Communication

Peptide Mapping of Phosphorylated Vimentin

EVIDENCE FOR A SITE-SPECIFIC ALTERATION IN MITOTIC CELLS*

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Vimentin, the subunit protein of one type of intermediate filament, has been isolated from 32P-labeled nonmitotic and mitotic mouse L-929 cells. Analysis of tryptic phosphopeptides by two-dimensional maps indicates that vimentin is phosphorylated at multiple sites in mitotic cells. Comparison of nonmitotic and mitotic vimentin phosphotryptic peptides indicated that in addition to the 6–7 major phosphorylated tryptic peptides found in nonmitotic cells, vimentin isolated from mitotic cells contained an additional 2 distinct phosphorylated peptides following trypsin digestion. Partial acid hydrolysis and one-dimensional phosphoamino acid analysis indicates that phosphoserine is present in all 8 major phosphopeptides. Treatment of L-929 cells with 8-bromo-cAMP did not result in a qualitative change in the phosphopeptide map of vimentin isolated from a normal cell population. These results suggest that the reorganization of vimentin filaments during mitosis is accompanied by a site-specific change in phosphorylation.

Intermediate filaments are a major component of the cytoskeleton of eukaryotic cells. Although there appear to be at least five distinct classes of intermediate filaments, cells of mesenchymal origin and most cells in culture contain 7–11 nm filaments composed of the subunit protein vimentin (1). A prominent feature of vimentin, as well as other intermediate filament types that have been studied, is the phosphorylation of the protein subunits (1, 2–4). It has been observed that the organization of vimentin filaments is profoundly changed during cell division with the characteristic wavy filament bundles forming a cage-like structure around the mitotic spindle (5–9). This change in filament organization is temporally related to an increase in vimentin phosphorylation (10, 11).

Vimentin filaments appear to provide a structural framework involved in cytoplasmic organization, but a precise function remains to be elucidated (1). The cellular mechanisms which influence intermediate filament assembly, disassembly, and organization are not known. The present studies were intended to further characterize the change in the phosphorylation of vimentin which occurs during cell division. In this communication, data are presented which show a site-specific phosphorylation of serine residues which occurs in vimentin at mitosis.

MATERIALS AND METHODS

Cell Culture—Mouse L-929 cells were grown in monolayer culture in a 1:1 mixture of F-12 Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum and 10 μg/ml of gentamicin at 37 °C. Cultures were radiolabeled with 0.5–1.0 μCi/ml of (32P)orthophosphoric acid (carrier-free, ICN) for 2 h in medium containing 1% normal phosphate concentration and 1% dialyzed fetal bovine serum. In studies involving cell synchrony, cells were incubated twice in complete medium containing 2 mM thymidine for 18 h, with a 12-h release period between thymidine blocks (12). Twelve hours after release from the second thymidine block, cells were labeled with 32P orthophosphoric acid for 2 h and a mitotically enriched population of cells was obtained by the procedure of Terasima and Tilmach (13) as previously described (10). The mitotic fraction of cell populations was determined by Giemsa staining and phase-contrast microscopy.

Preparation of 32P-labeled Vimentin—Following radiolabeling with 32P, cells were rinsed in a cold solution containing 145 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 10 mM NaF, 1 μg/ml of leupeptin and 0.5 mM phenylmethylsulfonyl fluoride (Tris-buffered saline). A Triton-insoluble residue was then prepared essentially as described by Zackroff and Goldman (14). The resulting precipitate was rinsed three times in Tris-buffered saline and centrifuged at 3000 × g for 5 min. The precipitate was resuspended in a small volume of a solution containing 2% sodium dodecyl sulfate and 1 mM EDTA. This solution was brought to approximately 8 M urea with the addition of solid urea and subjected to preparative two-dimensional gel electrophoresis (15) as previously described (16). The gels were covered with plastic wrap and autoradiographed at 4 °C. In some experiments, gels were stained in a solution containing 36% methanol, 5% acetic acid, 0.1% Coomassie blue and destained in 20% methanol, 5% acetic acid. Using autoradiographs or stained gels as a guide, the phosphorylated vimentin spots were cut from the gel. In the case of stained and unstained gels, the removed gel pieces were soaked for 30 min in 0.05 M ammonium bicarbonate, pH 8.0. The 32P-labeled protein was then eluted by shaking in a solution containing 0.05 M ammonium bicarbonate, 0.05% sodium dodecyl sulfate, pH 8.0, at 37 °C. The supernatant protein was then precipitated with trichloroacetic acid (20%, final concentration) and the precipitate was washed twice with ethanol:ether (1:1). The gel-purified 32P-labeled vimentin was then dried under a stream of nitrogen and used for subsequent peptide mapping studies. Re-electrophoresis of gel-purified vimentin (Fig. 1) shows that the recovered protein migrated identically to vimentin in Triton-insoluble residues.

Peptide Mapping—32P-labeled vimentin was incubated in 0.5 ml of a solution containing 0.05 M ammonium bicarbonate, pH 8.0, 0.05% sodium dodecyl sulfate and 100 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin (Sigma) for 20 h at room temperature. An additional 5 μg of trypsin was then added and the sample was incubated for 4 h. The samples were lyophilized, resuspended in 100 μl of water, and lyophilized again. Tryptic phosphopeptides were resuspended in a small volume of water and applied to thin layer cellulose sheets (20 × 20 cm) (Eastman Kodak). The 32P-peptides were analyzed essentially as described by O’Connor et al. (17) by electrophoresis at 1,000 V for 1 h in acetic acid:formic acid:water (11:4:6:370), pH 1.9, followed by chromatography in n-butanol:acetic acid:pyridine:water (60:12:40:48). The cellulose sheets were dried overnight and autoradiographed at −70 °C using Kodak X-AR film. In some experiments, using the autoradiograph as a guide, the spots containing individual phosphopeptides were scraped from the sheet, resuspended in a small volume of water, and centrifuged at 12,000 × g for 1 min in an Eppendorf centrifuge. The resulting supernatant was recovered and the peptides were hydrolyzed in 6 N HCl at 105 °C for 90 min. Phosphoamidino acids were analyzed on thin layer cellulose.
Phosphorylation of Vimentin in Mitotic Cells

by electrophoresis at pH 3.5 at 1,000 V for 1 h. Authentic unlabeled phosphoamino acids were included in each sample.

RESULTS

Tryptic phosphopeptides of vimentin isolated from L-929 cells were analyzed by two-dimensional mapping as shown in Fig. 2. Vimentin isolated from untreated 32P-labeled cells exhibited 6–7 major 32P-labeled peptides, indicating that vimentin in these cells is normally phosphorylated at multiple sites (Fig. 2A). Vimentin isolated from double thymidine blocked, 32P-labeled cells exhibited an identical pattern of 32P-labeled tryptic peptides, indicating that the thymidine block does not qualitatively alter sites of phosphorylation (Fig. 2B). Analysis of vimentin phosphopeptides from cells after release from the block, 32P-labeling, and mitotic selection (approximately 80% mitotic cells) is shown in Fig. 2C. In addition to the major 32P-labeled peptides found in nonmitotic cells, at least two additional phosphorylated peptides are present. Analysis of a mixture of the phosphopeptides from mitotic (Fig. 2C) and nonmitotic cells (Fig. 2B) is shown in Fig. 2D. Enzymatic digestion of similar samples for longer times (48 h) or with increased amounts of trypsin produced identical major 32P-peptides (data not shown). In addition, [32P]vimentin from cells treated for 16 h with 1 μg/ml of colcemid and then labeled with 32P; produces similar phosphopeptide maps to mitotic cells selected after thymidine block (Fig. 2C) (data not shown).

In vimentin, phosphoserine is believed to be the phosphorylated amino acid, although small amounts of phosphotyrosine have been reported to be associated with vimentin following viral transformation (18). However, as shown in Fig. 3, phosphoserine is the only detectable phosphoamino acid present in the phosphotryptic peptides isolated from peptide maps.

The differences in vimentin tryptic phosphopeptides between mitotic and nonmitotic cells shown in Fig. 2 are qualitative. The gels were purposely overexposed beyond the linear...
response of the film. In addition, significant variations in the relative intensity of individual phosphopeptides have been observed between experiments. In order to better ascertain the relative contribution of each phosphopeptide under the labeling conditions used in these studies, individual phosphopeptides were removed from tryptic maps and the 32P radioactivity was determined as shown in Table I.

In some cell culture systems, the phosphorylation of vimentin is sensitive to exogenous cAMP (19) or homones which increase cellular cAMP levels (19, 20). However, experiments with other cultured cell types have been unable to detect effects on total vimentin phosphorylation by exogenous cAMP (2, 10). In order to investigate the possibility that the additional sites of phosphorylation were due to increased cAMP, L-929 cells were labeled with 32Pi in the presence of 1 mM 8-bromo-CAMP for 2 h. [32P]vimentin isolated from these cells did not yield additional phosphopeptides when compared with untreated cells (data not shown).

### DISCUSSION

Vimentin is a prominent phosphoprotein found in most cells in culture. It is clear from two-dimensional gel analysis that vimentin exists in cells in nonphosphorylated as well as phosphorylated forms (2, 3, 10, 17), but the effect of phosphorylation on intermediate filament organization is not understood. Previous studies have demonstrated an increased phosphorylation of this protein in mitotic cells when filament organization is dramatically altered (10, 11). Peptide mapping studies indicate that this phenomenon involves at least in part a qualitative change in the sites at which the vimentin molecule is phosphorylated.

Vimentin is normally multiply phosphorylated, apparently at a minimum of 6-7 distinct serine sites (Fig. 2, A and B). This observation is similar to the previously described multiple phosphorylation of the related intermediate filament protein, desmin (17, 19). It is also consistent with measurements of the amount of protein substituted with phosphate. While the reported molar substitution of vimentin is nearly 1 mol of phosphate/mol of protein (21), studies of [32P]methionine-labeled vimentin using two-dimensional gel electrophoresis to separate phosphorylated and nonphosphorylated forms indicate that less than 10% of the vimentin molecules appear to be phosphorylated in a normal population of L cells.

Although there are apparent differences in the amount of 32P incorporated into the major tryptic phosphopeptides of

| Peptide | Experiment 1 | Experiment 2 |
|---------|--------------|--------------|
|         | cpm | % total | cpm | % total |
| 1       | 683 | 9.5    | 576 | 12.2   |
| 2       | 420 | 5.8    | 124 | 2.6    |
| 3       | 1107 | 15.3 | 856 | 18.2   |
| 4       | 1541 | 21.3 | 912 | 19.4   |
| 5       | 466 | 6.5    | 209 | 4.4    |
| 6       | 329 | 4.6    | 358 | 7.2    |
| 7       | 308 | 4.3    | 165 | 3.5    |
| 8       | 1219 | 16.7 | 643 | 13.7   |
| 9       | 1157 | 16.0 | 881 | 18.7   |

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Phosphorylation of Vimentin in Mitotic Cells

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Peptide mapping of phosphorylated vimentin. Evidence for a site-specific alteration in mitotic cells.

R M Evans

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