Pertussis Toxin Inhibits Retinoic Acid-induced Expression of Tissue Transglutaminase in Macrophages*

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Retinoic acid rapidly induces the accumulation of a specific enzyme, tissue transglutaminase (EC 2.3.2.13), in mouse macrophages. We have used the induction of tissue transglutaminase to study the regulation of gene expression by retinoic acid. In this study we report that pertussis toxin can inhibit retinoic acid-induced expression of tissue transglutaminase in mouse resident peritoneal macrophages. This inhibition is paralleled by the ADP-ribosylation of 41,000-dalton macrophage membrane protein.

MATERIALS AND METHODS

All-trans-retinoic acid was the generous gift of Hoffmann-La Roche. NAD, thymidine, arginine, creatine phosphate, creatine phosphokinase, isonitros, and CsCl were purchased from Sigma. [3H]Putrescine, [α-32P]NAD (28 Ci/mmol), and an in vitro translation kit were purchased from New England Nuclear. [35S]Methionine and [125I]protein A were purchased from ICN (Irvine, CA). Pertussis toxin was obtained from List Biologicals (Irvine, CA). Rabbit anti-goat antibodies used have been described in detail (6). Delipidized mouse serum was obtained by the procedure of Rothblat et al. (7) from frozen commercial serum (Pel-Freez Biologicals, Rogers, AR). Adult ICR strain mice were obtained from Harlan Sprague-Dawley (Houston, TX).

Mouse peritoneal macrophages were obtained from lavages of ICR strain mice in a manner already described (6). Cell viability was determined by trypan blue exclusion. Transglutaminase activity was determined by measuring the Ca\(^{2+}\)-dependent incorporation of [3H]putrescine into N,N-dimethyl casein as described previously (6). Protein concentrations were determined by the method of Bradford (8). Determinations of the levels of tissue transglutaminase polypeptides in macrolephage extracts were performed by solubilizing monolayers in boiling SDS\(^+\) electrophoresis sample buffer, fractionating on 10% discontinuous SDS-polyacrylamide gels (9) and electroblotting to nitrocellulose filters as previously described (6, 10). After saturating the filters with protein, the nitrocellulose was probed with anti-tissue transglutaminase antibody, washed, and then treated with affinity purified rabbit anti-goat IgG and 125I-protein A. The filters were extensively washed and autoradiographed.

To determine overall protein synthetic rates, cells were incubated with [35S]methionine in methionine-free media for 1 h, washed, solubilized in 0.05% Triton X-100 in 20 mM Tris-HCl, pH 7.5, twice co-precipitated with bovine serum albumin in 20% trichioroacetic acid at 4 °C, taken up in 0.1 N NaOH, and counted. Background levels were determined in wells treated with 50 μg/ml cycloheximide.

Macrophages (0.5 × 10⁶/well) were metabolically labeled by incubating with 10 μCi of [35S]methionine in methionine-free media for 40 min. The cells were washed and harvested or incubated in prelabeling medium. Immunoprecipitation was performed as described previously (14).

Macrophage RNA was isolated and translated as described previously (5) by CsCl gradient centrifugation and differential precipitation. Total RNA was translated using a commercial kit with reticulocyte lysate.

Macrophage membranes were prepared as follows. Monolayers were washed with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, placed at 4 °C for 15 min, scraped, and pelleted at 400 × g for 10 min. Cell pellets were resuspended in 5 mM Tris-HCl, pH 7.8, 5 mM NaCl, 0.1 mM EDTA, 1 mM DTT and incubated 3 min. MgCl₂ was added to 5 mM for 2 min, and the cells were disrupted with 45 strokes of a glass homogenizer and centrifuged at 400 × g for 15 min. Supernatants were centrifuged at 40,000 × g for 15 min, washed, taken up in homogenization buffer, and stored at −70 °C (11). In vitro labeling was performed using a modification of the procedure of Katada and Ull (12).

Macrophage membranes (20 μg) were incubated with 10 μM [α-32P]NAD, 1 mM ATP, 0.1 mM GTP, 5 mM MgCl₂, 10 mM thymidine, 10 mM arginine, 5 mM creatine phosphate, 1 unit/ml creatine phosphokinase, 1 mM EDTA, 25 mM HEPES, pH 7.2, 25 mM isonitros, and 1 μg/ml pertussis toxin (activated by treatment with 100 mM DTT for 15 min at 30 °C) for 30 min at 30 °C. A second aliquot of [α-32P]NAD to 10 μM was added after 15 min. The reaction was stopped with 2 ml of ice-cold 25 mM HEPES, pH 7.7, and centrifuged 15 min at 40,000 × g. The pellets were dissolved in SDS sample buffer, fractionated on 10% discontinuous SDS-polyacrylamide gels, and autoradiographed.
RESULTS

Pertussis Toxin Inhibits the Induction of Tissue Transglutaminase by Retinoic Acid in Macrophages—To examine the effect of pertussis toxin on retinoic acid-induced expression of tissue transglutaminase in macrophages, mouse resident peritoneal macrophages were preincubated for 4 h in RPMI 1640 media and 1% delipidized mouse serum (DLMS) with varying concentrations of pertussis toxin, and then transglutaminase was induced by the addition of 10 nM trans-retinoic acid for 20 h. The basal level of transglutaminase activity in untreated cells was 54 pmol/min/10^6 cells, and the addition of 10 nM retinoic acid increased this activity by 170 pmol/min/10^6 cells (Fig. 1A). Cells exposed to pertussis toxin showed a dose-dependent inhibition of the induction of the enzyme; 0.1 ng/ml produced a significant decrease in the level of transglutaminase activity and 10 ng/ml gave a maximal effect. Pertussis toxin did not produce a complete block; in the presence of 100 ng/ml pertussis toxin, 10 nM retinoic acid induced a small increase (70 pmol/min/10^6 cells) in macrophage transglutaminase activity.

We next examined the effect of a high level of pertussis toxin (50 ng/ml) on the induction of macrophage transglutaminase by different levels of retinoic acid. Macrophages were preincubated for 4 h with pertussis toxin in 1% DLMS and then transglutaminase was induced by the addition of varying levels of trans-retinoic acid in 1% DLMS. As we have reported previously (3), resident peritoneal macrophages respond to very low levels of trans-retinoic acid with a marked induction of tissue transglutaminase (Fig. 1B, open circles). Addition of 0.1 nM retinoic acid to the medium resulted in a doubling of transglutaminase activity, and increasing concentrations of retinoic acid up to 100 nM induced a dose-dependent accumulation of the enzyme in these cells. If the cells were first incubated with pertussis toxin the induction of transglutaminase was reduced (Fig. 1B, closed circles). In cells preincubated with pertussis toxin, 0.1 nM trans-retinoic acid had no effect on macrophage transglutaminase activity and 10–100 nM retinoic acid was required to induce a doubling of the enzyme activity of the cells.

Pertussis toxin also inhibits the induction of transglutaminase by retinoic acid in the absence of delipidized serum (Fig. 1C). The basal and induced levels of transglutaminase are lower than those observed in cells incubated in DLMS, but 50 and 100 nM free retinoic acid each gave a greater than 3-fold increase in transglutaminase activity. When cells were first preincubated with 50 ng/ml pertussis toxin, the induction of transglutaminase by 50 and 100 nM retinoic acid was inhibited 80% and 79%, respectively.

To differentiate between an effect of pertussis toxin on transglutaminase induction and inhibition of enzyme activity, we used a specific anti-tissue transglutaminase antibody to measure the levels of the protein in control and pertussis toxin-treated cells (Fig. 2A). Lane 1 shows the immunoblot of control cells incubated in 1% DLMS alone. The band of tissue transglutaminase in retinoic acid-treated cells (lane 2) is due to the marked induction of the enzyme by retinoic acid and DLMS. Lane 3 shows that the effect of retinoic acid in the cells that have been pretreated with pertussis toxin. Retinoic acid induced a small increase in the levels of tissue transglutaminase, but the accumulation is much less than in the cells which had not been pretreated with pertussis toxin. Comparison of the intensity of the transglutaminase band in the control and pertussis toxin-treated cells (lanes 2 and 3) shows that the induction of tissue transglutaminase was 60% inhibited by pertussis toxin. This reduced level of the enzyme matched the effect of the toxin on the induction of transglutaminase activity.

Inhibition of transglutaminase induction appears to be a very selective effect of pertussis toxin. Treating macrophages with 10 ng/ml pertussis toxin for up to 20 h had no significant effect on the morphology, viability, or overall rate of protein synthesis. In a representative experiment, pretreatment with 10 ng/ml pertussis toxin for 14 h produced no significant inhibition of protein synthesis (10 ± 15%) while greatly inhibiting the induction of transglutaminase (74 ± 5%). A more stringent test of the selectivity of the effect was to compare the rate of synthesis of a large number of proteins from control and pertussis toxin-treated cells. Fig. 2B, lanes 1 and 2, compares the profile of radiolabeled polypeptides from control and pertussis toxin-treated cells pulse labeled with [35S]methionine. The identical patterns of polypeptides indicates the synthesis of a large number of macrophage.
proteins is unaffected by pre-exposure of the cells to even high levels of pertussis toxin.

To demonstrate that the effect of pertussis toxin on transglutaminase accumulation is due to inhibition of tissue transglutaminase synthesis and not increased degradation of the polypeptide, we compared the rate of turnover of tissue transglutaminase by pulse-labeling macrophages with [35S]methionine and chasing with unlabeled methionine (Fig. 3). Immunoprecipitated tissue transglutaminase (arrowhead) that accumulated during 40-min pulse and 0-h chase was increased in cells incubated with 10 nM retinoic acid for 10 h (lane 2, 0) over the basal synthesis observed in control cells (lane 1, 0) and the level of synthesis observed in cells preincubated with 50 ng/ml pertussis toxin and treated with 10 nM retinoic acid for 10 h (lane 3, 0). The levels of labeled tissue transglutaminase remaining after 24-h chase were found by densitometric scanning to be 17 ± 4% for control cells (lanes in set 1), 49 ± 5% in cells treated with retinoic acid for 10 h (lanes in set 2), and 82 ± 2% in cells preincubated with 50 ng/ml pertussis toxin and treated with 10 nM retinoic acid for 10 h (lanes in set 3).

Inhibition of transglutaminase synthesis by pertussis toxin could be due to lower rates of translation from the same amount of tissue transglutaminase mRNA or to inhibition of mRNA accumulation. To assess whether pertussis toxin inhibits the accumulation of tissue transglutaminase mRNA, we prepared RNA from treated macrophages and translated it in vitro. Immunoprecipitation of the translated products allows us to determine the amount of translatable tissue transglutaminase mRNA present with each treatment (Fig. 4). (5). Control macrophages treated with 1% DLMS for 14 h showed no translated tissue transglutaminase (lane 1). Macrophages treated with 1% DLMS for 4 h followed by 10 nM retinoic acid in 1% DLMS for 10 h showed a prominent band of tissue transglutaminase (lane 2), and the macrophages treated with 50 ng/ml pertussis toxin in 1% DLMS for 4 h followed by 10 h in 10 nM retinoic acid in 1% DLMS showed a much lower level of translated enzyme (lane 3). Densitometric scanning of these bands revealed that pertussis toxin treatment inhibited the accumulation of translatable message by 70%. Pertussis toxin had no effect on the abundance of the other translatable messages detected by fractionation of total in vitro translation products on SDS-polyacrylamide gels (data not shown).

Pertussis Toxin ADP-ribosylates a 41,000-Dalton Protein in...
Macrophage Membranes—Pertussis toxin contains a mono-ADP-ribosyltransferase activity that is thought to be responsible for its effects on signal transduction. To identify substrates of the toxin in mouse macrophage membranes we incubated a crude preparation of macrophage membranes with pertussis toxin and [α-32P]NAD. Initially the very high levels of NAD-glycohydrolase activity in macrophage membranes prevented ADP-ribosylation (13). Under standard assay conditions (50 μg of membrane protein, 10 μM [α-32P]NAD, 30 °C), more than 99% of the NAD was hydrolyzed in 1 min. We found that the addition of 25 mM isoniazid to the assay blocked the breakdown of NAD (<2% of the NAD was destroyed) without reducing the catalytic activity of the toxin. In the presence of isoniazid we were then able to show that pertussis toxin catalyzed the incorporation of [α-32P]NAD into a single polypeptide band (Mr, 41,000) in mouse macrophage membranes (Fig. 5, lanes 1 and 2). This band had the same electrophoretic mobility (shown by the arrowhead) as Ns from mouse lymphoma cell (S-49) membranes.

To determine whether the same protein could be modified by pertussis toxin in intact macrophages, we preincubated macrophages with varying concentrations of pertussis toxin for 4 h. The cells were then disrupted, a crude membrane fraction was prepared, and the endogenous 41,000-dalton protein in these membranes was ADP-ribosylated by incubation with fresh toxin and [α-32P]NAD. The control for these studies, membranes prepared from cells not exposed to the toxin (Fig. 5, lane 2), demonstrated the incorporation of [32P]ADP-ribose into the 41,000-dalton protein. There was much less labeling of the 41,000-dalton protein in the cells that had been preincubated with the toxin. Lane 3 shows that treating macrophages for 4 h with 0.1 ng/ml of toxin greatly reduced the susceptibility of the macrophage 41,000-dalton protein for subsequent in vitro labeling. Treating the cells with 10 ng/ml pertussis toxin completely blocked the subsequent labeling of this protein (lane 4). These results indicate that the toxin can modify the 41,000-dalton protein in intact macrophages.

FIG. 5. Labeling of pertussis toxin substrates in macrophages. Membrane fractions were prepared from untreated mouse resident peritoneal macrophages (lanes 1 and 2) or macrophages which had been treated for 4 h with 0.1 ng/ml (lane 3) or 10 ng/ml (lane 4) pertussis toxin. Each of these sets of membrane fractions was subjected to in vitro labeling with [α-32P]NAD and pertussis toxin. Activated pertussis toxin at 1 μg/ml was included in each of the incubations save for those represented by lane 1. After labeling, membranes were pelleted, solubilized, and fractionated on a 10% discontinuous SDS-polyacrylamide gel. The arrow shows the position of mouse Ns on these gels. Molecular weight standards are 94 kDa, phosphorylase b, 67 kDa, bovine serum albumin, 43 kDa, ovalbumin, and 13.7 kDa, ribonuclease.

Discussion

Our interest in the effects of pertussis toxin on transglutaminase gene expression was an unanticipated outgrowth of studies on the effects of agents that elevate intracellular levels of cyclic AMP on retinoic-induced gene expression in myeloid cells. Previous work from our laboratory had shown retinoic acid was an acute and specific inducer of tissue transglutaminase expression (3, 4) and that analogues of cyclic AMP or agents that elevate intracellular cyclic AMP had a synergistic effect on the induction of the enzyme (14). Cholera toxin and pertussis toxin are bacterial toxins that can elevate intracellular cyclic AMP levels by modifying the activity of the GTP-binding subunits of adenylate cyclase (15). Cholera toxin ADP-ribosylates and activates the stimulatory subunit of the cyclase (Ns) and thereby increases intracellular cyclic AMP levels (16). Pertussis toxin ADP-ribosylates the inhibitory subunit of the cyclase (Nis) and by inactivating this protein can increase intracellular levels of cyclic AMP in some cells (12). As expected, we found that pretreatment of macrophages with cholera toxin greatly increased the transglutaminase-inducing activity of low levels of retinoic acid (14). To our surprise, however, we found that pertussis toxin actually inhibited the induction of tissue transglutaminase.

Our first concern was that this inhibition might represent some generalized inhibitory effect of the toxin on macrophage function. The levels of the toxin (0.1–1.9 ng/ml) that were effective, however, are far below the levels associated with nonspecific (B-subunit) effects of the toxin. Furthermore, using a number of indices including cell morphology, viability, and the rate of synthesis of a large number of specific proteins, we saw no generalized effect of the toxin. The toxin appeared to exert a very selective effect on the expression of the enzyme tissue transglutaminase. Pertussis toxin also inhibited the induction of transglutaminase by retinoic acid in the absence of serum proteins. The effect of pertussis toxin was found to be the result of decreased synthesis of tissue transglutaminase due to inhibition of tissue transglutaminase mRNA accumulation. Pertussis toxin did not enhance the rate of degradation of tissue transglutaminase. Thus, we believe that pertussis toxin is a selective and specific antagonist of retinoic acid-induced transglutaminase expression in macrophages.

Two features of the inhibitory effect of the toxin are striking. The first is that the toxin had little or no effect on the basal levels of transglutaminase expression in freshly isolated cells. We do not know the mechanisms that control the expression of the enzyme in unstimulated cells but this process is apparently refractory to toxin-induced suppression. Secondly, it is significant that even at high levels of toxin the inhibition of transglutaminase expression was not complete, and the actual extent of suppression is diminished in the presence of high levels of retinoid. We interpret these findings as suggesting that the primary effect of the toxin is to reduce the sensitivity of the cells to retinoid-induced gene expression rather than produce an absolute block on the action of the retinoid. It is also possible the incomplete inhibition reflects
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the presence of more than one mode of transglutaminase induction, one of which is inhibited by pertussis toxin. In several other experimental systems it has been reported that pertussis toxin may produce incomplete inhibition of hormone action (17-21) and it has been observed that high levels of certain agonists can produce a partial reversal of the inhibitory effects of the toxin (19-21). There is as yet no general explanation for this particular feature of pertussis toxin action.

We do not as yet know the molecular events that account for the inhibitory effect of pertussis toxin on transglutaminase induction in macrophages, but our data suggest that ADP-ribosylation of a 41,000-dalton macrophage membrane protein is likely to be involved in the process. Certainly this protein is the only substrate for pertussis toxin-catalyzed ADP-ribosylation that we can find in macrophage membranes, and furthermore, this protein becomes ADP-ribosylated in intact cells under conditions that parallel the suppression of tissue transglutaminase expression. The 41,000-dalton macrophage protein has the same electrophoretic mobility as mouse Nm, but we do not know whether this is the protein that interacts with adenylate cyclase or some different but related polypeptide.

There is considerable evidence to suggest that retinoids play an important role in regulating the growth and differentiation of both normal and malignant cells. Several laboratories, including our own, have suggested that abnormalities in retinoid responsiveness, particularly the loss of sensitivity to physiological levels of retinoic acid, may be an important contributor to abnormalities in growth control in malignant cells (4,22). The studies that we are reporting here demonstrate that the ADP-ribosylation of one or more membrane-associated proteins can result in a loss of sensitivity of macrophages to retinoic acid-induced gene expression. Since this pertussis toxin substrate is presumptively a GTP-binding protein, we are currently investigating the possibility that abnormalities in GTP-binding protein activity can modify the sensitivity of malignant cells to retinoids and contribute to the abnormal growth properties of leukemic cells.

Finally, one intriguing possibility is the existence of a membrane receptor for retinoid acid. Recently, the β-adrenergic receptor has been found to be homologous to the retinoid binding photoreceptor, rhodopsin (23). Signalling through each of these plasma membrane receptors requires GTP-binding proteins. We are currently carrying out studies to determine whether retinoid action is mediated through an as yet unidentified member of this family.

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