Phosphorylation at a tyrosine residue of lipomodulin in mitogen-stimulated murine thymocytes (transformation/tyrosine-specific protein kinase/Ca^{2+}-dependent protein kinase/cyclic AMP-dependent protein kinase/phospholipase inhibitory protein)

FUSAO HIRATA, KEICHI MATSUDA, YOSITADA NOTSU, TOSHIO HATTORI, AND RENATA DEL CARME

ABSTRACT When murine thymocytes were stimulated by mitogens such as concanavalin A, the Ca^{2+} ionophore A23187, or 4α-phorbol 12-myristate 13-acetate, there was a marked increase of 32P incorporation into immunoprecipitable lipomodulin, a phospholipase inhibitory protein. These compounds enhanced 45Ca^{2+} influx into thymocytes, which, in turn, increased protein phosphorylation, probably by Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C). Cyclic 8-bromo-AMP, an inhibitor of lymphocyte mitogenesis, blocked the mitogen-stimulated phosphorylation of lipomodulin, although it stimulated the protein phosphorylation via cyclic AMP-dependent kinase (protein kinase A). On electrophoresis, the hydrolysates of 32P-labeled lipomodulin showed a single radioactive spot, which comigrated with authentic phosphotyrosine. The partially purified middle-sized tumor antigen was able to phosphorylate lipomodulin after being phosphorylated by protein kinase C but not by the catalytic subunit of protein kinase A. Our findings suggest that the activity of a tyrosine-specific kinase, which phosphorylates lipomodulin in vivo as well as in vitro, is stimulated by protein kinase C and inhibited by protein kinase A.

Phosphorylation of Lipomodulin in Vitro. Protein phosphorylation was assayed as described (16, 17). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.4), 10% ethylene glycol, 0.2% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 mM MgCl2, 1 mM CaCl2, 0.2 mM ZnCl2, 10 mM [γ-32P]ATP (10,000 cpm/pmol), and 5 μg of lipomodulin in a total volume of 10 μl. To measure in vivo phosphorylation.

COMMUNICATED BY JULIUS AXELROD, APRIL 20, 1984

MATERIALS AND METHODS

Thymocytes. Thymocytes from C3H/HeN (6 weeks old, female) mice were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 15 mM Hepes buffer (pH 7.2), 100 units of penicillin per ml, and 100 μg of streptomycin per ml (complete medium) (11). [3H]Thymidine incorporation was assayed 48 hr later after adding a mitogen (11). 45Ca^{2+} influx and release of [1-14C]acridionate were measured 5 min and 30 min, respectively, after stimulation as described (12, 13). Lipomodulin was purified from the media of rabbit neutrophils treated with fluocinolone acetonide as described (10). Ascites fluids from nu/nu mice bearing a hybridoma cell line, 4-4C3, were used as anti-lipomodulin antibody (14).

Preparation of Various Kinases. The middle-sized tumor (MT) antigen was partially purified from polyoma virus-infected mouse 3T6 cells (15). Briefly, the plasma membranes from these fibroblasts were solubilized with 10 mM sodium phosphate buffer, pH 7.4/150 mM NaCl/1% Nonidet P-40/30% ethylene glycol. After centrifugation at 27,000 × g for 60 min, aliquots were applied to a column of anti-MT antigen antibody-coupled agarose (25 ml). The column was washed extensively with 0.5% Nonidet P-40 in 30% ethylene glycol, and the MT antigen was eluted with 0.5% Nonidet P-40/30% ethylene glycol/0.01 M acetic acid. After neutralization with 0.1 M NaOH, the concentrates were applied to a Biosead SM-2 column (1 × 5 cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.4/10% ethylene glycol. The fractions containing the MT antigen, as measured by autophosphorylation (15), were collected and used as partially purified MT antigen. Ca^{2+}-dependent kinase (protein kinase C) was purified from rat brains as described (16, 17). The preparation obtained by DEAE-cellulose column chromatography was applied to a Sephadex G-75 column. The fractions containing the Ca^{2+}- and phosphatidylinerine-dependent kinase activity were used as protein kinase C. The catalytic subunit of cyclic AMP-dependent kinase (protein kinase A) from bovine hearts was purchased from Sigma and used without further purification.

Abbreviations: MT antigen, middle-sized tumor antigen; Con A, concanavalin A; PMA, phorbol 12-myristate 13-acetate.

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of lipomodulin, thymocytes (10⁶ cells) were first incubated with 0.1 mCi of ^32P/m of complete medium (1 Ci = 37 GBq) for 1 hr and then stimulated with concanavalin A (Con A; 25 μg/ml), phorbol 12-myristate 13-acetate (PMA; 100 ng/ml), 0.1 mM A23187, or 1 mM cyclic 8-bromo-AMP. Phosphorylation was measured as described (16, 17), except that the trichloroacetic acid precipitates were extracted once with 3 ml of chloroform/methanol and then dissolved in 500 μl of 0.6 M NaOH. To measure lipomodulin phosphorylation in intact cells, 100 μl of 2% Nonidet P-40 and 0.1% sodium deoxy sulfate in 90% ethylene glycol and 100 μl of 5 mM phenylmethylsulfonyl fluoride and 2% aprotinin mixture were added to 1 ml of the incubation mixture. After centrifugation at 27,500 × g for 60 min, immunoprecipitation was carried out by adding 100 μl of anti-lipomodulin antibody to 1 ml of the aliquots as described (10).

Identification of Phosphorylated Amino Acids. To identify the phosphorylated amino acid residues, the immunoprecipitates were washed first with 2 ml of chloroform/methanol, 2:1 (vol/vol), and then with 1 ml of acetone. The proteins were hydrolyzed with 100 μl of 6 M HCl at 130°C for 3 hr. After lyophilization overnight, the residues were dissolved in 20 μl of an aqueous solution containing phosphotyrosine, phosphoserine, and phosphothreonine (100 μg/ml). The hydrolysates thus obtained were analyzed by electrophoresis at 800 V for 120 min in pyridine/acidic acid/water, 5:35:960 (vol/vol) (5).

RESULTS

Effect of Cyclic AMP on Mitogenic Responses of Murine Thymocytes. When murine thymocytes were cultured in the presence of mitogens such as Con A, the Ca²⁺ ionophore A23187, or 4β-phorbol 12-myristate 13-acetate (PMA), a marked increase in [³H]thymidine uptake by the thymocytes was observed in 48 hr (Table 1). These mitogens induced ⁴⁵Ca²⁺ influx into the cells and [¹⁴C]arachidonate release in the early stage of stimulation, whereas cyclic 8-bromo-AMP-inhibited [¹⁴C]arachidonate release stimulated by these mitogens. In addition, this nucleotide reduced the responses of thymocytes to these mitogens as measured by [³H]thymidine uptake. Although Ca²⁺ is necessary for mitogenesis and arachidonate release (12, 13), the inhibition of Ca²⁺ influx by cyclic AMP did not necessarily parallel those of arachidonate release and thymidine uptake, suggesting that an elevated concentration of Ca²⁺ is not sufficient to initiate the mitogenic response.

Protein Phosphorylation in Mitogen-Stimulated Thymocytes. PMA and cyclic AMP have been reported to stimulate protein phosphorylation by protein kinases C and A, respectively (16–18). When protein phosphorylation in thymocytes labeled with ³²P was measured, mitogenic stimulation with Con A, A23187, or PMA increased ³²P incorporation into the proteins (Table 2). Cyclic AMP alone also stimulated protein phosphorylation. However, the degrees of protein phosphorylation by the mitogens and by cyclic AMP were not additive, and the nucleotide rather inhibited the mitogen-stimulated phosphorylation. It should be noted that these compounds did not increase or decrease ³²P incorporation into the ATP pool of thymocytes as measured by chromatography on a Dowex 1 × 8 formate column (data not shown).

To examine the effect of these mitogens on phosphorylation of lipomodulin in lymphocytes, ³²P-labeled lipomodulin was immunoprecipitated using anti-lipomodulin antibody (Table 2). In nonstimulated cells, the apparent amount of phosphorylated lipomodulin was less than 0.5% of the total phosphorylated proteins. The mitogens increased lipomodulin phosphorylation by 5- to 8-fold (1.5% of total phosphorylated protein). Cyclic AMP did not change the phosphorylation of lipomodulin in nonstimulated lymphocytes, but it blocked the mitogen-induced increase in the phosphorylation.

Identification of Phosphorylated Lipomodulin by NaDodSO₄/PAGE. To confirm the phosphorylation of lipomodulin, the immunoprecipitable ³²P-labeled lipomodulin was analyzed by NaDodSO₄/PAGE. Lipomodulin immunoprecipitated from the incubation mixture had ³²P-labeled proteins of M₉, 36,000, 24,000, and 14,000 (Fig. 1, lane a). These phosphorylated proteins could not be detected in the nonstimulated cells and were not precipitated by control sera obtained from mice bearing Py3 × 63Ag8 or by antibody that had been preabsorbed with partially purified lipomodulin (data not shown). Prolonged incubation of thymocytes with Con A resulted in increasing the ³²P radioactivity in the smaller peptides (lane b). Since only the M₉, 36,000 peptide was detected in the membrane fractions (data not shown), we assume that

Table 1. Effect of various mitogens on ⁴⁵Ca²⁺ uptake, [¹⁴C]arachidonate release, and [³H]thymidine uptake by murine thymocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>⁴⁵Ca²⁺ uptake, cpm per 10⁶ cells</th>
<th>[¹⁴C]Arachidonate released, cpm per 10⁶ cells</th>
<th>[³H]Thymidine uptake, cpm per 10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>432 ± 30</td>
<td>316 ± 20</td>
<td>1,500 ± 160</td>
</tr>
<tr>
<td>Con A</td>
<td>1250 ± 180</td>
<td>920 ± 85</td>
<td>24,650 ± 1620</td>
</tr>
<tr>
<td>PMA</td>
<td>820 ± 90</td>
<td>820 ± 60</td>
<td>12,650 ± 1220</td>
</tr>
<tr>
<td>A23187</td>
<td>ND</td>
<td>1,360 ± 110</td>
<td>11,650 ± 1326</td>
</tr>
<tr>
<td>Cyclic AMP</td>
<td>238 ± 20</td>
<td>320 ± 50</td>
<td>963 ± 232</td>
</tr>
<tr>
<td>Cyclic AMP plus Con A</td>
<td>820 ± 84</td>
<td>560 ± 32</td>
<td>6,523 ± 1860</td>
</tr>
<tr>
<td>Cyclic AMP plus PMA</td>
<td>680 ± 72</td>
<td>480 ± 40</td>
<td>3,520 ± 962</td>
</tr>
<tr>
<td>Cyclic AMP plus A23187</td>
<td>ND</td>
<td>740 ± 62</td>
<td>3,820 ± 867</td>
</tr>
</tbody>
</table>

Thymocytes (10⁶ cells per ml) were stimulated with Con A (0.25 μg per 10⁶ cells), PMA (100 ng/ml), A23187 (0.1 μM), or cyclic 8-bromo-AMP (1 mM) or combinations thereof. Values are expressed as mean ± SD of triplicate assays. ND, not determined. The amount of [¹⁴C]arachidonate released by A23187 treatment was approximately 6% of the total radioactivity incorporated.
Phosphorylation of Lipomodulin in mitogen-stimulated lymphocytes. Murine thymocytes (10⁶ cells per ml) were incubated with 0.1 mCi of [32P]P for 1 hr and then stimulated by addition of 25 µg of Con A. After 1 hr of incubation, cells and medium were separated by centrifugation at 27,000 x g for 30 min at 4°C. Lipomodulin was immunoprecipitated from incubation mixtures of cells stimulated with Con A for 2 hr (lane a) and for 8 hr (lane b). Data shown are for one of six experiments with similar results. p36, p24, and p14, peptides of Mr 36,000, 24,000, and 14,000.

the smaller species may be derived from the Mr 36,000 species, probably by the cleavage of lipomodulin (14).

Phosphorylated Sites of Lipomodulin. Lipomodulin isolated by Sephadex G-100 column chromatography from the media of the mitogen-stimulated lymphocytes did not inhibit porcine pancreas phospholipase A₂ in vitro (10). After this preparation was dephosphorylated by treatment with alkaline phosphatase (14), the capacity to inhibit phospholipase A₂ was restored with concomitant release of 32P (data not shown). Serine- and/or threonine-phosphorylated lipomodulin, obtained by in vitro treatment with protein kinase C or A (10), was less sensitive to such alkaline phosphatase treatment (unpublished data). Since alkaline phosphatase dephosphorylates phosphotyrosine faster than phosphoserine or phosphothreonine (19), these results suggest that lipomodulin is phosphorylated at a tyrosine residue. To obtain further evidence for tyrosine phosphorylation, 32P-labeled lipomodulin, immunoprecipitated from thymocytes, was hydrolyzed and subjected to electrophoresis (Fig. 2). The 32P radioactivity obtained from the Con A-stimulated cells comigrated with authentic phosphotyrosine (lane d). The tyrosine phosphorylation of lipomodulin was also enhanced by Ca²⁺ ionophore or PMA, activators of protein kinase C, and inhibited by cyclic AMP, an activator of protein kinase A (Table 1). These observations suggest that an activity of a tyrosine-specific protein kinase in thymocytes is regulated by protein kinases A and C.

Phosphorylation of Lipomodulin by Partially Purified MT Antigen. To confirm the phosphorylation of lipomodulin by a tyrosine-specific kinase in vitro, we partially purified the MT antigen from polyoma virus-infected fibroblasts, a protein that has been reported to have tyrosine-phosphorylating activity (15). When lipomodulin purified from rabbit neutrophils (Mr = 40,000) was used as a substrate for this MT antigen, no significant incorporation of [32P]P from [γ-32P]ATP was detected (data not shown). Since our findings have indicated that the tyrosine phosphorylation of lipomodulin in vivo is enhanced by protein kinase C but inhibited by protein kinase A, the MT antigen was first treated with these kinases, and the phosphorylated MT antigen was separated by HPLC (Fig. 3). The MT antigen thus obtained had a Mr of ~58,000 (Fig. 4). MT antigen previously treated with protein kinase C, but not that previously treated with protein kinase A, phosphorylated lipomodulin in vitro. To exclude the possibility that protein kinase A or C, eluted in the fractions together with the MT antigen, phosphorylates lipomodulin, the corresponding fraction from MT antigen-free reaction mixtures was incubated with lipomodulin, but no phosphorylated lipomodulin was detected. The phosphorylated amino acid residue in the Mr 40,000 protein (which corresponds to rabbit lipomodulin) was identified as tyrosine by two-dimensional electrophoresis (data not shown). Since our preliminary results had shown that MT antigen phosphorylated by protein kinase C together with protein kinase A (an activity identical to that of protein kinase C) had approximately 60% of the full activity obtained with protein kinase C alone, we conclude that the activity of a tyrosine-specific protein kinase is stimulated by protein kinase C and inhibited by protein kinase A.

**DISCUSSION**

The data in this communication show that lipomodulin is a substrate for a tyrosine-specific protein kinase whose activity is regulated by protein kinases A and C. Increased activity of a tyrosine-specific kinase has been reported to be associated with the onc gene products of tumorigenic viruses and stimulation of the receptors for insulin and epidermal growth factor (1–6). These tyrosine-specific kinases are often found
were collected.

(1) protein kinase C for used (50 Ag) to intact findings that may nase C kinases due lipomodulin though serine kinase a contains cells normal The (28).

FIG. 3. Separation of MT antigens. Partially purified MT antigen (50 μg) was incubated with 5 μg of protein kinase A (A) or 100 μg of protein kinase C (B) and then applied to a SynChropak GPC 300 column (1 m x 4.6 mm). Elution was carried out with 10 mM sodium phosphate buffer, pH 7.5/150 mM NaCl. One-milliliter fractions were collected. Radioactivity in fractions prepared with (c) and without (e) MT antigen was measured. Arrows indicate fractions used for MT antigen phosphorylation.

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Fig. 4. In vitro phosphorylation of purified lipomodulin by MT antigen. MT antigen (5 μg) prepared as described in the legend to Fig. 3 was incubated with 5 μg of lipomodulin partially purified from rabbit neutrophils. After 20 min of incubation, the reaction was stopped by adding 10 μl of 20% NaDodSO4, and the mixture was electrophoresed. Lanes: a, MT antigen phosphorylated by protein kinase A; b, fraction of protein kinase A equivalent to the MT antigen; c, MT antigen phosphorylated with protein kinase C; d, fraction of protein kinase C equivalent to the MT antigen. As expected, phosphorylated MT antigen was detected in lanes a and c but not in lanes b and d. The preparation of lipomodulin used in this experiment contained proteins other than lipomodulin, which were also phosphorylated under these conditions. p58 and p40, peptides of M, 58,000 and 40,000.

inhibited by purified lipomodulin (29). These findings suggest that lipomodulin can inhibit a step in mitogenesis by inhibiting a phospholipase(s). The phosphorylation and dephosphorylation of lipomodulin, mediated by a tyrosine-specific protein kinase and alkaline phosphatase, could regulate phospholipid metabolism in lymphocytes. Once lipomodulin is phosphorylated, the phospholipases, especially phospholipase A2, will be allowed to be fully active (10). Consequently, lysophosphatidylcholine, a product by phospholipase A2, would accumulate and, thus, enhance Na+ permeability and glucose uptake (30, 31). Alternatively, this compound could affect the Na+/K+-ATPase and serve as an acceptor of unsaturated fatty acids such as arachidonic acid and oleic acid by the action of acyl-CoA transferase, whose activity is also dependent on the level of lysophosphatidylcholine (32). All these events are reported to occur after stimulation of lymphocytes with mitogens. Thus, the phosphorylation of lipomodulin appears to play a key role in the mitogenic events taking place in lymphocytes.

We wish to thank Dr. Y. Itoh of the National Cancer Institute for supplying polyoma virus-infected fibroblasts and sera from guinea pigs bearing the tumor.