cells; one group found several low molecular weight GTP-binding proteins in the cytosol and particulate fractions of undifferentiated MEL cells that were isoprenylated and carboxymethylated (41, 42). One of these proteins was subsequently found to be G25K/CDC42Hs, but the others were not characterized immunologically nor were they studied during differentiation of MEL cells (42, 43). It is difficult to compare our results with their work because of technical differences, but two of the proteins they identified may represent pp 21–1 and 21–2 (42).

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