Dual Regulation of Intermediate Filament Phosphorylation

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ABSTRACT Intermediate filament proteins have been isolated from ME-180, cells of a human cervical carcinoma. Eight of these proteins have been identified as keratins by immunologic cross-reactivity to antibodies raised against authentic human epidermal keratins. The ME-180 keratin proteins consist of two major subunits designated MEK-1 and MEK-2 with approximate molecular weights of 58,000 and 53,000, respectively, and six minor subunits of 59, 57, 52.5, 50.5, 45, and 40 kilodaltons. When ME-180 cells were incubated for 2–24 h in the presence of [^{32}P]orthophosphate, MEK-1 and MEK-2 as well as the 52.5- and 40-kilodalton keratins were phosphorylated at their serine residues. V8 protease digests revealed that phosphorylation of MEK-2 is restricted to one peptide representing approximately half the molecule.

Regulation of MEK-1 and MEK-2 phosphorylation has been studied by prelabeling the cells for 2 h in ^{32}P-labeled medium. This was followed by up to 2 h of continued incubation in the same medium after the addition of a variety of perturbing agents. The phosphorylation of MEK-2 increased in the presence of 10^{-4} M dibutyryl cyclic AMP (twofold), 1 mM methylisobutylxanthine (2.5-fold), 10^{-5} M isoproterenol (fivefold), and 10^{-9} M cholera toxin (sevenfold). In contrast, MEK-1 phosphorylation was unaffected by these agents. Neither cyclic GMP, Ca^{++}, hydrocortisone, nor epidermal growth factor had any effect on the phosphorylation of MEK-1 or MEK-2. The results indicate that the phosphorylation of these two keratins is independently controlled by cyclic AMP–dependent kinase for MEK-2 and by cyclic nucleotide–independent kinase for MEK-1. The observed differences in control suggest distinct functions for MEK-1 and MEK-2 within the cytoskeletal network.

Desmin, vimentin, glial acidic fibrillary protein, and neurofilament protein are the monomers of the intermediate filament systems of muscle, mesenchyme, glial cells, and neurons, respectively (1). These proteins are distinct biochemically although immunologic and structural homologies have been described (2, 3). They are each modified posttranslationally by phosphorylation and in some cases cyclic AMP (cAMP)[1] stimulation of phosphorylation has been demonstrated (4). The relationship of this modification to filament function is not known.

In contrast, the intermediate filaments of epithelial cells are composed of polymeric subunit proteins designated collectively as keratins. We have previously demonstrated that rat epidermal keratins are phosphorylated in vivo and in vitro (5, 8). Keratin phosphorylation has also been described in human, mouse, and bovine epidermis (5, 6), in keratinocytes in culture (7), and in esophageal epithelium (8).

The mechanism by which keratin phosphorylation is regulated in these tissues is not known. To examine the cellular control of this process, we have chosen to study the epithelial cell line ME-180 derived from a human cervical carcinoma (9). Use of this cell culture system has allowed us to screen a variety of potential regulatory agents.

In this communication we demonstrate that MEK-1 and MEK-2, the two predominant keratin filament subunits of ME-180 cells, are both phosphoproteins. The phosphorylation of MEK-1 is regulated by cyclic nucleotide–independent kinase(s) while phosphorylation of MEK-2 is stimulated by cAMP.

MATERIALS AND METHODS

Cell Culture: Initial cultures of ME-180 cells were a gift of Dr. John Sykes (Southern California Cancer Center) (9). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% bovine calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY). Cultures were maintained at 37°C in an atmosphere of 5% CO_2, 95% air.

[1] *Abbreviations used in this paper:* cAMP, cyclic AMP; BME, β-mercaptoethanol; MIX, methylisobutylxanthine; NP-40, Nonidet P-40; PAP, peroxidase-antiperoxidase; PMSF, phenylmethylsulfonyl fluoride.

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Isolation of ^{32}P-labeled Intermediate Filament Proteins: Cells were grown in 60-mm dishes to ~75% confluence and labeled for the times indicated below at 37°C in medium containing 100 μCi/ml carrier-free [^{32}P]orthophosphate (Amersham Corp., Arlington Heights, IL). ME-180 cells were harvested by being scraped with a rubber policeman and washed in PBS to remove residual medium. The packed cell volume was noted and the cells were processed at 4°C. They were lysed in 20 vol of Tris-Triton buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride [PMSF], 1% Triton X-100) and the buffer-insoluble pellet was collected by centrifugation at 12,000 *g* for 5 min. The pellet was washed three times in 50 vol of wash buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF) and re-extracted with Tris-Triton buffer. The insoluble pellet was again washed as described above and resuspended in 1 M KCl, 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF and incubated at 37°C for 30 min with intermittent agitation. The insoluble material was collected by centrifugation and washed three times. The pellet was extracted at 37°C for 30 min in 9 M urea, 100 mM β-mercaptoethanol (BME), 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF. Insoluble material was pelleted and the supernatant was analyzed as described below.

Electrophoresis and Autoradiography: The urea-BME-soluble proteins of ME-180 cells were analyzed by one-dimensional gel electrophoresis in the system of Laemmli (10) (SDS PAGE) and by two-dimensional electrophoresis as described by O'Farrell (11). Gels were stained in 0.2% Coomassie Brilliant Blue (Bio-Rad Laboratories, Richmond, CA) in 25% methanol and 10% acetic acid, destained in methanol-acetic acid, and dried in a Hoefer gel dryer. Gels were autoradiographed using Kodak XAR film to localize radioactivity.

Peptide Mapping: Individual keratin proteins were excised from duplicate tracks of an SDS polyacrylamide gel and subjected to limited proteolysis according to the procedure described by Cleveland et al. (12). The gel slices were washed extensively in water to remove residual methanol acetic acid and then incubated in 0.4 M Tris-HCl, pH 6.8, 7.5% dithiothreitol, 10% SDS, and 50% glycerol for 30 min at room temperature.

Two excised gel slices containing 10 μg of protein per slice were placed in each of four wells of a 14 × 22 cm Cleveland gel (12) and overlayered with 25 μl of a solution of 80 mM Tris-HCl, pH 6.8, 1.5% dithiothreitol, 2% SDS, and 10% glycerol containing 0, 10, 50, 500, or 1000 ng of *Staphylococcus aureus* V8 protease. The samples were electrophoresed at 20 mA per gel until the tracking dye reached the interface of the stacking and resolving gels. Current was stopped for 30 min for continued digestion and then electrophoresis was resumed. The gel was stained, destained, dried, and autoradiographed as described above.

Immunologic Characterization of ME-180 Keratins: Keratin subunits solubilized in urea-BME were identified by immunoprecipitation and immunoblot analysis as described below. All antisera were raised against the keratins of human callus.

For immunoprecipitation we used polyclonal antikeratin antiserum raised in guinea pig (13). Preimmune serum was from the same guinea pig that subsequently produced the antiserum.

For immunoblot analyses we used a commercial antikeratin antiserum (polyclonal, Lot #006C, Transformation Research, Inc., Framingham, MA). We confirmed the specificity of this rabbit anti-human keratin and found that there was no cross-reactivity with vimentin or actin in immunoblot analysis or immunoprecipitation (data not shown). We also used the monoclonal antikeratin antisera designated AE1 and AE3 (14), which were generous gifts of Dr. T.-T. Sun (New York University Medical Center).

Immunoprecipitation of ME-180 Keratins: ME-180 cells were incubated for 48 h in medium containing [^{35}S]methionine at 30 μCi/ml (Amersham Corp., 1135 Ci/mmol). Urea-BME-soluble proteins were isolated as described above. The immunoprecipitation procedure is a modification of that of Kessler (15). Each 5-μl aliquot contained ~10^5 cpm of trichloroacetic acid–precipitable protein (30–50 μg).

Duplicate aliquots of 5 or 10 μl were incubated for 16 h at 4°C in the presence of 50 μl of buffer (0.1% SDS, 0.5% Nonidet P-40 (NP-40), 10 mM Tris-HCl, pH 7.4) and 5 μl of preimmune guinea pig serum. Incubation was continued for 30 min at 0°C after the addition of 50 μl of a 10% suspension of Protein A (IgGsorb, The Enzyme Center, Inc. (IgGsorb, The Enzyme Center, Inc., Boston, MA) in buffer (0.1% SDS, 5 mM EDTA, 0.15 M NaCl, and 50 mM Tris-HCl, pH 7.4). Protein A was pelleted by centrifugation for 1 min in an Eppendorf microfuge (15,000 *g*). The supernatant was collected into tubes containing 5 μl of antikeratin antiserum or preimmune serum and incubated for 1 h at 0°C. 50 μl of Protein A was added and incubation was continued for 30 min at 0°C. Protein A was collected by centrifugation and the supernatants were collected and stored. This Protein A pellet was washed twice in each of the following sequence of buffers: (*a*) 0.1% SDS, 0.5% NP-40, 10 mM Tris-HCl, pH 7.4; (*b*) 0.5% NP-40, 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 5 mM EDTA; 0.5% NP-40, 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM EDTA.

The final pellet was resuspended in 20 mM Tris-HCl, pH 7.4, 2% SDS and 20 mM BME and heated at 100°C for 3 min. After centrifugation, the supernatant was collected and analyzed by SDS PAGE.

Immunoblot Analysis of ME-180 Keratins: The procedure is essentially that described by Towbin et al. (16). The urea-BME-soluble proteins were separated by two-dimensional PAGE before electrophoretic transfer to nitrocellulose paper (Millipore Corp., Bedford, MA; HAWP-304-FO). Transfer was accomplished in 2–3 h at 300 mA at 4°C using a model EC215 Electroblot System (E-C Apparatus Corp., St. Petersburg, FL).

We visualized the transferred proteins by staining the blot for 10 min in 0.1% fast green in 40% methanol-10% acetic acid and destaining it in methanol-acetic acid. Residual methanol acetic acid was removed by washing in PBS and the blot was photographed using a Kodak No. 25 red filter (14).

To localize the keratin proteins, we processed the fast green–stained blot as described by Sternberger (17) and Glass et al. (18). The blot was incubated at 37°C for 30 min in 3% BSA (Sigma Chemical Co., St. Louis, MO) in PBS and then in 4% goat serum (Gibco Laboratories) in BSA/PBS for 30 min. After being washed in three changes of PBS at room temperature, the paper was exposed to an appropriate dilution of polyclonal or monoclonal antikeratin antiserum or to preimmune serum. Following a 60-min incubation at 37°C, the blot was washed in PBS and immersed in a 1/20 dilution of either goat anti-rabbit or goat anti-mouse IgG (Miles-Yeda, Miles Laboratories Inc., Elkhart, IN) for 30 min at 37°C. After being washed in PBS, the paper was incubated in a 1/80 dilution of rabbit (Miles-Yeda) or mouse (Sternberger-Meyer, Jarrettsville, MD) peroxidase-antiperoxidase (PAP) for 60 min at 30°C. Unbound PAP was removed by washing in 50 mM Tris-HCl, pH 7.4. The antigen-antibody complexes were visualized by exposing the blot to a solution of 0.5% 3,3′-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl, pH 7.4, containing 8 μl of 30% H_2O_2 per 50 ml. The blot was washed in running water, air dried, and photographed using a Kodak No. 47-B filter.

Analysis of Phosphoamino Acids: The ^{32}P-urea-BME-soluble proteins were freed of nonprotein phosphate as described by Britte and Kabat (19). The resulting protein precipitate was subjected to limited acid hydrolysis in 6 N HCl (Pierce Chemical Co., Rockford, IL) at 105°C for 2 h. The hydrolysate was evaporated to dryness and the residue was resuspended in 2.5% formic acid, 7.8% acetic acid, pH 1.9. A 5-μg aliquot was electrophoresed on Whatman 3MM paper (Whatman Laboratory Products Inc., Clifton, NJ) at 2,000 V for 2 h. 5-μg aliquots of *O*-phosphoserine and *O*-phosphothreonine (Sigma Chemical Co.) served as standards and were localized by spraying the chromatogram with cadmium-ninhydrin (20).

Perturbation of Intermediate Filament Phosphorylation: ME-180 cells were prelabeled with [^{32}P]orthophosphate for 2 h in serum-free medium as described above. The cells were then exposed to a single concentration of a given perturbant for periods of up to 2 h. The concentrations at which each perturbant was tested are as follows: N^6, O^2-dibutyryladenosine 3′,5′-cyclic phosphate (Sigma), 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M; 8-bromoguanosine 3′,5′-cyclic phosphate (P-L Biochemicals, Inc., Milwaukee, WI), 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} M; methylisobutylxanthine (MIX, Sigma), 0.2, 0.5, 1, 2 mM; cholera toxin (Sigma), 10^{-7}, 10^{-9}, 10^{-11} M; isoproterenol (Sigma), 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M; hydrocortisone (Sigma), 0.1, 0.5, 1, 5 μg/ml; epidermal growth factor (gift of Dr. Lloyd King, Vanderbilt University School of Medicine), 0.1, 1, 10, 100 ng/ml; and calcium chloride (Fisher Scientific Co., Pittsburgh, PA), 0.075, 0.15, 1.0, 2.0 mM. When calcium was the perturbant, the cells were grown in calcium-depleted medium as described by Hennings et al. (21).

Solutions of perturbants were prepared in calcium and magnesium-free Hanks' basic salt solution (Gibco). Control cultures received equivalent volumes of this solution. Incubation was continued and duplicate 60-mm Petri dishes were processed at 0, 15, 30, 60, and 120 min after addition of the perturbant. To stop the reaction, the labeled medium was removed, the culture dishes were rapidly washed in PBS, and the cells were lysed in buffer (0.1 M Na_2HPO_4, pH 7.5, 1% Triton X-100, 0.1 M NaF, 1 mM EDTA, 1 mM PMSF). The buffer-insoluble proteins (filament fraction) were collected by centrifugation at 15,000 *g* for 1 min in an Eppendorf microcentrifuge, resuspended in SDS (2% SDS, 5% BME, 0.06 M Tris-HCl, pH 6.8), heated to 100°C for 3 min, and analyzed by SDS PAGE and autoradiography as described below.

Quantitation of Changes in Keratin Phosphorylation: The specific activity of the radiolabeled phosphokeratins in the presence or absence of each perturbant was determined. The urea-BME extract of ME-180 cells was subjected to SDS PAGE and the Coomassie Blue–stained gel was scanned using a Beckman spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) equipped with a Gilford linear transport densitometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). The peak area corresponding to a ^{32}P-labeled protein band was determined. The gel was dried and autoradiographed to obtain a visual record of the phosphorylation patterns. The protein bands were excised from the dried gels and analyzed in Econofluor in a Packard Prias liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, IL) to quantitate the incorporated radioactivity. Specific activities were

calculated as counts per minute per unit area of protein. Values were determined at each time point for experimental and control samples. The change in phosphorylation has been expressed as the ratio of specific activities of experimental to control values.

RESULTS

The filament subunits of ME-180 cells were isolated by extracting the cells sequentially in nonionic detergent, 1 M KCl and urea-BME, at pH 7.4. The keratins were identified by immunoprecipitation. Fig. 1 displays the autoradiographic pattern of [^{35}S]methionine-labeled ME-180 proteins and the components that react with antikeratin antiserum. Keratins of 59, 58, 57, 53, 50.5, 45, and 40 kdaltons were precipitated. The major keratins have been designated MEK-1 (58,000 daltons) and MEK-2 (53,000 daltons).

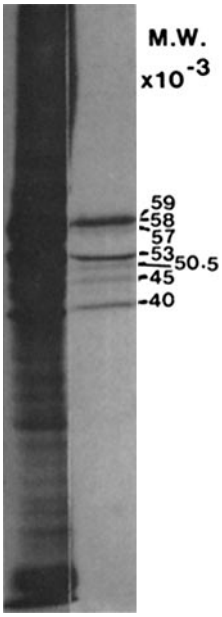

FIGURE 1 Immunoprecipitation of ME-180 keratins. ME-180 cells were labeled with [^{35}S]methionine and fractionated as described in Materials and Methods. (*left lane*) Autoradiogram of an aliquot of the urea-BME-soluble proteins. Approximately 105,000 trichloroacetic acid-precipitable cpm were loaded in 5 μl. (*right lane*) Autoradiogram of the ^{35}S-labeled keratins precipitated from the urea-BME proteins with guinea pig antiserum against the keratins of human callus. The precipitate represents 4% of the cpm of the starting aliquot.

Two-dimensional electrophoretic analysis was undertaken to further define the ME-180 keratins and to make certain that we could separate the keratins from other intermediate filament proteins such as vimentin. Fig. 2*a* indicates that several keratins of ME-180 cells exhibit isoelectric heterogeneity similar to that described for keratin subunits from other tissues (23–26). Immunoblot analysis (Fig. 2, *b* and *c*) of the two-dimensional gel demonstrates that the multiple isoelectric variants of the 58- (MEK-1), 53- (MEK-2), 52.5- (not resolved from the 50.5-kdalton keratin in Laemmli gels), and 40-kdalton proteins as well as the single component at 50.5 kdaltons react with antikeratin antisera.

Two-dimensional electrophoretic analysis of ^{32}P-labeled keratins of ME-180 cells (Fig. 3) demonstrates that [^{32}P] phosphate is associated with variants of MEK-1 (58 kdalton), MEK-2 (53 kdalton), and the 52.5- and 40-kdalton keratins. [^{32}P]phosphoserine was the only labeled amino acid detected in hydrolysates of the keratin proteins (Fig. 4). The 59-, 57-, 50.5-, and 45-kdalton keratins incorporate little if any $^{32}PO_4$. The 59- and 57-kdalton keratins that migrate as several isoelectric variants, some of which are more basic than the 58-kdalton keratins, are not detectable by Coomassie Blue staining at the protein concentrations used in Figs. 2 and 3.

To define the relationship between MEK-1 and MEK-2, we separated the proteins by SDS PAGE and compared them by peptide mapping (Fig. 5). MEK-1 (Fig. 5, *a* and *b*) is readily digested by 10 ng of V8 protease into two sets of peptides, one ranging from 22–28 kdaltons and the other from 3 to 15 kilodaltons. At 500 ng of V8 protease, all resultant peptides are in the 3–15-kdalton range and, as can be seen in Fig. 5*b*, the phosphate is localized primarily to the 15-kdalton peptide.

Digestion of MEK-2 with 10 ng of *S. aureus* V8 protease (Fig. 5*c*) yielded two peptides with apparent molecular weights of 25,000 and 24,000. The 24-kdalton peptide was resistant to further digestion by amounts of protease up to 500 ng but was hydrolyzed to small species by a dose of 1 μg. The low molecular weight proteolytic products of the 1-μg

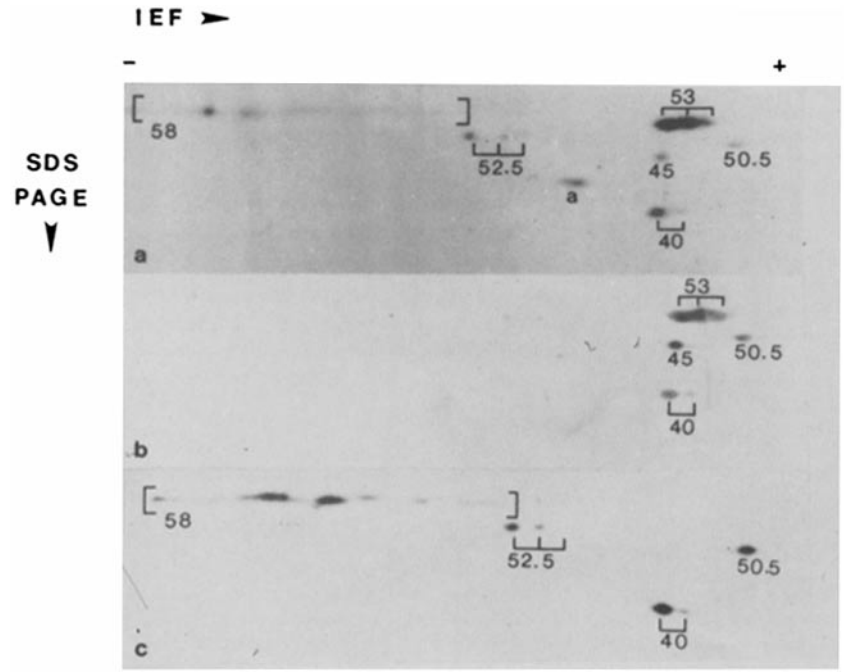

FIGURE 2. Immunoblot analysis of ME-180 keratins. The urea-BME-soluble proteins of ME-180 were resolved by two-dimensional PAGE. The pH gradient extended from pH 4.0 to pH 7.0. Proteins from duplicate gels were transferred electrophoretically to nitrocellulose paper. (*a*) Electroblot stained with fast green. The 53-kdalton protein (MEK-2) isolates as three variants between pI 5.3–5.5 The 52.5-kdalton protein, which cannot be resolved from MEK-2 on SDS PAGE, also isolates as three variants. The 40-kdalton protein focuses as one major and one minor variant. The 58-kdalton protein (MEK-1) isolates as multiple variants, the concentration of most of which are below the sensitivity of the fast green stain. (*b*) The same electroblot exposed to the commercial antikeratin antiserum and PAP. The blot was photographed to show only the PAP-reactive proteins as described in Materials and Methods. The antiserum reacts with all variants of the 53- (MEK-2), 50.5-, 45-, and 40-kdalton proteins. (*c*) A duplicate electroblot exposed to a mixture of the two monoclonal antikeratin antisera, AE1 and AE3. The antisera react with multiple variants of the 58-(MEK-1), 52.5-, and 40-kdalton proteins and with the single component at 50.5 kdaltons. Note that actin, seen in *a*, co-isolates with the ME-180 keratins but does not cross-react with any of the antikeratin antisera. *IEF*, isoelectric focusing. Molecular weight × 10^{-3}.

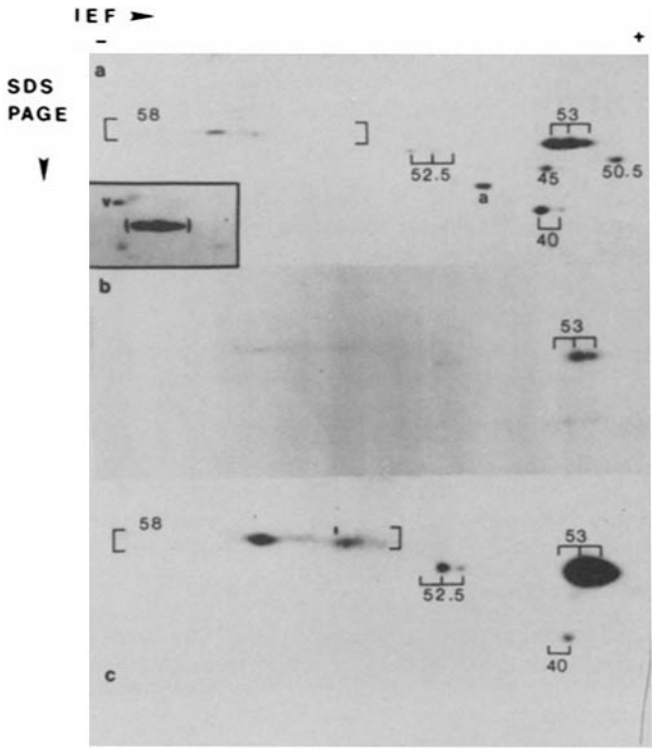

FIGURE 3 Two-dimensional electrophoretic analysis of ^{32}P-ME-180 phosphokeratins. (a) Coomassie Blue-stained gel of the urea-BME-soluble fraction of ^{32}P-labeled ME-180 cells subjected to isoelectric focusing (*IEF*) over a pH gradient from 4.0(+) to 7.0(−). *b* and *c* are two autoradiographic exposures of *a*. The short exposure (*b*) shows that the phosphorylation of the 53,000-dalton keratin (MEK-2) is localized to the two most acidic variants. The longer exposure (*c*) visualizes phosphorylated variants of the 58- (MEK-1), 52.5-, and 40-kdalton keratins. *Inset:* position of migration of vimentin (*V*) relative to the 53-kdalton keratin. Molecular weight × 10^{-3}.

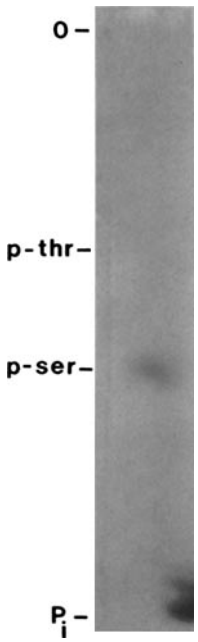

FIGURE 4 The urea-BME proteins of ^{32}P-labeled ME-180 cells were freed of nonprotein phosphate and hydrolyzed as described in Materials and Methods. High voltage electrophoresis and autoradiography revealed the presence of [^{32}P]phosphoserine (*p-ser*) but neither phosphothreonine (*p-thr*) nor phosphotyrosine, which co-migrate in this system.

digestion were clearly visualized in the radioautograms of protease-digested, ^{14}C-labeled MEK-2 shown in Fig. 5*c*. The autoradiogram of ^{32}P-labeled MEK-2 (Fig. 5*d*) indicated that the phosphate associated with this keratin is initially localized to the 25-kdalton, protease-sensitive peptide which represents approximately one-half of the molecule. When the dose of enzyme is raised, the phosphate is on three low molecular weight peptides.

To study the control of phosphorylation in these cells, we cultured ME-180 cells in the presence of agents that modulate the activity of protein kinases and determined the effect upon the extent of MEK-1 and MEK-2 phosphorylation. Phosphorylation of ME-180 keratins in the presence of 10^{-4} M cAMP and 0.2 mM MIX indicated an apparent stimulation of MEK-2 phosphorylation (Fig. 6). To quantitate this change, we have calculated the specific activity of MEK-2 and MEK-1 in the presence or absence of cAMP and other perturbants. The data summarized in Table I indicate that cAMP and compounds that potentiate cAMP effected increased phosphorylation of MEK-2.

Other compounds were tested that affect cell morphology, growth, or differentiation in a number of systems (27–30). None of these agents altered keratin phosphorylation although we observed other effects on ME-180 cells. For example, the phosphorylation of a 170-kdalton ME-180 protein was stimulated in the presence of epidermal growth factor (L. King and M. Gilmartin, unpublished observation) although keratin phosphorylation was not affected. ME-180 cells cultured in calcium-depleted medium exhibited altered morphology com-

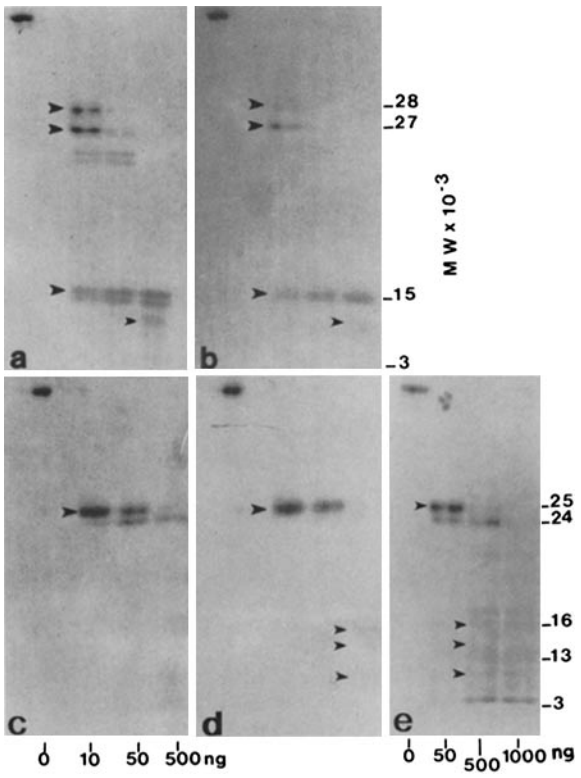

FIGURE 5 *S. aureus* V8 protease digests of MEK-1 and MEK-2. The ME-180 cells were labeled with [^{32}P]orthophosphate or ^{14}C-amino acid mix. The cells were extracted and the urea-BME fraction was analyzed by SDS PAGE. MEK-1 and MEK-2 were excised and subjected to the peptide mapping procedure described in Materials and Methods. (*a*) Coomassie Blue-stained gel of digestion products of ^{32}P-MEK-1. (*b*) Autoradiogram of *a*. Arrows indicate corresponding labeled peptides. (*c*) Coomassie Blue-stained gel of ^{32}P-MEK-2. (*d*) Autoradiogram of *c*. (*e*) Autoradiogram of ^{14}C-MEK-2. The map of MEK-2 labeled with ^{14}C-amino acids permits visualization of the low molecular weight peptides generated by doses of 500 and 1,000 ng of protease.

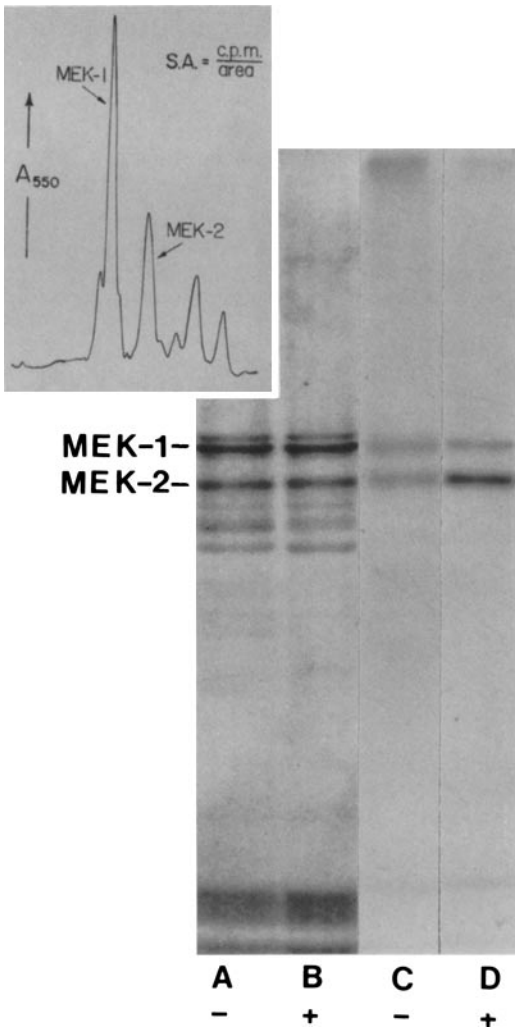

FIGURE 6 ME-180 cells were labeled with [^{32}P]orthophosphate in the presence (+) or absence (–) of 10^{-4} dibutyryl cAMP and 0.2 mM MIX. Equal amounts of the SDS-BME-solubilized filament protein (see Materials and Methods) from control (*A*) and treated (*B*) cells were compared by SDS PAGE. The corresponding autoradiograms (*C* and *D*) demonstrate increased phosphorylation only of MEK-2. Specific activities of ^{32}P-MEK-1 and ^{32}P-MEK-2 were calculated as the ratio of cpm of the excised gel slice to the corresponding area of the densitometric tracing (*inset*). The increase of MEK-2 phosphorylation was quantitated (see Table I) as the ratio of specific activities between experimental and control samples.

pared with control cultures but did not show altered keratin phosphorylation (data not shown).

DISCUSSION

The studies detailed in this communication were accomplished with ME-180 cells, a line that has ultrastructural characteristics of epithelial cells including desmosomes and intracellular 10-nm filament bundles. The presence of both keratin and vimentin filament systems has been demonstrated in several transformed lines (30) and, in fact, Franke et al. (31) have decorated filaments in ME-180 cells with antivimentin antibody. We have performed similar studies, and the cytoskeleton of the ME-180 cells we carry in our lab also can be decorated with antikeratin and antivimentin antisera (data not shown). However, the filament proteins we have isolated in the urea-BME fraction from ME-180 cells consisted predominantly of keratins. The identity of the filament proteins was confirmed by immunoprecipitation (Fig. 1) and immunoblot analysis (Fig. 2) using antibodies raised against the keratins of human callus. In addition, exogenously added vimentin is isoelectrically distinct from ME-180 cells keratins (Fig. 3).

The major keratins of ME-180 cells appear to correspond in molecular weight and isoelectric distribution to several of the keratins isolated from human keratinocytes. Using the catalog of Moll et al. (32), the corresponding proteins are ME-180, 58 kdaltons: human keratin No. 5; ME-180, 52.5 kdaltons: No. 8; Me-180, 53 kdaltons: No. 13; ME-180, 45 kdaltons: No. 17; and ME-180, 40 kdaltons: No. 19. As with other epithelial keratins, the ME-180 keratins can be divided into at least two isoelectrically distinct groups each containing one major and multiple minor subunits (Fig. 3). Those of lower molecular weight have more acidic isoelectric points than the higher molecular weight keratins. We have named the major keratin subunits in each molecular weight class MEK-1 (58,000) and MEK-2 (53,000). These two keratins are immunologically distinct since, as we have shown in Fig. 2, they react to two different antibodies. The peptide maps (Fig. 3) indicate that they are biochemically distinct as well since they have very different peptide patterns and sensitivities to proteolytic digestion. Both are phosphoproteins and in each case the phosphate is localized to the protease-sensitive portion of the molecules. This localization is consistent with Steinert's model for keratin structure in which the phosphate is localized to the nonhelical, protease-sensitive portions of these proteins (6, 32). As would be expected, the two-dimensional electrophoretograms (Fig. 4) indicate that in MEK-1 and MEK-2 as well as in the 52.5- and 40-kdalton keratins, the phosphate is associated with the most acidic variants.

TABLE I

Stimulation of Keratin Phosphorylation in ME-180 Cells

Perturbant	Concentration	Specific activity* MEK-1 (*n*)	Specific activity* MEK-2
Control	—	1.00 ± 0.23 (10)	1.00 ± 0.19
Dibutyryl cAMP	10^{-4} M	0.73 (3)	2.00
MIX	1 mM	0.78 (2)	2.68
Dibutyryl cAMP + MIX	10^{-4} M + 2 mM	0.99 (1)	3.26
Isoproterenol	10^{-5} M	0.96 (1)	5.15§
Cholera Toxin‡	10^{-9} M	1.21 (2)	7.04‖
8-Bromo cyclic GMP	10^{-6} M	1.01 (2)	0.92
EGF	10 ng/ml	1.22 (3)	1.15
Hydrocortisone	1 ng/ml	0.80 (1)	0.96
Calcium	1 mM	1.07 (2)	1.17

Incubation conditions are described in Materials and Methods. Data reported are for concentrations and time points that yielded maximal stimulation. (*n*) indicates the number of experiments performed with each perturbant. Average values are reported where possible. *EGF*, epidermal growth factor.

* Specific activities of protein bands, expressed as the ratio of specific activities of experimental to control values, were determined as described in Materials and Methods.

‡ The specific activity of the trichloroacetic acid-soluble label (nucleotide triphosphate pools) was followed in control and cholera toxin-treated cells. No significant change in the specific activity of acid-soluble label was observed.

§ Specific activity was calculated for the 120-min time point.

‖ Specific activity was calculated for the 60-min time point. All other values are for 30-min time points.

Our analysis of the effect on keratin phosphorylation of several compounds that modify the activity of phosphokinases has indicated stimulation of MEK-2 phosphorylation in the presence of dibutyryl cAMP and those compounds that enhance intracellular cAMP concentration (Table I and Fig. 6). Phosphorylation of MEK-1 was not altered by any of the perturbants tested. These data demonstrate that the controls of MEK-1 and MEK-2 phosphorylation are independent. Furthermore, the ability of cAMP to modulate phosphorylation of MEK-2 implies a response, either direct or indirect, of this filament protein to events at the cell membrane. We have not yet determined to what extent the phosphate content of MEK-2 is regulated by cAMP nor whether the observed stimulation of phosphorylation is due to altered kinase or phosphatase activities or both.

There is as yet no information regarding the functional significance of keratin phosphorylation. In intact cells such as ME-180, individual keratin proteins have not yet been localized within the cytoskeletal network. MEK-1 and MEK-2 may be parts of the same filament structure or may exist in different subsets of filaments. Alterations in charge density of filament subunits through phosphorylation could affect in vivo filament polymerization. When tested in vitro, both phosphorylated and partially dephosphorylated filament subunits were found capable of repolymerization (6).

If, in fact, the phosphorylation of intermediate filaments is not required for interactions among filament proteins, phosphorylation may still influence filament interaction with other cellular components. An example would be the reorganization of keratin filaments into filament bundles during terminal differentiation of mammalian epidermis (33). In several systems, the presence of a matrix protein, filaggrin, is required for such reorganization (34–36) and it is known that active filaggrin is processed by dephosphorylation from its precursor (35). The phosphorylation of keratin subunits could prevent their premature association with phosphorylated filaggrin species.

The extent to which the keratins are modified is unclear. Keratins are acetylated (37) and phosphorylated. We have recently demonstrated that in ME-180 cells, MEK-2 is glycosylated (38). Individual molecules of MEK-2 may be both glycosylated and phosphorylated or these modifications may mark distinct subsets of MEK-2 with distinct localizations and/or functions.

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