Dual Regulation of Intermediate Filament Phosphorylation

MARY E. GILMARTIN, JOHN MITCHELL, ALDA VIDRICH, and IRWIN M. FREEDBERG
Department of Dermatology, New York University Medical Center, New York, New York 10016

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 against authentic human epithelial 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 [32P]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 32P-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 choleratoxin (sevenfold). In contrast, MEK-1 phosphorylation was unaffected by these agents. Neither cyclic GMP, Ca^{2+}, 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.

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, N.Y.). Cultures were maintained at 37°C in an atmosphere of 5% CO_{2}, 95% air.

Abbreviations used in this paper: cAMP, cyclic AMP; BME, β-mercaptoethanol; MIX, methylisobutylxanthine; NP-40, Nonidet P-40; PAP, peroxidase-antiperoxidase; PMSF, phenylmethylsulfonyl fluoride.
Isolation of 32P-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 [32P]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 measured and the cells were processed 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 re-suspended 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 above.

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 twodimensional 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 Hoefler gel dryer. Gels were autoradiographed using Kodak XAR film to localize radioactive bands.

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% diethylthiourea, 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 Gel grid (12) and overlaid with 25 μl of a solution of 80 mM Tris-HCl, pH 6.8, 1.5% diethylthiourea, 2% SDS, and 10% glycerol containing 0, 10, 50, 100, 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 cell lines.

For immunoprecipitation we used polyclonal antikeratin antiserum raised in guinea pig (13). Preimmune serum was from the same guinea pig that subcultivated the antisera. For immunoblot analyses we used a commercial antikeratin antiserum of T.-T. Sun (New York University Medical Center). 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 Electrophoretic 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 then photographed using a Kodak No. 25 red filter (14).

To localize the keratin proteins, we processed the fast green–stained blot as described by Sternerberger (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 excised and processed to an appropriate level of polyvalent or monoclonal antikeratin antiserum or to preimmune serum. Following a 60-min incubation at 37°C, the blot was washed in PBS and immobilized in a 1/5 dilution of 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 1/5 dilution of rabbit (Miles-Yeda) or mouse (Sternerberger-Meyer, Jarretsville, MD) peroxidase-antiperoxidase (PAP) for 60 min at 37°C. Unbound PAP was removed by washing in 37°C Tris-HCl, pH 7.4, and 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% H2O2 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 32P-urea-BME–soluble proteins were freed of nonprotein phosphate as described by Britte and Kabet (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 Laboratories 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 preincubated with [32P]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: N6, 0.2-dibutyryladenosine 3′,5′-cyclic phosphate (Sigma), 10−6, 10−5, 10−4, 10−3 M; 8-bromoguanosine 3′,5′-cyclic phosphate (P-L Biochemicals, Inc., Milwaukee, WI), 10−6, 10−5, 10−4 M; sodium butyrate (Sigma), 10−6, 10−5, 10−4 M; phenylalanine (Sigma), 10−6, 10−5, 10−4 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 Hank’s 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 Na2HPO4, pH 7.5, 0.15 M Tris-X100, 0.1 M EDTA, 1 mM PMSF). The buffer-insoluble proteins (filament fraction) were collected by centrifugation at 10,000 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 phosphorylated keratins 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 gels were stained as described above. The gel was cut into sections, and the sections were treated with 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 32P-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 gel and the radiolabeled proteins were eluted from the gel in a Packard Trias 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 [35S]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).

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. 2a 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 32P-labeled keratins of ME-180 cells (Fig. 3) demonstrates that [32P]phosphate is associated with variants of MEK-1 (58 kdalton), MEK-2 (53 kdalton), and the 52.5- and 40-kdalton keratins. [32P]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 [32P]PO4. 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. 5b, the phosphate is localized primarily to the 15-kdalton peptide.

Digestion of MEK-2 with 10 ng of S. aureus V8 protease (Fig. 5c) 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 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-, 52.5-, and 40-kdalton keratins. Inset: position of migration of vimentin (V) relative to the 53-kdalton keratin. Molecular weight $\times 10^{-3}$.

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. 5c. The autoradiogram of $^{32}$P-labeled MEK-2 (Fig. 5d) 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 $10^{-6}$ 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 6 ME-180 cells were labeled with [32P]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 32P-MEK-1 and 32P-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 pre-dominantly 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.

| Perturbant                  | Concentration | MEK-1 (n) | 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              | 10−3 M        | 0.96 (1)  | 5.15* |
| Cholera Toxin*             | 10−9 M        | 1.21 (2)  | 7.04* |
| 8-Bromo cyclic GMP         | 10−5 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 phosphory kinases 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 epidermal matrix proteins, filaggrin, is required for intermediate-sized filamentous filaments of the vimentin type in cultured cells. Biochemistry. 21:177-183.

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