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INHIBITION OF LEUKOCYTE CHEMOTAXIS BY Glu-Glu-Glu-Glu-Tyr-Pro-Met-Glu AND Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Gln-Gly

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SUMMARY: Chemotaxis of rabbit peritoneal leucocytes stimulated by fMet-Leu-Phe, a synthetic chemoattractant, was inhibited by Glu-Glu-Glu-Glu-Tyr-Pro-Met-Glu (MT peptide) and Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Glu-Gly (Src peptide). Both peptides did not inhibit the binding of [^{3}H] formyl-NLe-Leu-Phe, a chemoattractant, to neutrophils, suggesting that the peptides inhibit the events distal to the chemotactic receptors. These peptides blocked the release of arachidonic acid from phospholipids in neutrophils stimulated with chemoattractants, whereas they had no effect on phospholipase A_2 activity itself. The peptides markedly reduced the phosphorylation of lipomodulin, a phospholipase inhibitory protein, in either intact cells or isolated plasma membranes. Lipomodulin immunoprecipitated by monoclonal anti-lipomodulin antibody had phosphorylserine and phosphoryltyrosine as analyzed upon electrophoresis. The MT peptide which does not contain threonine or serine was phosphorylated by isolated plasma membranes. These results, taken together, suggest that a tyrosine phosphorylating kinase is involved in biochemical events of chemotactic receptors, and that lipomodulin is a substrate for this kinase.

Activation of phospholipase(s) appears to be an important step for the biochemical events occurring after the stimulation of receptors for chemotaxis since inhibitors of phospholipase(s) can inhibit chemotaxis (1,2). The inhibition of leukocyte chemotaxis by glucocorticoids has been proposed to be associated with the induction of the synthesis of phospholipase inhibitory protein(s), such as lipomodulin, in these cells (3). We have previously reported that successive phosphorylation and dephosphorylation of lipomodulin apparently regulate the release of arachidonic acid from rabbit neutrophils (4). The *in vitro* phosphorylation of lipomodulin can be catalyzed by cyclic AMP-dependent kinase (protein kinase A), whereas the entry of Ca^{++} enhances the phosphorylation of lipomodulin

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in intact cells (4). To study the involvement of membrane protein phosphorylation in the processes of chemotaxis, we initiated the search for kinases which phosphorylate lipomodulin. We find that phosphorylated lipomodulin isolated by immunoprecipitation with anti-lipomodulin antibody contains phosphorylserine and phosphoryltyrosine. It is also shown that the phosphorylation of lipomodulin in intact rabbit neutrophils and isolated plasma membranes can be inhibited by the peptides whose sequences match with those of the tyrosine phorphorylating sites of the middle sized tumor (MT) antigen and $pp60^{src}$. Since these peptides inhibit epidermal growth factor recepter kinase (5,6,7), a tyrosine phosphorylating kinase has been suggested as a protein kinase for lipomodulin in stimulated rabbit neutrophils.

METHODS AND MATERIALS

Neutrophils - Rabbit peritoneal neutrophils were obtained as described previously (1) and were suspended in Gey's balanced solution containing 2% bovine serum albumin. Chemotaxis was assayed with Boyden chamber technique, utilizing the chemoattractant, fMet-Leu-Phe, at 37°C for 2 hrs (1).

Binding assay of fNle-Leu-[^{3}H]Phe - Neutrophils (4.4×10^6/ml) were incubated at 4°C for 1 hr in Gey's solution containing 0.1 mM tosylamino-2-phenylethyl chloromethyl ketone and 0.2 µCi fNLe-Leu-[^{3}H]Phe (New England Nuclear, Boston, MA) (8). Nonspecific binding of fNLe-Leu-[^{3}H]Phe was measured in the presence of 10 µM fMet-Leu-Phe.

Phosphorylation of lipomodulin - Plasma membranes of rabbit neutrophils were isolated by the methods previously described for the separation of plasma membranes from hepatocytes (9). The membrane fraction located between 50% and 60% sucrose upon a density gradient was collected and used as plasma membranes. The specific activity of 5'-nucleotidase in the homogenates (0.5 nmol/mg protein/min) increased 6-fold by this purification. Phosphorylation of plasma membranes was carried out in a reaction mixture containing 10 nM [γ -^{32}P]ATP (10×10^4 cpm/pmol), 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 50 mM Tris-Cl, pH 7.4, 0.5 mM PMSF, 0.2 mM $ZnCl_2$ and 200 µg of membranes in a total volume of 50 µl. The incubation was run at 30° for 10 min and terminated by adding 50 µl of 2% SDS. Phosphorylation of lipomodulin in intact neutrophils was performed as described previously (4). Phosphorylated lipomodulin was immunoprecipitated using 100 µl of monoclonal anti-lipomodulin antibody, instead of sera from patients with systemic lupus erythematosus in the previous report (4). Neither peptide, MT or Src peptide, interfered the immunoprecipitation of lipomodulin.

Phosphorylation of MT peptide - Ten µg of MT peptide was incubated with 0.2 mg neutrophil plasma membranes in the presence of 10 nM 4β-phorbol-12-myristate-13-acetate (PMA) under the conditions described above. After 30 min incubation, the reaction was terminated by the addition of 100 µl of 1 N formic acid. The mixture was passed through Dowx-1-X8 formate column (1 x 5 cm). The phosphorylated peptide was eluted with 10 ml of distilled water. After lyophilization, the residues were dissolved into 50 µl of pyridine/acetic acid/water (35/5/960, v/v) and applied on cellulose thin layer plates. The plate was immersed with the same buffer solution and was electrophoresed at 800 V for 90 min.

Identification of phosphorylated amino acid - The immunoprecipitated lipomodulin was washed with 0.5 ml of acetone and was suspended into 100 µl of 6 N HCl followed by hydrolysis at 130°C for 4 hr. After lyophilization overnight, the residues were dissolved in 20 µl of pyridine/acetic acid/water (35

5/960, v/v) solution containing 10 μg of phosphorylserine, phosphorylthreonine and phosphoryltyrosine and were electrophoresed upon a cellulose plate with a buffer system of pyridine/ acetic acid/ water (35/5/960, v/v) at 800 V for 90 min. Phosphorylated amino acids were detected by ninhydrin solution.

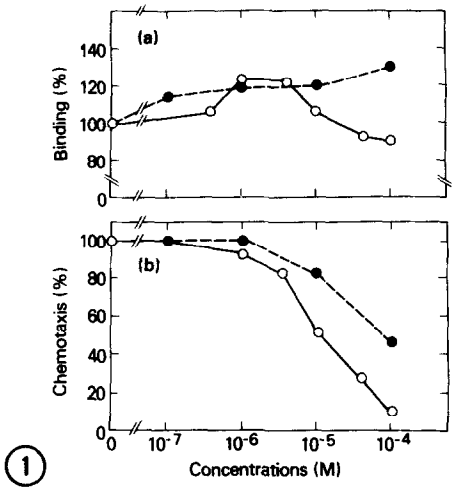
The measurement of [1-^{14}C]arachidonic acid release - Neutrophils were labeled with [1-^{14}C]arachidonic acid (55 μCi/μmol) for 1 hr as described (4). To prevent reacylation of released arachidonate, the cells were preincubated with 0.1 mM p-mercuribenzylsulfonic acid for 30 min at 37°C prior to stimulation with 5 nM fMet-Leu-Phe. The release of arachidonic acid was measured as described previously (4).

Synthesis of peptides - The MT and Src peptides were synthesized by Dr. M. Hashimoto of Fujisawa Pharmaceutical Company Ltd., Osaka, Japan and was kindly provided. The details of synthesis and analysis of these peptides will be published elsewhere.

RESULTS

Inhibition of chemotaxis by MT and Src peptides - Chemotaxis of rabbit neutrophils induced with fMet-Leu-Phe was dose-dependently inhibited by the MT and Src peptides (Fig. 1b). Although these peptides contain high amounts of Glu or Asp, it is unlikely that the inhibition of chemotaxis is attributable to their chelating action towards Ca^{++}. The medium employed for the assays contained 5 mM Ca^{++}, which was sufficiently higher than the concentration of peptides employed (up to 0.1 mM). In addition, peptides such as Glu-Glu-Glu-Glu-Glu-Glu and Asp-Asp-Asp-Asp-Asp-Asp had no effect on the chemotaxis of neutrophils (data not shown). Since any concentration range of peptides which resulted in a substantial inhibition of chemotaxis, did not affect the binding of [^{3}H]fNLe-Leu-Phe, to neutrophils (Fig. 1a). These results suggest that the MT and Src peptides inhibit chemotaxis by blocking some biochemical events occurring distally after binding of chemotactic peptides to the receptors.

Inhibition of arachidonic acid release by MT and Src peptides - When rabbit neutrophils labeled with [1-^{14}C]arachidonic acid were stimulated with fMet-Leu-Phe, the MT and Src peptides markedly suppressed the release of [1-^{14}C]arachidonic acid (and its metabolites) (Fig. 2a). The dose response curve for inhibition of arachidonate release paralleled that for inhibition of chemotaxis. However, these peptides failed to inhibit porcine pancreatic phospholipase A_2 (Fig. 2b). No inhibition was detected even with a partially purified preparation of phospholipase A_2 from rabbit neutrophils, a key enzyme for the release of arachidonic acid in these cells (1,2,10). Furthermore, these peptides had



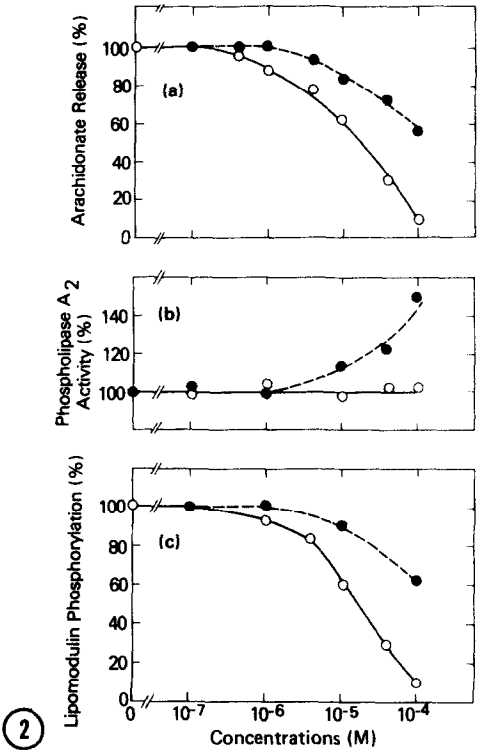


Figure 1. Effects of Src and MT peptides on (a) binding of chemoattractant and (b) chemotaxis.

Binding of chemoattractant and chemotaxis were measured with MT (O) and Src (●) peptides as described in "Methods and Materials". Bindings of fNle-Leu[^{3}H]Phe in the absence and presence of 10 μM fMet-Leu-Phe were 4500 and 800 cpm, respectively, in the control cells (4.4×10^6 cells). Data are shown from one of 5 different experiments with similar results. Standard errors were within 7 %.

Figure 2. Effects of Src and MT peptides on (a) release of arachidonate from neutrophils stimulated by fMet-Leu-Phe, (b) porcine pancreas phospholipase A_2 and (c) phosphorylation of lipomodulin in intact neutrophils.

Release of arachidonic acid from neutrophils (2200 and 250 cpm in the stimulated and nonstimulated cells,respectively) and phosphorylation of lipomodulin in intact cells (1160 and 158 cpm in the stimulated and nonstimulated cells, respectively) were assayed in the presence of Src (●) and MT (O) peptides as described in "Methods and Materials". Neutrophils were stimulated by 5 nM fMet-Leu-Phe. The activity of 1μg porcine pancreas phospholipase A_2 (specific activity; 17 μmol/min/mg protein) was measured at varying concentrations of Src (●) and MT (O) peptides. All determinations were carried out in triplicates. Data shown are from one of 4 experiments with similar results. Standard errors were within 8 %.

no effects on phosphatidylinositol phospholipase C partially purified from rat brains (data not shown). These results suggest that the inhibitory effect of these two peptides on arachidonate release and chemotaxis are not due to the direct inhibition of phospholipases in rabbit neutrophils.

Inhibition of lipomodulin phosphorylation by MT and Src peptides - The MT and Src peptides inhibited the chemoattractant-stimulated phosphorylation of lipo-

modulin in intact neutrophils (Fig. 2c). The dose-response curve for inhibition of lipomodulin phosphorylation was well corelated with those for inhibition of arachidonate release and chemotaxis. When ^{32}P-labeled immunoprecipitated lipomodulin was hydrolyzed and then analyzed by electrophoresis, two amino acids, tyrosine and serine, were found to be labeled with ^{32}P (Fig. 3). Carbobenzoyl-Phe-Met, an antagonist of the formyl peptides, inhibited the phosphorylation

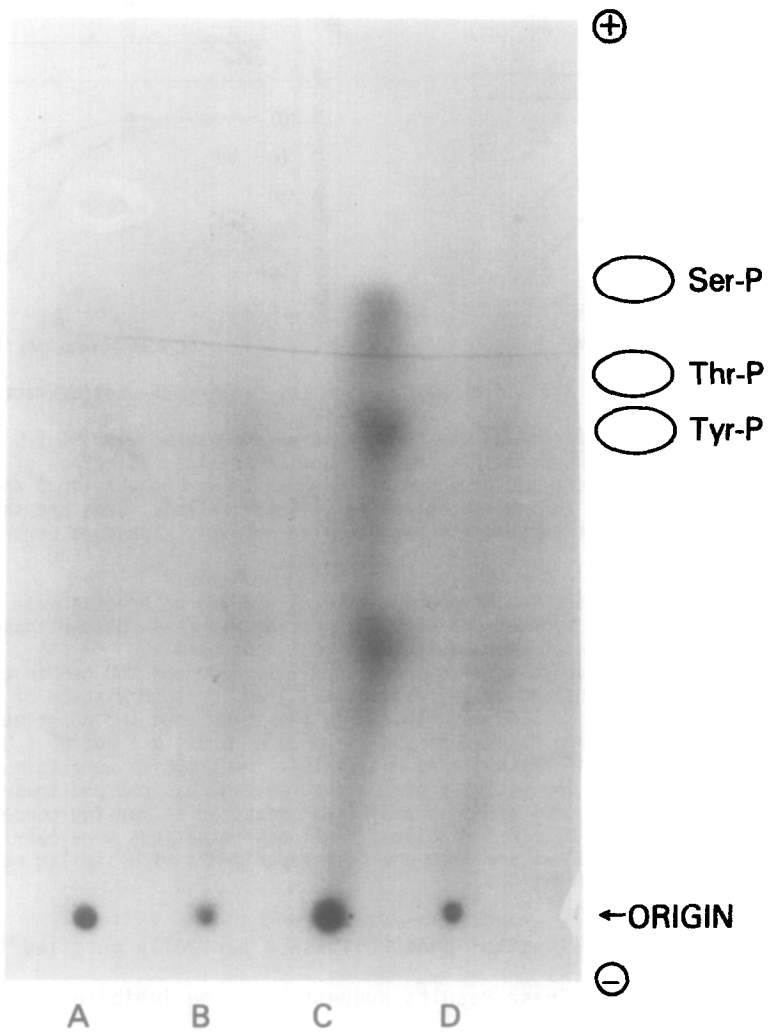


Figure 3. Electrophoresis of phosphorylated amino acids in lipomodulin. In this experiment, the volumes of the reaction mixtures were 5 fold scaled up. ^{32}P labeled lipomodulin was obtained using anti-lipomodulin antibody. The immunoprecipitates (5,000 cpm in the lane C) were hydrolyzed and electrophoresed as described in the "Methods and Materials". Lane A: no stimulation, Lane B: 10^{-4}M ZPhe-Leu, Lane C: 10^{-8}M fMet-Leu-Phe, and Lane D: 10^{-4}M ZPhe-Leu plus 10^{-8}M fMet-Leu-Phe.

at the both residues, suggesting that the phosphorylation of these amino acids in lipomodulin is the consequence of chemotaxis receptor stimulation.

Involvement of a tyrosine phosphorylating kinase in phosphorylation of lipomodulin - To obtain additional evidence showing the inhibitory effect of the peptides on the phosphorylation of lipomodulin, we isolated the plasma membranes of neutrophils and incubated them with [γ-^{32}P] ATP. In the absence of Ca^{++}, no significant phosphorylation was observed (Fig. 4). Cyclic AMP could not stimulate the protein phosphorylation in these plasma membranes. On the other hand, the addition of Ca^{++} to the reaction mixture resulted in the phosphorylation of four proteins, whose molecular weights were 57, 55, 46 and 40 K, respectively (Fig. 4). PMA, an activator of protein kinase C enhanced the phosphorylation of 40 K protein. Monoclonal anti-lipomodulin antibody immuno-

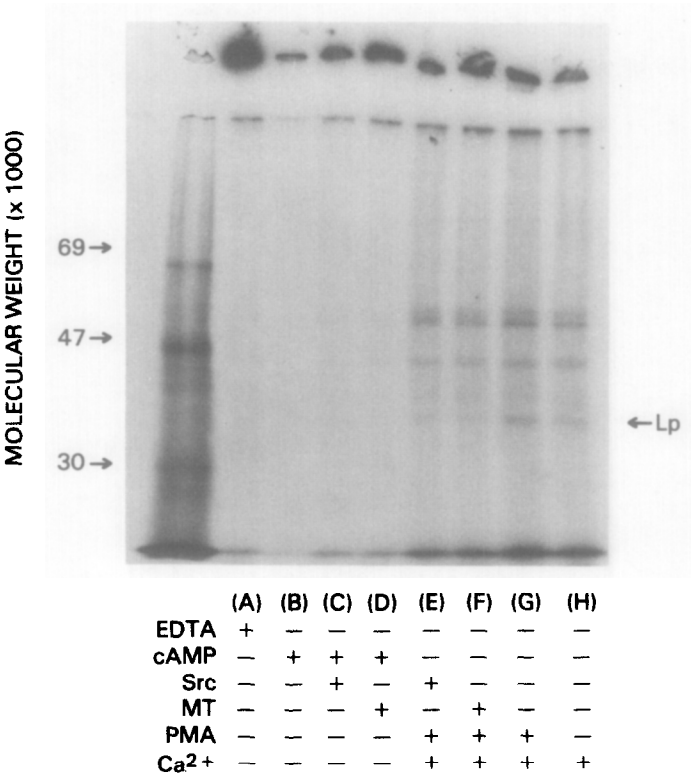


Figure 4. Protein phosphorylation of neutrophil plasma membranes. Neutrophil plasma membranes (0.2 mg) were incubated with [γ-^{32}P] ATP as described in the "Methods and Materials". The concentrations of EDTA, PMA, MT peptide and Src peptide were 1 mM, 10 ng/ml, 0.2 mM and 0.2 mM, respectively. The total incorporation of ^{32}P into membranes was 2,100 cpm in lane 6. SDS-PAGE (10%) was performed as described previously (4).

precipitated this 40 K phosphorylated protein (data not shown). The MT and Src peptides inhibited the phosphorylation of 40 K protein, and were concomitantly phosphorylated by the plasma membranes. The phosphorylation of the MT peptide was identified by Dowex-1 (x8) formate column chromatography and electrophoresis (Fig. 5). Since the MT peptide does not contain serine or threonine, the phosporylated site of this peptide should be tyrosine. These results suggest that the plasma membranes of rabbit neutrophils contain a tyrosine phosphorylating kinase which can phosphorylate lipomodulin.

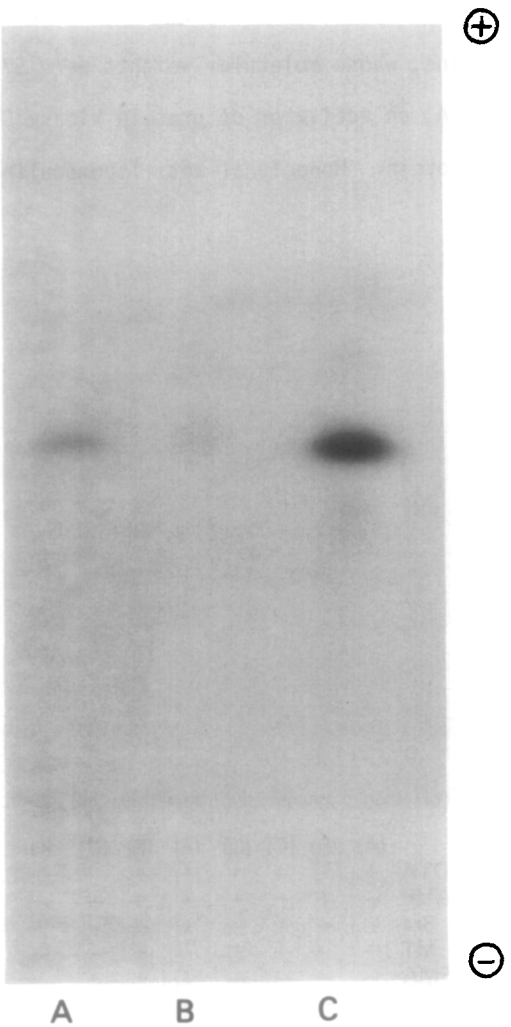


Figure 5. Electrophoresis of phosphorylated MT peptide.
MT peptide (0.2 mM) was incubated with neutrophil plasma membranes under the conditions described in the legend for Fig. 4. Phosphorylated MT peptide (2,400 cpm) was isolated by Dowex-1-X8 formate column and electrophoresed as described in "Methods and Materials". Lane A; membranes only, Lane B; MT peptide without membranes, and Lane C; membranes plus MT peptide.

DISCUSSION

The data in the present communication showed that the peptides whose sequences match with the phosphorylating sites of the MT antigen and $pp60^{src}$ can inhibit chemotaxis of rabbit neutrophils by competing with a tyrosine phosphorylating kinase for the phosphorylation of lipomodulin, a phospholipase inhibitory protein. A tyrosine phosphorylating kinase has been reported to be associated with the receptors for epidermal growth factor and insulin (12, 13), and the MT and Src peptides have been found to inhibit the autophosphorylation of epidermal growth receptors, and to be phosphorylated by this receptor kinase (6,7). Since these peptides can be phosphorylated by the plasma membranes of neutrophils, a tyrosine phosphorylating kinase appears to exist in rabbit neutrophils and to phosphorylate lipomodulin. On the other hand, phorbol esters such as PMA can enhance chemotaxis, probably due to the activation of phospholipase A_2 in neutrophils (1) and can also stimulate the phosphorylation of lipomodulin (Fig. 4). Since these compounds enhance the activity of protein kinase C (11), another protein kinase which phosphorylates lipomodulin in neutrophils appears to be protein kinase C. Indeed, phosphorylated lipomodulin contained phosphorylserine in addition to phosphoryltyrosine. Protein kinase A, which can phosphorylate lipomodulin *in vitro* (4), has been reported to increase phospholipase A_2 activity in homogenates of macrophages (14). Although the addition of cyclic AMP to intact neutrophils or plasma membranes did not increase significantly the phosphorylation of lipomodulin, the exogenously added catalytic unit of protein kinase A was able to phosphorylate proteins in membranes including lipomodulin (data not shown). This might result from the compartmentalization between lipomodulin and protein kinase A in the cells. Thus, protein kinase C and a tyrosine pohosphorylating kinase have been suggested to be involved in the biochemical events occuring after binding of chemoattractants to the receptors, and one *in situ* substrate for these kinase has been demonstrated as lipomodulin. How these kinases are related to the chemotactic receptors, or how the phosphorylation of lipomodulin is modulated by these two protein kinases still remains to be established.

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