Tyrosine Phosphorylation of a 94-kDa Protein of Human Fibroblasts Stimulated by Streptococcal Lipoteichoic Acid*

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Lipoteichoic acid (LTA) is an amphipathic component of Gram-positive bacteria. Previous studies from this laboratory have shown that at low concentrations, ranging from 0.1 to 10.0 μg/ml, LTA binds to mammalian cells and stimulates mitogenic responses as demonstrated by increased DNA and RNA synthesis. Tyrosine kinase appears to be involved in the action of a number of mitogens including epidermal growth factor, platelet-derived growth factor, and insulin. In the present study, we report the novel finding that tyrosine protein kinase activity is increased in human fibroblasts treated with LTA. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography of the whole cell lysate of fibroblasts cultured with 32P, showed increased phosphorylation of a 94-kDa polypeptide. Alkali treatment of the gel resulted in a decreased intensity of the 94-kDa phosphorylated protein in control cells, but not in LTA-treated cells, suggesting the addition of phosphate groups to threonine or tyrosine residues. High voltage electrophoresis of the acid hydrolysate of the excised and eluted 94-kDa protein revealed that LTA stimulated the phosphorylation of tyrosine but not threonine residues. These results suggest that LTA acts on mammalian cells by phosphorylating tyrosine residues of certain proteins and thereby may regulate diverse functions of these cells.

Lipoteichoic acids (LTAs) are amphipathic molecules synthesized and excreted by many Gram-positive bacteria. As a surface component of certain bacterial cells such as Streptococcus pyogenes, LTA has been shown to mediate the adherence of these organisms to mammalian epithelial cells (1–3). In its free form, LTA exhibits other biological activities including the stimulation of bone resorption in tissue culture (4), the inhibition of platelet aggregation by collagen (5), and the production of nephrocalcinosis in rabbits (6). In addition, several reports have suggested that LTA may exert a mitogenic or cytotoxic effect on mammalian cells in culture, depending on the concentrations of LTA used (7–11). It is interesting to note that lipopolysaccharide (LPS), an amphipathic molecule from Gram-negative bacteria, also acts as a mitogen on bone marrow-derived mouse lymphocytes (12). LPS stimulates macrophages to release prostaglandins (13) and neutral proteinases (14, 15); the biological actions of LPS have been attributed to the lipid A moiety (16, 17). Recently, free lipid A has been shown to stimulate protein kinase C of macrophages (18). Thus, the mitogenic properties of LPS may reside in the capacity of the molecule to induce the phosphorylation of biologically active proteins.

Covalent modification of tyrosine residues of proteins by phosphorylation induced by tyrosine kinase is thought to play a central role in the action of a number of mitogens including epidermal growth factor, insulin, and platelet-derived growth factor (19–26). These factors bind to their respective membrane receptors and thereby stimulate the autophosphorylation of the receptor proteins at tyrosine residues (20, 27–29). Tyrosine kinase-induced phosphorylation also has been implicated in the transformation of infected cells by Rous and Fujinami viruses (30, 31).

In this paper, we report studies of the effect of LTA in mitogenic doses on human foreskin fibroblasts in tissue culture. We show that LTA stimulates phosphorylation of the tyrosine residues of a 94-kDa protein produced by these cells. These studies provide new insights into the mechanism of the biological activity of streptococcal lipoteichoic acids.

EXPERIMENTAL PROCEDURES

Materials—32P, was obtained from ICN Radiochemical. [3H]Leucine and [3H]thymidine were purchased from New England Nuclear. All tissue culture reagents were from Gibco Laboratories (Grand Island, New York). Cellulose-precoated TLC plates (0.1 mm) were purchased from Merck. All other reagents were obtained from Sigma. Solvents were from Mallinckrodt Chemical Works and were of analytical grade.

Cell Culture—Human foreskin fibroblasts (primary culture, HF33c, used between passages 10–30) were cultured in Eagle’s minimum essential medium, supplemented with 10% fetal calf serum (FCS), 50 IU/ml penicillin, 5 μg/ml streptomycin, and appropriate concentrations of LTA. Each plastic dish (100 mm, Costar 3100) was seeded with 4 × 10⁵ cells and was used after the cells had grown to confluency (3–4 days).

Determination of DNA Synthesis and Protein Synthesis—DNA and protein synthesis were measured in stimulated as well as unstimulated cells by determining the incorporation of [3H]thymidine and [3H]leucine, respectively. Cells to be assayed were pulse-labeled for 18 h with 1 μCi of [3H]thymidine in the standard incubation medium or with 1 μCi of [3H]leucine in leucine-free minimum essential me-
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RESULTS AND DISCUSSION

In order to study the mitogenic effect of LTA on human foreskin fibroblasts, DNA and protein synthesis were assayed in unstimulated and LTA-stimulated cells. In a typical experiment, the cells were treated with LTA at concentrations of 1, 10, and 100 μg/ml of culture medium for 72 h, and incorporation of [3H]thymidine and [3H]leucine was measured. The maximum incorporation of [3H]thymidine was observed in cells treated with 1 μg/ml LTA (Fig. 1); incorporation decreased as the concentration was increased to 10–100 μg/ml. The incorporation of [3H]leucine followed a similar pattern (Fig. 1). The incorporation of the radiolabeled amino acid reached a maximum at 1 μg/ml LTA but was suppressed to the untreated control level at 100 μg/ml. The total incorporation of 32P into the cellular proteins reached a maximum at a concentration of 5 μg/ml LTA (Fig. 1, inset). At higher concentrations of LTA, the uptake of 32P into the trichloroacetic acid-precipitable proteins was suppressed in parallel with the suppression of DNA and protein synthesis. The absolute level of phosphorylation varied from experiment to experiment probably because of variations in the state of confluency and the number of passages the test cells had undergone before each experiment.

Previous studies from this laboratory and other groups have demonstrated that at low concentrations, LTA acts as a mitogen, whereas at high concentrations it acts as a cytotoxic agent (7–9). The molecular mechanism(s) of mitogenicity of LTA were not explored in these reports. Several reports indicate that the mitogenic effects of various biological polypeptides including epidermal growth factor, platelet-derived growth factor, and insulin are associated with phosphorylation of cellular proteins at tyrosine residues (19–29). Since tyrosine-specific protein kinase activities are increased in concert with the phosphorylation of growth factor receptors, it is believed that the biological effects of these agents require the covalent modifications of certain polypeptides.

In order to determine whether or not LTA stimulates the phosphorylation of cellular proteins, human foreskin fibroblasts were incubated with 32P, in the absence or presence (0.01–25 μg/ml) of LTA. SDS-polyacrylamide gel electrophoresis and autoradiography of the 178,000 × g supernatant of whole cell lysate showed that LTA stimulated an increased phosphorylation of two proteins migrating at 98 and 94 kDa (Fig. 2). Phosphorylation of the 94-kDa protein was maximal (2.5-fold) at 5 μg/ml LTA, as shown by densitometric scanning of the autoradiogram, whereas that of the 98-kDa polypeptide was random (data not shown).

It is known that the insulin receptor (350 kDa) is composed of two α-subunits (120–130 kDa) and two β-subunits (90–95 kDa) which are linked by disulfide bonds (38–40). In addition,
the β-subunit of the receptor undergoes phosphorylation in the presence of insulin (21, 41–44). Like insulin, type 1 insulin-like growth factor is composed of two α-subunits (130 kDa) and two β-subunits (98 kDa) and are linked by sulfhydryl groups (45, 46). Since the β-subunit of insulin receptor and the type-1 insulin-like growth factor have a molecular mass of approximately 94 kDa, it was of interest to explore the identity of the 94-kDa phosphorylated polypeptide present in LTA-stimulated fibroblasts. Electrophoresis of 32P-labeled whole cell lysate from fibroblasts under reducing and nonreducing conditions showed no discernible difference in the migration or intensity of the 94-kDa phosphorylated polypeptide (data not shown), indicating that the 94-kDa polypeptide is not part of the insulin receptor complex.

It has previously been shown that a 98-kDa cellular protein (NCP 98) from normal chicken bone marrow cell extracts cross-reacts and is functionally homologous with the Fujinami sarcoma virus transforming protein PI40 (47). NCP 98 is a phosphoprotein with associated kinase activity capable of phosphorylating itself and other protein substrates specifically at tyrosine residues. Therefore, attempts were made to identify the phosphoamino acids of the 98-kDa as well as the 94-kDa protein stimulated by LTA.

In order to determine the degree of phosphorylation of serine as opposed to tyrosine or threonine residues, SDS gels containing the separated cellular proteins were exposed to alkali (35). The phosphate-ester linkages of serine are more labile in alkali than are those of tyrosine and threonine. Alkali treatment did not alter the relative intensities of the 98-kDa band either from unstimulated or from LTA-stimulated cells (Fig. 3). In contrast, the phosphorylated 94-kDa protein band in unstimulated control cells disappeared completely after alkali treatment, whereas that of the LTA-stimulated cells remained intense (Fig. 3). These results suggested that LTA stimulated phosphorylation of the 98-kDa protein mainly at serine residues, while stimulating that of the 94-kDa protein at threonine or tyrosine residues.

In order to distinguish the degree of phosphorylation of serine and threonine as opposed to tyrosine in the 94-kDa protein, we performed high voltage electrophoresis of the acid-hydrolyzed proteins precipitated with acetone from lysates of cells stimulated with LTA. LTA stimulated a dramatic increase in phosphorytrosine but only a slight increase in phosphothreonine residues (Fig. 4). It should be noted that the absolute stimulation of tyrosine kinase activity varied slightly from experiment to experiment with a resultant change in the ratio of serine to tyrosine phosphate residues. The degree of stimulation by LTA of tyrosine phosphorylation appeared to be related to the number of cell passages the fibroblasts had undergone before the experiments were performed (48).

**FIG. 2.** Phosphorylation pattern of cellular proteins in LTA-stimulated fibroblasts. Confluent cultures of human foreskin fibroblasts were labeled with 32P; at 37°C for 18 h as described under "Experimental Procedures." The cells were removed by scraping in buffer A and lysed with buffer B (see "Experimental Procedures"). The 178,000 × g supernatant was boiled with SDS sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 10% glycerol and electrophoresed in a 6–15% gradient SDS gel. The gels were stained, destained, dried, and exposed to XAR-5 (Kodak) film for autoradiography. The molecular weight standards used were: myosin, Mr = 200,000; β-galactosidase, 116,000; phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 45,000; carbonic anhydrase, 31,000; and soybean trypsin inhibitor, 21,000.

**FIG. 3.** Alkali treatment of SDS-polyacrylamide gel. The 178,000 × g supernatants from LTA-treated and control cells labeled with 32P, were electrophoresed in a 6–15% gradient SDS gel. The gel was stained with Coomassie Brilliant Blue R-250 and treated with 1 N NaOH for 1 h at 40°C. The gel was rinsed in 7.5% (v/v) acetic acid, 10% (v/v) methanol and subjected to autoradiography as described previously except that the exposure time was doubled. The standards used were the same as for Fig. 2.

**FIG. 4.** Analysis of phosphoamino acids in LTA-stimulated fibroblasts. The cells were labeled with 32P in the presence of different concentrations of LTA as described under "Experimental Procedures." The cells were lysed in buffer B and subjected to centrifugation. The supernatant from the 178,000 × g centrifugation was precipitated in the presence of cold acetone and washed as described under "Experimental Procedures." The precipitate was hydrolyzed in 6 N HCl and hydrolyzed for 2 h at 110°C under N2. The hydrolysate was dried and dissolved in water. The high voltage electrophoresis was carried out as described under "Experimental Procedures." The phosphoamino acids were identified by co-migration of the radioactivity with 10 μl of internal standards of phosphoserine (Ser(P)) (1 mg/ml), phosphothreonine (Thr(P)) (1 mg/ml), and phosphotyrosine (Tyr(P)) (1 mg/ml). The standards were detected by spraying with 0.2% ninhydrin in acetone.
tyrosine residues in the 94-kDa protein (Fig. 5); phosphotyrosine could not be detected in the proteins obtained from unstimulated control cells, and phosphothreonine was not detectable in this protein obtained from either unstimulated or LTA-stimulated cells (Fig. 5).

In order to correlate increases in DNA synthesis in the presence of LTA with the phosphorylation of the cellular proteins, simultaneous time course experiments were performed (Fig. 6). Cells were stimulated with 5 μg/ml LTA since at this concentration the incorporation of 32P into proteins was evidenced by trichloroacetic acid precipitation was maximum (Fig. 1). Following the addition of LTA, cells were labeled with [3H]thymidine or 32P; at 24, 48, and 72 h (Fig. 6). [3H]Thymidine uptake was maximum at 72 h, as was phosphorylation of the 94-kDa protein. The incorporation of 32P into fibroblast proteins was analyzed by lysing the cells and electrophoresing the 178,000 × g supernatant on a 6–15% gradient gel. The autoradiogram of the dried gel showed a consistent increase in the phosphorylation of the 94-kDa polypeptide with time, whereas in the control cells the phosphorylation of this protein remained the same as determined by densitometric scanning.

Preliminary studies indicate that the 94-kDa protein is located in the cell membrane. When LTA-stimulated and unstimulated cells were lysed by Dounce homogenization in 0.25 M sucrose, 10 mM Tris-HCl, 0.2 mM MgCl2, 5 mg/ml bovine serum albumin, pH 7.4, followed by sucrose density gradient centrifugation, the 94-kDa protein was found in the membrane particulate fraction but not in the supernatant. Because of its membrane location, this protein becomes a candidate as a membrane receptor for the binding of LTA.

Although the mitogenic effects of amphipathic molecules, including LTA and LPS, have been investigated, no molecular mechanism of these actions has been elucidated. Recently, it has been reported that biologically active lipid moieties of LPS activate protein kinase C (18), indicating that the phosphorylation of certain polypeptides may be relevant to the biological activity of LPS. The studies of LPS were carried out by using partially purified protein kinase C. In the present study, enhancement of tyrosine kinase activity as well as phosphorylation of a cellular protein at tyrosine residues was observed in LTA-stimulated cells. Although there is no direct proof that enhancement of the phosphotyrosine level in the presence of this amphipathic molecule is sufficient to bring about the mitogenic effect of LTA, it is, nevertheless, possible that the covalently modified protein(s) plays an important role in the biochemical events associated with the biological properties of streptococcal lipoteichoic acid.

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