COOH-terminal Methylation of Lamin B and Inhibition of Methylation by Farnesylated Peptides Corresponding to Lamin B and Other CAAX Motif Proteins*

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Previous reports from this laboratory have demonstrated that lamin B is reversibly methylesterified in a cell cycle-dependent manner. The site of this methylation, however, was not identified. In this report, we describe a single major methylated product obtained following reversed-phase high-performance liquid chromatographic analysis of peptides generated by proteolytic digestion of lamin B from rat liver nuclear envelopes. This peptide was retained on a lamin B COOH-terminal-specific antibody-affinity column, and COOH-terminal localization was confirmed by amino acid sequencing. Two other COOH-terminal peptides were found but were not methylated and differed in sequence by at least a single residue from the methylated peptide, indicating the existence of two lamin B gene products.

Tetrapeptides, representing the putative mature COOH termini of lamin B, K-ras-2A, and unprocessed lamin A, were synthesized with or without farnesyl modification of the COOH-terminal cysteines. All three farnesylated peptides served as substrates for the partially purified lamin B methyltransferase with apparent Kₘ values of 4.5, 0.69, and 21 μM, respectively. Nonfarnesylated peptides were not substrates for the enzyme. The three farnesylated peptides were also effective to varying degrees in inhibiting the methylation of lamin B and other cellular proteins in cell lysates.

Methylesterification of lamin B, an intermediate filament protein and major component of the nuclear lamina, has been shown to be a prerequisite for reassembly of the nuclear envelope following mitosis in mammalian cells (1). Methylesterification occurs in other proteins as well, including the γ-subunit of brain G-proteins (2, 3), Saccharomyces cerevisiae Ras2 protein (4) and γ-factor mating pheromone (5), human platelet protein smg p21B (6), and mammalian ras proteins (7, 8). In all of these proteins, this modification has been localized to the COOH-terminal cysteine (2, 5–7, 9) which has been shown to be isoprenylated as well (5, 6, 9–11). These proteins share a common COOH-terminal CAAX motif in which C is cysteine, A represents an aliphatic amino acid and X represents serine, methionine, or leucine among other amino acids. The proposed chain of events in CAAX protein processing includes isoprenylation of the cysteine residue through a thioether linkage followed by removal of the three COOH-terminal amino acids and methylesterification of the resulting terminal cysteine α-carboxyl group (12, 13).

Lamin B, which contains the CAAX motif, has been shown to be farnesylated at or near the COOH terminus (14) and to be methylesterified (15). We have now localized the methylesterification site of lamin B to the COOH terminus of the protein by digestion with V8 protease and purification of the resulting peptides. We also show that synthetic farnesylated tetrapeptides, representing the putative processed COOH termini of lamin B and related proteins, can be methylesterified. The ability of these peptides to act as methyl acceptors, as well as their ability to inhibit lamin B methylation, is dependent on farnesylation of the COOH-terminal cysteine.

MATERIALS AND METHODS

Peptides—All peptides were manually synthesized using solid phase methods and FMoc (N-(9-fluorenylmethoxycarbonyl) chemistry (16, 17). Peptides were N-acetylated using acetic anhydride in dimethylformamide. After deprotection and cleavage from the resin, all peptides were purified by precipitation in ether followed by high-performance liquid chromatography (HPLC) on a Vydac reversed-phase C₁₈ semipreparative column (Rainin Instrument Co.). Typically, a linear gradient of 0–36% acetonitrile in 0.1% trifluoroacetic acid over 20 min was used for peptide purification. The flow rate was 1 ml/min. Identity of purified peptides was confirmed by fast atom bombardment mass spectrometry. Peptides were modified with farnesyl bromide, prepared by the method of Ruegg et al. (18) from all-trans-farnesol (Aldrich Chemical Co.). Peptides (150 μmol) were dissolved in 0.75 ml of 50% aqueous dimethylformamide plus 20 μg of magnesium oxide with addition of one equivalent farnesyl bromide in 0.43 ml of isopropyl ether. The mixture was rotated at room temperature overnight in the dark. The farnesylated peptide products were purified by reversed-phase HPLC using a Beckman Ultrasphere column (2 mm × 25 cm) with a linear gradient of 0–90% acetonitrile in 0.1% trifluoroacetic acid over 35 min at 0.25 ml/min.

Antibody Production—The peptide used for antibody production, CEERFQQAGPR, was coupled to Keyhole limpet hemocyanin (Sigma). Keyhole limpet hemocyanin (5 mg) in 0.38 ml of 0.1 M sodium phosphate buffer, pH 7, was added to 1.5 mg of the cross-linker sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce Chemical Co.) and incubated for 2 h at 23 °C. The reaction was loaded on a 6 mm × 12-cm Sephadex G-50 column and 0.6 ml fractions were collected. The first peak to absorb at 280 nm, which eluted in 1.2 ml, was added to 4.5 mg of purified peptide and incubated for 2 h at 23 °C and 18 h at 4 °C. Rabbits were initially immunized with 1.5 mg of the protein-peptide conjugate in complete Freund’s adjuvant (Difco Laboratories, Detroit, MI) and boosted with 0.75 mg of conjugate in incomplete Freund’s adjuvant (GIBCO) biweekly for 4 weeks, and then monthly.

Preparation of Immunofluorescence Column—For affinity column pro-

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1 The abbreviations used are: HPLC, high-performance liquid chromatography; CLAP, a protease inhibitor mixture (chymostatin, leupeptin, antipain, and pepstatin); SDS, sodium dodecyl sulfate; Hepes, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; PBS, phosphate-buffered saline; AdoMet, S-adenosyl-L-methionine.
duction, the peptide used to immunize rabbits was coupled to chicken serum albumin (Cappel Laboratories, West Chester, PA) using sul-

purified by a modification of the method originally described by Kaufmann and modified as described (15). Farnesylated peptides were evaluated as methyl acceptors using methyltransferase assays, were then assayed for base-labile methyl ester radioactivity.

RESULTS

A Single Methylated Peptide from Lamin B—Lamin B is the only detectably methylated protein in a mouse liver nuclear pore complex-lamina fraction (15). This fraction was methylated with [3H]AdoMet and digested with V-8 protease. The resulting crude digest was fractionated by reversed-phase HPLC. Only one methylated peptide, containing 48% of the original lamin B-associated radioactivity, was observed (Fig. 1). This recovery is very close to what we typically experience (50–70%) with synthetic peptides on this column. The experiment was then repeated with additional purification steps to isolate the methylated peptide.

Antibody against the COOH Terminus of Lamin B—Work with other CAAX motif proteins suggested that the methylated peptide isolated from lamin B may have derived from its COOH terminus. This portion of the protein was therefore targeted in our purification scheme by producing antisera against a peptide representing a region adjacent to the COOH terminus of mouse lamin B1 (23). Affinity-purified antisera specifically recognizes lamin B in mouse and rat liver nuclear envelopes, as determined by Western blot analysis (Fig. 2) and immunofluorescence (not shown). This antisera was used to prepare a lamin B COOH-terminal-specific immunoaffinity column for peptide purification.

Characterization of the Lamin B-methylated Peptide—Lamin B from rat liver nuclear pore complex-lamina was labeled with [3H]AdoMet and digested with V8 protease. COOH-terminal peptides were isolated by immunopurification as described under “Materials and Methods.” Recoveries of base-labile radioactivity for each of the six purification steps ranged from 68 to 76% with an overall recovery of 13% (Table I). Rat liver was used due to the low recoveries obtained with mouse liver during purification. Reversed-phase HPLC, the last step in the rat liver peptide purification scheme, yielded a series of peptide peaks (Fig. 3) eluting in a range of 12–40% acetonitrile (Table II). All major peptide peaks, one of which contained essentially all of the base-labile radioac-

FIG. 1. Base-labile radioactivity detected in a V8 protease digest of methylated lamin B fractionated by reversed-phase HPLC. A nuclear pore complex-lamina fraction derived from mouse liver was digested overnight with V8 protease; the digested material was acidified to 0.1% trifluoroacetic acid and fractionated by reversed-phase HPLC as described under “Materials and Methods” except that a Vydac C18 column (4.6 mm × 25 cm) was used at a flow rate of 1 ml/min. Each fraction was assayed for base-labile radioactivity.
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Fig. 2. Immunoreactivity of the anti-lamin B peptide antibody. Western blot analysis of mouse liver nuclei on a 12.5% polyacrylamide-SDS gel. Rabbit immune serum, purified by ammonium sulfate precipitation and immunoaffinity purification, was tested for purity and immunoreactivity (at a 1:10,000 dilution) prior to its use in preparation of the antibody immunoaffinity column. Lamin B is migrating as a 67-kDa protein. Bound antibody was visualized with 125I-protein A and autoradiography. Bovine serum albumin (68 kDa; Integrated Separation Systems, Hyde Park, MA) is used as the molecular mass marker.

Table I

<table>
<thead>
<tr>
<th>Recovery</th>
<th>cpmp</th>
<th>Each step</th>
<th>Overall %</th>
</tr>
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<tr>
<td>Rat liver nuclear</td>
<td>1,604,733</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>complex-lamina</td>
<td>fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V8 digest</td>
<td>1,173,667</td>
<td>73</td>
<td>73</td>
</tr>
<tr>
<td>12,000 x g supernatant of</td>
<td>841,006</td>
<td>72</td>
<td>52</td>
</tr>
<tr>
<td>trichloroacetic acid precipitation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-10 column pool</td>
<td>569,650</td>
<td>68</td>
<td>35</td>
</tr>
<tr>
<td>Soluble fraction after</td>
<td>431,175</td>
<td>76</td>
<td>27</td>
</tr>
<tr>
<td>concentration</td>
<td>Ab affinity column eluate</td>
<td>320,480</td>
<td>74</td>
</tr>
<tr>
<td>HPLC peak (72 min)</td>
<td>216,500</td>
<td>68</td>
<td>13</td>
</tr>
</tbody>
</table>

Fig. 3. HPLC purification of COOH-terminal lamin B peptides. Rat liver nuclear pore complex-lamina fraction was digested using V8 protease with subsequent immunoaffinity purification of lamin B peptides (see "Materials and Methods"). Fractionation of the peptides on a reversed-phase HPLC column was monitored for both protein and base-labile radioactive methyl esters (open triangles).

The two unlabeled peptides for which sequence data was obtained have apparently identical sequences with the exception that one of the two is extended by an additional arginine. The methylated peptide has a sequence which extends 3 residues beyond the longer of the two unlabeled peptides. It also has a threonine in place of an alanine at one position (Table II). Both sequences are nearly identical to the inferred COOH-terminal regions from the cloned mouse and human lamin B genes (Table II). Chicken lamin B1 and B2 share identity with 6 residues in this region. While informative sequence data obtained for the methylated peptide ends short of the expected cysteine by three amino acids, the methyl group appears to be located at or very near to the COOH terminus and is associated with only one of the two rat lamin B sequences isolated.

Methylation of Synthetic Peptides—Three synthetic peptides were used to study the substrate requirements for the lamin B methyltransferase. This enzyme catalyzes the transfer of a methyl group from AdoMet to a carboxylic acid to form a methyl ester. The activity studied here copurifies with the radioactive peptide, as well as their nonfarnesylated precursors, were assayed for their ability to accept a methyl group using rat liver nuclear envelope as the source of methyltransferase. Methylation of the nonfarnesylated peptides was not detectable. The farnesylated peptides, however, were substrates for the methyltransferase (Table III). The range in apparent K_m values for the three sequences, determined by Lineweaver-Burk plots, is approximately 30-fold.

In the methylation assay, unmodified peptides were analyzed for methyl ester content following HPLC purification.
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TABLE II
Characterization of COOH-terminal lamin B peptides

Amino acid sequence, HPLC elution, and base-labile radioactivity data of peptides obtained from V8 protease digestion of lamin B and subsequent immunopurification on a lamin B COOH-terminal peptide column. Shaded areas indicate regions of identity with known COOH-terminal sequences for lamin B proteins from mouse, human, and chicken.

<table>
<thead>
<tr>
<th>Rat Lamin B</th>
<th>Sequence</th>
<th>Elution time</th>
<th>% Acetonitrile</th>
<th>Total base labile CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Experiment</td>
<td>XFHQQGTTRAXN</td>
<td>72 min.</td>
<td>40</td>
<td>216500</td>
</tr>
<tr>
<td></td>
<td>GEERFHOQQGPR</td>
<td>34 min.</td>
<td>14</td>
<td>490</td>
</tr>
<tr>
<td></td>
<td>XEERFHOQQGAP</td>
<td>30 min.</td>
<td>12</td>
<td>355</td>
</tr>
<tr>
<td>2nd Experiment</td>
<td>XFHQQGTTRAXN</td>
<td>69 min.</td>
<td>38</td>
<td>126670</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Human Lamin B</th>
<th>Sequence</th>
<th>Elution time</th>
<th>% Acetonitrile</th>
<th>Total base labile CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Lamin B</td>
<td>EEELFHOQQGTTRASNRC</td>
<td>12 min.</td>
<td>40</td>
<td>216500</td>
</tr>
<tr>
<td>Mouse Lamin B</td>
<td>EEERFHOQQGAFRANWCKSC</td>
<td>12 min.</td>
<td>40</td>
<td>216500</td>
</tr>
<tr>
<td>Chicken Lamin B</td>
<td>LEDVIFHQGGQRFFERS</td>
<td>12 min.</td>
<td>40</td>
<td>216500</td>
</tr>
<tr>
<td>Chicken Lamin B</td>
<td>EEGLENGQGGQRFFERS</td>
<td>12 min.</td>
<td>40</td>
<td>216500</td>
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TABLE III
Methylation and HPLC elution of farnesylated and nonfarnesylated tetrapeptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Methylation, K_m</th>
<th>% acetonitrile at HPLC elution</th>
<th>Unmethylated peptide</th>
<th>Methylated peptide</th>
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</thead>
<tbody>
<tr>
<td>N-Acetyl-S-farnesyl-NKSC</td>
<td>4.5</td>
<td>44</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-NKSC</td>
<td>ND*</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-S-farnesyl-PQNC</td>
<td>21</td>
<td>51</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-PQNC</td>
<td>ND</td>
<td>13</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-S-farnesyl-IKKC</td>
<td>0.69</td>
<td>44</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-IKKC</td>
<td>ND</td>
<td>14</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Farnesol</td>
<td>69</td>
<td>59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* ND, not detectable.

The farnesylated peptides, however, were extracted in butanol and assayed directly. This was possible due to the hydrophobic nature of the farnesyl modification and greatly facilitated the assay. In order to assure that the radioactivity measured in the butanol-extracted samples was entirely associated with the farnesylated peptides, aliquots of the extracted samples were also fractionated by HPLC in the presence of excess unmethylated farnesylated peptide. In each case, all of the radioactivity was recovered in a single peak which consistently eluted approximately 2-3 min after the unmethylated peptide standard (Fig. 4). This increase in retention time is expected for the methyl derivative.

Peptide Inhibitors of Lamin B Methylation—Farnesylated peptides were incubated with the rat liver nuclear pore complex-lamina fraction, which contains lamin B and its methylesterase, in the presence of [3H]AdoMet. At the highest concentration tested (200 μM), the farnesylated K-ras-2A and lamin B peptides were able to inhibit lamin B methylation by 88% while the farnesylated lamin A peptide inhibited by 68% (Fig. 5). IC_{50} values were 24, 41, and 154 μM, respectively. The experiment was repeated using nonfarnesylated peptides (132-192 μM) with no inhibition of lamin B methylation detected (data not shown).

Inhibition of Methylation in a Total Cell Lysate—When [3H]AdoMet is added to a total cell lysate, a reproducible set of proteins is methylesterified, as determined following gel electrophoresis (28). In this study, lamin B (67 kDa), as well as a soluble 36-kDa protein and a cluster of proteins in the 23-26-kDa range incorporated base-labile radioactivity fairly equally (Fig. 6A). Addition of any of the three farnesylated peptides to the labeling reaction resulted in a substantial reduction in lamin B methylation (Fig. 6, B and C; Table IV). The farnesylated peptides were also effective at inhibiting the 23-26-kDa methylations, completely blocking them in the case of the lamin B peptide (Fig. 6C). The 36-kDa protein was more variably affected by the three peptides (Table IV).

DISCUSSION

Lamin B, a member of the COOH-terminal CAAX motif family, is the predominant methylesterified protein of the nuclear envelope. We have localized the site of this methylation to the COOH terminus of the protein by proteolytic digestion of a radiolabeled rat liver nuclear envelope preparation with subsequent purification of peptides on a lamin B COOH-terminal-specific immunoaffinity column. This procedure resulted in the isolation of a single radiolabeled, methylated peptide and two unmethylated peptides. Analysis of the isolated peptides reveals two different sequences that are very similar to other mammalian lamin B sequences but differ from each other by a single amino acid, suggesting the presence of two lamin B proteins in rat. One of the peptides is associated with most, if not all, of the methylation attributed to lamin B in these in vitro labelings. The methylated peptide consistently produces the sequence FHQQGTTRAXN. While this peptide is clearly proximal to the COOH terminus, based on mouse and human lamin B c-DNA sequences (23, 29, 30), we cannot assume that the labeled peptide ends at the asparagine since the amount of material recovered in the last sequencing cycle was at the detection limit of the sequencer. This was not the case for the two unmethylated COOH-terminal peptides which contain an alanine in place of the threonine and end six and seven amino acids short of the...
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**Fig. 4.** HPLC elution and base-labile radioactivity profiles of methylated farnesylated synthetic peptides. Farnesylated peptides were incubated with nuclear envelopes as a source of methyltransferase and [3H]AdoMet as the methyl donor. These peptides were separated from the reaction mixture by extraction with butanol (see "Materials and Methods"). Half of each sample was mixed with 2-4 nmol of the corresponding unmethylated farnesylated peptide and analyzed by HPLC. A, no peptide; B, N-acetyl-S-farnesyl-NKSC; C, N-acetyl-S-farnesyl-PQNC; D, N-acetyl-S-farnesyl-IKKC. Absorbance peaks at 3 and 36 min are solvent-related. Open circles represent base-labile radioactivity.

**Fig. 5.** Inhibition of lamin B methylation by farnesylated peptides. Farnesylated peptides in Me2SO were added to 0.1-ml aliquots of the nuclear pore complex-lamine fraction (derived from 0.13 g of rat liver) in label buffer and incubated in the presence of 8 μCi of [3H]AdoMet for 30 min at 37°C. Final Me2SO concentration was 2%. Nonfarnesylated peptides, dissolved in water (132-192 μM final concentration) were also tested, but no inhibition was detected. Samples were fractionated on a 12.5% polyacrylamide-SDS gel and the lamin B region in each lane was assayed for base-labile radioactivity. □, N-acetyl-S-farnesyl-PQNC; ■, N-acetyl-S-farnesyl-NKSC; ○, N-acetyl-S-farnesyl-IKKC.

predicted mature COOH terminus of lamin B. The methylated peptide described here corresponds to human lamin B in this region, while the nonmethylated peptides with the threonine to alanine substitution, match the sequence determined for mouse lamin B1. The existence of two lamin B proteins is consistent with results obtained in both chicken (31,32) and mouse (23, 30). While nonspecific proteolysis could explain the unexpectedly short sequences of the unmethylated alanine peptides, it is also possible that one of the lamin B genes may code for a truncated, unmodified, version of the protein.

By analogy with other lamin B sequences as well as with the ras proteins, which are also farnesylated and methylesterified, we expect that the methylated peptide actually contains three more amino acids. These are possibly KSC, RSC, or RGC (Table I), the cysteine being a prerequisite for the farnesyl modification and subsequent carboxymethylation (33). In addition to comparison with published data on ras and lamin B farnesylation, we suspect the presence of the farnesyl group based on retention of this peptide on a reversed-phase HPLC column. The methylated peptide eluted at an acetonitrile concentration 3-fold that of the unmodified peptides. This is not attributable to a difference in sequence or to methylation. It is consistent with the presence of a hydrophobic moiety, however, and parallels the difference in elution observed between unmodified synthetic peptides and their farnesylated counterparts.

Synthetic peptides were modeled on the predicted COOH terminus of processed CAAX motif proteins in which the cysteine is farnesylated and the last three amino acids are removed. Tetrapeptides ending in a cysteine were evaluated as substrates for the lamin B methyltransferase with or without the farnesyl thioether modification. While the unmodified peptides were inactive as methylation substrates, the farnesylated peptides were good substrates with apparent $K_v$ values ranging from 0.69 to 21 μM. Farnesylation as a prerequisite for methylation also argues for the presence of such a modification on the methylated lamin B peptide.

The farnesylated peptides were also tested for their ability to competitively inhibit the methylation of lamin B and other cellular proteins in partially purified nuclear envelope extracts and in crude cell lysates. Nonfarnesylated peptides were ineffective at inhibiting methylation in either preparation. In
nuclear envelope extracts, the farnesylated peptides were able to compete with lamin B in a concentration-dependent manner. The mouse K-ras-2A and lamin B COOH-terminal peptides were the most effective, while the lamin A peptide, consistent with its weaker substrate results, was less effective as an inhibitor. The ability of these peptides to inhibit methylation of proteins in a cell lysate varied with each methylated protein observed. However, all peaks of protein methylation, fractionated by SDS gel electrophoresis, were affected to some extent. This suggests the existence of a class of methyltransferases which recognize a similar structure, presumably including an isoprenoid moiety. Lamin B methylation was consistently reduced by about two-thirds with all three peptides tested. This was essentially the same result as seen for nuclear envelopes at the same inhibitor concentration. Proteins in the 23–26-kDa range were more substantially affected, with methylation completely blocked by the peptide corresponding to the lamin B COOH terminus. This region contains the farnesylated (Cpr) ras proteins but is thought to be made up of many methylated geranylgeranylated (Cpr) ras-related proteins as well (6, 34, 35).

The lamin B methyltransferase appears to be sequence-selective based on the 30-fold range in \( K_a \) values for methylation of the three farnesylated peptides tested. Inhibition data from the same peptides, however, suggests that it may be difficult to selectively inhibit the methylation of a specific protein. This may be true even if the proteins are modified by different isoprenyl groups given that methylation of all 23–26-kDa proteins, presumably including both farnesylated and geranylgeranylated proteins, was inhibited by a single farnesylated peptide. Whether this is due to a single methyltransferase being responsible for modifying these isoprenylated proteins or to a similar sensitivity of several methyltransferases to the inhibitors is not clear. This issue is currently being addressed.

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REFERENCES