Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 4717–4721, August 1984
Biochemistry

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)

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Communicated by Julius Axelrod, April 20, 1984

ABSTRACT **When murine thymocytes were stimulated by mitogens such as concanavalin A, the Ca^{2+} ionophore A23187, or 4β-phorbol 12-myristate 13-acetate, there was a marked increase of ^{32}P incorporation into immunoprecipitable lipomodulin, a phospholipase inhibitory protein. These compounds enhanced $^{45}Ca^{2+}$ 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 ^{32}P-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.**

Protein phosphorylation is one of the major mechanisms by which intracellular events in many cells are controlled by various physiological stimuli (1). Most proteins are phosphorylated at serine or threonine residues to change their biological activities, while a minor portion of proteins is phosphorylated at tyrosine residues (2). The phosphotyrosine content of cells can be increased up to 10-fold after transformation by sarcoma viruses (2). The product of the transforming gene of these viruses has been identified as a tyrosine-specific protein kinase (3). Similar gene products can be detected in nontransformed cells, although their contents are very small (4). Similarities are reported in kinases of epidermal growth factor and insulin receptors (5, 6). Although several proteins have been found to be phosphorylated at tyrosine residues, their biologial functions have not been determined (2, 7). Demonstration of substrates with known functions is essential to understanding the mechanisms of cellular transformation, mediated by a tyrosine-specific kinase(s). Cells that are stimulated with mitogens such as platelet-derived factor or insulin, or cells that are transformed by tumor viruses, produce larger amounts of prostaglandins, probably by increased activity of phospholipase(s) in these cells (8, 9). Since activity of phospholipase(s) in neutrophils is dependent on the phosphorylation of lipomodulin, a phospholipase inhibitory protein (10), we searched for kinases that phosphorylate lipomodulin in mitogen-stimulated cells. We report here that lipomodulin is phosphorylated at a tyrosine residue in mitogen-stimulated murine thymocytes. Evidence is also presented suggesting that activity of a tyrosine-specific kinase is regulated by Ca^{2+}-dependent kinase (protein kinase C) and cyclic AMP-dependent kinase (protein kinase A).

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). [^{3}H]Thymidine incorporation was assayed 48 hr later after adding a mitogen (11). $^{45}Ca^{2+}$ influx and release of [1-^{14}C]arachidonate 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 Biobead 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 phosphatidylserine-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.

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 $MgCl_2$, 1 mM $CaCl_2$, 0.2 mM $ZnCl_2$, 10 nM [γ-^{32}P]ATP (10,000 cpm/pmol), and 5 μg of lipomodulin in a total volume of 100 μl. To measure *in vivo* phosphorylation

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

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of lipomodulin, thymocytes (10^8 cells) were first incubated with 0.1 mCi of ^{32}P/ml 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 nM 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 dodecyl 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/acetic 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 Ca^{2+} ionophore A23187, or 4β-phorbol 12-myristate 13-acetate (PMA), a marked increase in [^{3}H]thymidine uptake by the thymocytes was observed in 48 hr (Table 1). These mitogens induced $^{45}Ca^{2+}$ influx into the cells and [1-^{14}C]arachidonate release in the early stage of stimulation, whereas cyclic 8-bromo-AMP-inhibited [1-^{14}C]arachidonate release stimulated by these mitogens. In addition, this nucleotide reduced the responses of thymocytes to these mitogens as measured by [^{3}H]thymidine uptake. Although Ca^{2+} is necessary for mitogenesis and arachidonate release (12, 13), the inhibition of Ca^{2+} influx by cyclic AMP did not necessarily parallel those of arachidonate release and thymidine uptake, suggesting that an elevated concentration of Ca^{2+} is not sufficient to initiate the mitogenic response.

Protein Phoshorylation 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 ^{32}P was measured, mitogenic stimulation with Con A, A23187, or PMA increased ^{32}P 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 ^{32}P incorporation into the ATP pool of thymocytes as measured by chromatography on a Dowex 1 × 8 formate column (data not shown).

Table 2. Effect of various mitogens on protein phosphorylation in murine thymocytes

Treatment	Total protein, cpm × 10^{-3}	Lipomodulin, cpm
None	48.6 ± 0.9	240 ± 50
Con A	108.5 ± 1.1	1800 ± 126
PMA	96.9 ± 1.0	1090 ± 108
A23187	80.8 ± 0.7	660 ± 65
Cyclic AMP	98.1 ± 0.6	240 ± 50
Cyclic AMP plus Con A	75.5 ± 0.8	480 ± 35
Cyclic AMP plus PMA	51.9 ± 0.6	330 ± 50
Cyclic AMP plus A23187	52.4 ± 0.9	540 ± 50

C3H/HeN thymocytes (10^8 cells per ml) were first incubated with 0.1 mCi of ^{32}P/ml of RPMI 1640 medium for 1 hr at 37°C and then stimulated with various compounds for 4 hr as described in the legend to Table 1. Values are expressed as mean ± SD of triplicate assays.

To examine the effect of these mitogens on phosphorylation of lipomodulin in lymphocytes, ^{32}P-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 NaDodSO$_4$/PAGE. To confirm the phosphorylation of lipomodulin, the immunoprecipitable ^{32}P-labeled lipomodulin was analyzed by NaDodSO$_4$/PAGE. Lipomodulin immunoprecipitated from the incubation mixture had ^{32}P-labeled proteins of M_r 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 ^{32}P radioactivity in the smaller peptides (lane b). Since only the M_r 36,000 peptide was detected in the membrane fractions (data not shown), we assume that

Table 1. Effect of various mitogens on $^{45}Ca^{2+}$ uptake, [1-^{14}C]arachidonate release, and [^{3}H]thymidine uptake by murine thymocytes

Treatment	$^{45}Ca^{2+}$ uptake, cpm per 10^6 cells	[^{14}C]Arachidonate released, cpm per 10^6 cells	[^{3}H]Thymidine uptake, cpm per 10^6 cells
None	432 ± 30	316 ± 20	1,500 ± 160
Con A	1250 ± 180	920 ± 85	24,650 ± 1620
PMA	820 ± 90	820 ± 60	12,650 ± 1220
A23187	ND	1360 ± 110	11,650 ± 1326
Cyclic AMP	238 ± 20	320 ± 50	963 ± 232
Cyclic AMP plus Con A	820 ± 84	560 ± 32	6,523 ± 1860
Cyclic AMP plus PMA	680 ± 72	480 ± 40	3,520 ± 962
Cyclic AMP plus A23187	ND	740 ± 62	3,820 ± 867

Thymocytes (10^6 cells per ml) were stimulated with Con A (0.25 μg per 10^6 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 [^{14}C]arachidonate released by A23187 treatment was approximately 6% of the total radioactivity incorporated.

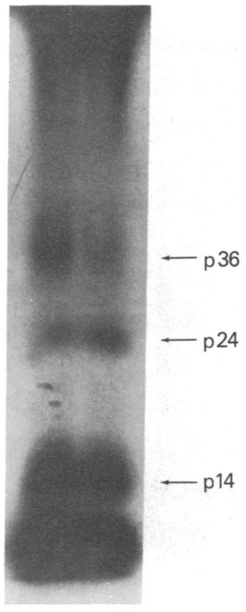

FIG. 1. Phosphorylation of lipomodulin in mitogen-stimulated lymphocytes. Murine thymocytes (10^8 cells per ml) were incubated with 0.1 mCi of ^{32}P for 1 hr and then stimulated by addition of 25 μg of Con A. After 1 hr of incubation, cells and medium were separated by centrifugation at 27,000 × *g* 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 M_r 36,000, 24,000, and 14,000.

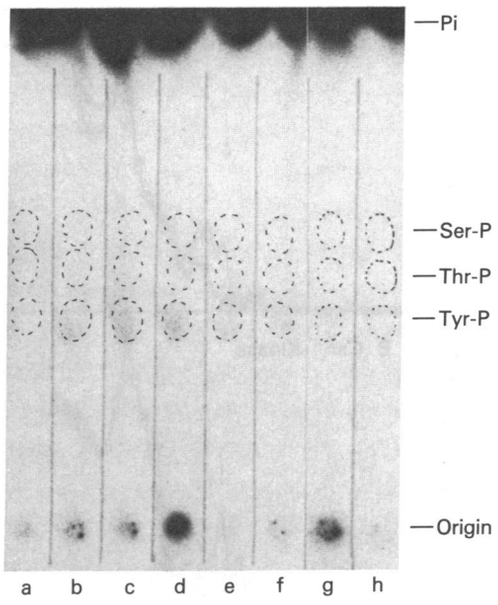

FIG. 2. Identification of phosphorylation sites in lipomodulin. Lipomodulin immunoprecipitated from unstimulated cells (lane a) and from cells stimulated with Ca^{2+} ionophore (lane b), PMA (lane c), Con A (lane d), Ca^{2+} ionophore plus cyclic 8-bromo-AMP (lane e), PMA plus cyclic 8-bromo-AMP (lane f), Con A plus cyclic 8-bromo-AMP (lane g), or cyclic 8-bromo-AMP (lane h) was hydrolyzed, lyophilized, and electrophoresed. In lane d, when 1200 cpm of immunoprecipitate hydrolysate was applied, 300 cpm was recovered in the phosphotyrosine spot and <20 cpm was detected in the phosphoserine and phosphothreonine spots. All samples were analyzed in parallel. Radioactivities in the phosphotyrosine spots were well correlated with those reported in Table 2. Data shown are for one of four experiments with similar results.

the smaller species may be derived from the M_r 36,000 species, probably by the cleavage with protease(s) (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 A_2 *in vitro* (10). After this preparation was dephosphorylated by treatment with alkaline phosphatase (14), the capacity to inhibit phospholipase A_2 was restored with concomitant release of ^{32}P (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, ^{32}P-labeled lipomodulin, immunoprecipitated from thymocytes, was hydrolyzed and subjected to electrophoresis (Fig. 2). The ^{32}P radioactivity obtained from the Con A-stimulated cells comigrated with authentic phosphotyrosine (lane d). The tyrosine phosphorylation of lipomodulin was also enhanced by Ca^{2+} 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 (M_r = 40,000) was used as a substrate for this MT antigen, no signifcant incorporation of ^{32}P from [γ-^{32}P]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 M_r 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 M_r 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

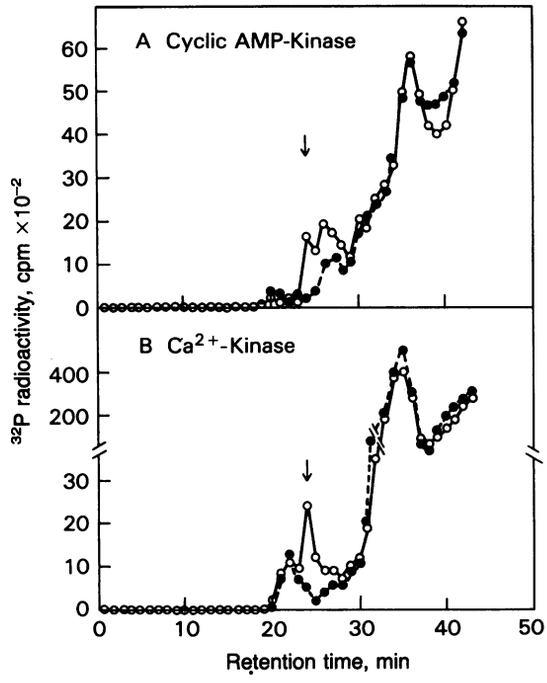

FIG. 3. Separation of MT antigens. Partially purified MT antigen (50 μg) was incubated with 5 μg of protein kinase A (*A*) or 100 μg of protein kinase C (*B*) and then applied to a SynChropak GPC 300 column (1 m × 4.6 mm). Elution was carried out with 10 mM sodium phosphate buffer, pH 7.5/150 mM NaCl.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

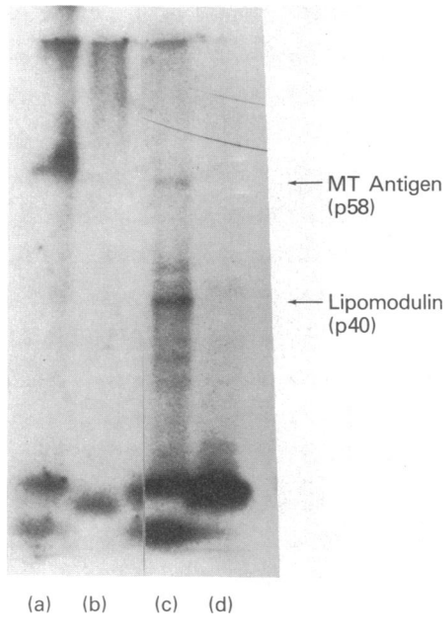

FIG. 4. *In vitro* phosphorylation of purified lipomodulin by MT antigen. MT antigen (5 μg) prepared as described in the legend to Fig. 3 was incubated with 5 μg of lipomodulin partially purified from rabbit neutrophils. After 20 min of incubation, the reaction was stopped by adding 10 μl of 20% $NaDodSO_4$ 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_r 58,000 and 40,000.

to be phosphorylated at serine residues in intact cells, while they can autophosphorylate tyrosine residues *in vitro* (6). Furthermore, phorbol esters can stimulate tyrosine phosphorylation in intact cells, although they activate protein kinase C *in vitro* (20, 21). Thus, it has been proposed that phosphorylation at a serine, but probably not at a tyrosine, residue may regulate the activity of tyrosine-specific protein kinases (22, 23). This proposal is supported by our present findings that partially purified MT antigen can phosphorylate lipomodulin after being phosphorylated at a serine residue by protein kinase C but not by protein kinase A. Therefore, protein kinases C and A appear to phosphorylate different serine and/or threonine residues of a tyrosine-specific protein kinase, as reported in the case of protein I, a specific neuronal phosphoprotein in the synaptosomes (24). Although serine phosphorylation of $pp60^{src}$ kinase has been suggested to be catalyzed by protein kinase A (25), phosphorylation of the β subunit of the insulin receptor, which contains a tyrosine kinase, is reported to be ehanced by PMA, an activator of protein kinase C (26). Recent work has shown that a $pp60^{src}$-like protein copurifies with the MT antigen (27) and that lymphoma cells contain a $pp60^{src}$-like protein (28). The content of $pp60^{src}$-like proteins is small but real in normal cells (2–4). Since a phosphorylated residue of lipomodulin in the stimulated lymphocytes has been identified as tyrosine (Fig. 2), these results suggest that a tyrosine-specific kinase is present in normal lymphocytes, even if it is not identical with $pp60^{src}$ or MT antigen.

Infection with tumorigenic viruses and stimulation of insulin or epidermal growth factor receptors generally result in increased mitogenic responsiveness of cells. These cells have a higher activity of a tyrosine-specific protein kinase as well as an increased capacity to release arachidonic acid (8, 9). Previous work from this laboratory has shown that the growth of U937 cells (a histiocytic leukemia cell line) can be 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 phoshorylated, the phospholipases, especially phospholipase A_2, will be allowed to be fully active (10). Consequently, lysophosphatidylcholine, a product by phospholipase A_2, 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.

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