Pasturella multocida Toxin, a Potent Intracellularly Acting Mitogen, Induces p125FAK and Paxillin Tyrosine Phosphorylation, Actin Stress Fiber Formation, and Focal Contact Assembly in Swiss 3T3 Cells*

Hadriano M. Lacerda‡§ Alistair J. Lax†, and Enrique Rozengurt‡

From the Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom and †Institute for Animal Health, Compton, Newbury, Berkshire RG20 7NN, United Kingdom

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Treatement of Swiss 3T3 cells with recombinant Pasteurella multocida toxin (rPMT), a potent intracellularly acting mitogen, stimulated tyrosine phosphorylation of multiple substrates including bands of M, 110,000–130,000 and M, 70,000–80,000. Tyrosine phosphorylation induced by rPMT occurred after a pronounced lag period (1 h) and was blocked by either lysosomotropic agents or incubation at 22 °C. Focal adhesion kinase (p125FAK) and paxillin are prominent substrates for rPMT-stimulated tyrosine phosphorylation. Tyrosine phosphorylation by rPMT could be dissociated from both protein kinase C activation and the mobilization of calcium from intracellular stores. rPMT stimulated striking actin stress fiber formation and focal adhesion assembly in Swiss 3T3 cells. Cytochalasin D, which disrupts the actin cytoskeleton, completely inhibited rPMT-induced tyrosine phosphorylation. In addition, tyrosine phosphorylation of p125FAK and paxillin in response to rPMT was completely abolished when cells were subsequently treated with platelet-derived growth factor at a concentration (30 ng/ml) that disrupted the actin cytoskeleton. Our results demonstrate for the first time that rPMT, a bacterial toxin, induces tyrosine phosphorylation of p125FAK and paxillin and promotes actin stress fiber formation and focal adhesion assembly in Swiss 3T3 cells.

The mechanism of action of bacterial toxins has provided insights into the control of cellular regulatory processes, including signal transduction and cell proliferation (1–3). Recombinant Pasteurella multocida toxin (rPMT) is an extremely potent mitogen for murine Swiss 3T3 cells, other fibroblast cell lines, and early-passage cultures and promotes anchorage-independent growth of Rat-1 cells (4, 5). The toxin is a 146-kDa protein that has been purified, cloned, and sequenced (6–13). The deduced amino acid sequence of PMT shows partial homology with CNF1 and CNF2, produced by some strains of pathogenic Escherichia coli (14, 15). It has been proposed that PMT enters the cells and acts intracellularly to initiate and sustain DNA synthesis.

Prior to the stimulation of DNA synthesis, rPMT stimulates the formation of inositol phosphates and mobilizes Ca2+ from an intracellular pool (16). Analysis of the inositol phosphate species generated in response to rPMT strongly suggests that the toxin stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate by activating cellular phospholipase C (16), a major transducer of transmembrane signaling (17). rPMT also increases the cellular content of diacylglycerol, causes translocation of PKC to cellular membranes, and stimulates the phosphorylation of 80K/MARCKS (18), a prominent substrate of PKC in cultured fibroblasts (19–22). The stimulation of these early events by rPMT, like its mitogenic action (5), requires cellular entry and activation of the toxin.

Changes in protein tyrosine phosphorylation are known to play a key role in the action of growth factors and oncogenes but have not been demonstrated in response to rPMT. Recently, tyrosine phosphorylation of the cytosolic protein kinase p125FAK (23, 24) and of the cytoskeleton-associated protein paxillin (25, 26) have been identified as early events in the action of diverse signaling molecules that mediate cell growth and differentiation including mitogenic neuropeptides (27–29), the bioactive lipids LPA and sphingosine (30–32), extracellular matrix proteins (33–37), PDGF at low concentration (38), and transforming variants of src (39). The increases in p125FAK and paxillin tyrosine phosphorylation are accompanied by profound alterations in the organization of the actin cytoskeleton and in the assembly of the focal adhesion plaques (31, 38, 40, 41), the distinct sites in the plasma membrane where both p125FAK and paxillin are localized (23, 24, 42). The effects of rPMT on protein tyrosine phosphorylation, actin stress fiber formation and focal adhesion assembly were unknown.

Here we report that rPMT stimulates tyrosine phosphorylation of multiple proteins including p125FAK and paxillin and induces a striking increase in stress fiber formation and focal adhesion assembly in Swiss 3T3 cells. The mode of action of rPMT differs from that of neuropeptides, growth factors, and extracellular matrix proteins in that the toxin appears to enter the cells to trigger protein tyrosine phosphorylation and cytoskeletal reorganization.

EXPERIMENTAL PROCEDURES

C6I Culture—Stock cultures of Swiss 3T3 fibroblasts were maintained in DMEM supplemented with 10% FBS in a humidified atm-
sphere containing 10% CO₂ and 90% air at 37 °C. For experimental purposes, cells were plated either in 30-mm Nunc Petri dishes at 10⁵ cells/dish, or in 90-mm dishes at 6 × 10⁵ cells/dish, in DMEM containing 10% fetal bovine serum and used after 6–8 days when the cells were confluent and quiescent (43).

Immunoprecipitation—Quiescent cultures of Swiss 3T3 cells (1–2 × 10⁵) were incubated twice with DMEM, treated with rPMT or other factors in 10 ml of DMEM/Waymouth 1:1 (v/v) for the times indicated, and lysed at 4 °C in 1 ml of a lysis buffer solution containing 10 mM Tris/HCl, pH 7.5, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na₃VO₄, and 1% Triton X-100. Proteins were immunoprecipitated at 4 °C overnight with agarose-linked mAbs directed against phosphotyrosine, paxillin, or p125FAK as indicated. Immunoprecipitates were washed three times with lysis buffer and extracted for 10 min at 95 °C in 2 × SDS-PAGE sample buffer (200 mM Tris/HCl, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8) and analyzed by SDS-PAGE.

Western Blotting—Treatment of quiescent cultures of cells with factors, cell lysis, and immunoprecipitations were performed as described above. After separation by SDS-PAGE, proteins were transferred to Immobilon membranes (44). Membranes were blocked using 5% nonfat dried milk in PBS, pH 7.2, and incubated for 3–5 h at 22 °C with a mixture of PY20 and 4G10 anti-Tyr(P) mAbs (1 μg/ml each). Immunoreactive bands were visualized using [125I]-labeled sheep anti-mouse IgG followed by autoradiography. Autoradiograms were scanned using an LKB Ultrascan XL internal integrator. The values expressed represent percentages of the maximum increase in tyrosine phosphorylation above control values.

32P Labeling of Cells and Analysis of 80K/MARCKS Phosphorylation—Quiescent and confluent cultures of Swiss 3T3 cells in 30-mm dishes were washed twice in phosphate-free DMEM and incubated at 37 °C with this medium containing 50 μCi/ml carrier-free [32P]Pi. After 12 h, various factors were added for the indicated times. The cells were subsequently lysed, and the lysates were immunoprecipitated with specific anti-80K/MARCKS antibody (45).

Immunostaining of Cells—Quiescent Swiss 3T3 cells were washed twice with DMEM and incubated for the indicated time in DMEM/Waymouth 1:1 (v/v) at 37 °C with the indicated concentration of rPMT or other factors. For actin staining, cells were washed once with PBS, fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, and permeabilized with PBS containing 0.2% Triton X-100 for 8 min at room temperature. The cells were then incubated with TRITC-conjugated phalloidin (0.25 μg/ml) in PBS for 10 min at room temperature and visualized using a Zeiss Axiohot immunofluorescence microscope. In experiments in which quiescent Swiss 3T3 cells were labeled with both TRITC-conjugated phalloidin and anti-vinculin antibody, the cells were fixed and permeabilized as described above and then stained with a mixture of TRITC-conjugated phalloidin (0.25 μg/ml) and anti-vinculin antibody (dilution 1:100) for 30 min at room temperature. Cells were subsequently washed three times in PBS and mounted with FITC-conjugated with TRITC-labeled with anti-mouse IgG and FITC-conjugated anti-vinculin antibody at a dilution of 1:100 for another 30 min at room temperature.

Microinjection—For C3 ecocymzme microinjection experiments Swiss 3T3 cells were plated in 30-mm dishes at 10⁵ cells/dish in DMEM containing 10% fetal bovine serum and used after 6–8 days when the cells were confluent and quiescent. To facilitate localization of microinjected cells, a circle was scored on the bottom of the dishes, and in each experiment approximately 60 adjacent cells in the circle were microinjected. The efficiency of injection was determined by co-injecting rabbit immunoglobulin at 0.5 mg/ml followed by staining of the cells with FITC-conjugated anti-rabbit IgG antibody. Cells were stained with 4G10 mAb directed against phosphotyrosine residues and visualized by confocal microscopy using Cy5-linked anti-mouse IgG.

Confocal Microscopy—Confocal imaging was performed using a Bio-Rad MRC 600 laser scanning head fitted to a Nikon Optiphot microscope. A 40× numerical aperture/1.4 planachromatic oil immersion lens (Nikon) was used for all imaging. FITC- and TRITC-conjugated phalloidin fluorescent were excited at 488 and 568 nm, respectively, using argon/krypton mixed gas laser (Bio-Rad). Two filter blocks were used, K1 and K2. K1 is a double dichromic filter enabling excitation at the wavelengths of 488 and 568 nm, whereas the K2 filter is a 560-nm dichromatic combined with 522-nm green emission and 585-nm red emission filters. Images were collected using the Kalman filter. Care was taken to ensure that the TRITC-conjugated phalloidin channel was sufficiently bright relative to the fluorescence signal to minimize the contribution of bleed-through from the green channel into the red channel (approximately 10%). Correction of images for bleed-through and other processing was carried out using COMOS and SOM programs (Bio-Rad) run on a Compaq Deskpro 6664 486 computer (66 MHz). Data are presented as projections of sequential optical sections. For Z-series, optical sections were recorded at 0.5 μm. Final images were photographed directly from the VDU screen (see Figs. 4, 6, and 8).

Materials—Phorbol 12,13-dibutyrate, bombesin, cytochalasin D, TRITC-conjugated phalloidin, monoclonal anti-vinculin antibody, and FITC-linked anti-mouse IgG were obtained from Sigma. The specific PKC-inhibitor GF109203X and thapsigargin were obtained from Calbiochem-Novabiochem Ltd. Nottingham, UK. Agarose-linked anti-Tyr(P) mAb was purchased from Oncogene Science Inc., Manhasset, NY. 2A7 anti-p125FAK mAb was from TCS Biologicals Ltd., Buckingham, UK. 4G10 anti-Tyr(P) mAb was from Upstate Biotechnology, Inc., Lake Placid, NY. Py20 anti-Tyr(P) mAb and the mAb directed against paxillin (mAb 165) were from ICN, High Wycombe, UK. 4G10 anti-Tyr(P) mAb was from Upstate Biotechnology, Inc., Lake Placid, NY. mAb 2A7 directed against p125FAK was from TCS Biologicals Ltd., Buckingham, UK. Confocal imaging was performed using a Bio-Rad MRC 600 laser scanning head fitted to a Nikon Optiphot microscope. A 40× numerical aperture/1.4 planachromatic oil immersion lens (Nikon) was used for all imaging. FITC- and TRITC-conjugated phalloidin fluorescent were excited at 488 and 568 nm, respectively, using argon/krypton mixed gas laser (Bio-Rad). Two filter blocks were used, K1 and K2. K1 is a double dichromic filter enabling excitation at the wavelengths of 488 and 568 nm, whereas the K2 filter is a 560-nm dichromatic combined with 522-nm green emission and 585-nm red emission filters. Images were collected using the Kalman filter. Care was taken to ensure that the TRITC-conjugated phalloidin channel was sufficiently bright relative to the fluorescence signal to minimize the contribution of bleed-through from the green channel into the red channel (approximately 10%). Correction of images for bleed-through and other processing was carried out using COMOS and SOM programs.
The pattern of tyrosine-phosphorylated proteins induced by rPMT is strikingly similar to that stimulated by bombesin and LPA in Swiss 3T3 cells (27, 31). Recently, the cytosolic tyrosine kinase p125FAK (23, 24) and the adaptor protein paxillin (25, 26, 46) have been identified as prominent tyrosine-phosphorylated proteins in bombesin and LPA-treated cells (27–29, 31). To determine whether these cellular proteins were also substrates for rPMT-induced tyrosine phosphorylation, lysates of Swiss 3T3 cells, incubated with 20 ng/ml rPMT for 6 h, were immunoprecipitated with mAbs that recognize either p125FAK or paxillin, and the immunoprecipitates were analyzed by Western blotting with a mixture of anti-Tyr(P) mAbs. Fig. 1A shows that rPMT markedly stimulated p125FAK and paxillin tyrosine phosphorylation. Thus, p125FAK is a component of the broad M, 110,000–130,000 band, whereas paxillin is a component of the diffuse tyrosine-phosphorylated band migrating with an apparent M, 70,000–80,000.

**TABLE I**

| Addition | rPMT antiserum | Tyr(P) M, 110,000–130,000 band |
|----------|----------------|---------------------------------|
|          |                | 19 ± 6                          |
|          |                | 16 ± 5                          |
| BOM      |                | 67 ± 4                          |
| BOM      |                | 63 ± 3                          |
| rPMT     |                | 100                             |
| rPMT     |                | 24 ± 3                          |

Parallel cultures of these cells incubated at 22 °C. (iv) Many bacterial toxins that enter the cells cannot be removed by extensive washing (1). Fig. 2C demonstrates that the increase in tyrosine phosphorylation in rPMT-treated cells persisted after removal of the toxin from the extracellular medium. A transient exposure of cells to the toxin for 3-h stimulated maximum tyrosine phosphorylation. Thus, the toxin appears to enter the cells via an endosomal/lysosomal pathway where it is processed and then released into the cytosol in an active form.

**Role of PKC and Ca**2+** on rPMT-induced Tyrosine Phosphorylation—**rPMT induces a striking mobilization of Ca2+.**

Fig. 2A demonstrates that the increase in tyrosine phosphorylation by 20 ng/ml rPMT of the M, 110,000–130,000 band quantified by scanning densitometry. Values (mean ± S.E.; n = 3) are shown as percentage of the maximum response.

![Fig. 2. Effect of methylamine, temperature, and exposure time on rPMT-induced tyrosine phosphorylation](image)
from intracellular stores by specifically inhibiting the endo-
tyrosine phosphorylation of p125FAK by rPMT.

Formation of new stress fibers was detected after a lag period of approxi-
mately 1 h. Quiescent Swiss 3T3 cells induced localization of vinculin into focal adhesions, actin stress fibers, and focal contacts, which form at the end of actin stress fibers, we examined the effect of rPMT on actin cytoskeleton organization and focal adhesion assembly. Actin filaments were visualized with TRITC-conjugated phalloidin, and vinculin was detected by immuno-
nofluorescence with an anti-vinculin mAb. As shown in Fig. 4, quiescent Swiss 3T3 cells have few actin stress fibers. Addition of 20 ng/ml rPMT to quiescent Swiss 3T3 cells induced a striking increase in the formation of actin stress fibers. Formation of new stress fibers was detected after a lag period of approximately 1 h and reached a maximum after 8 h when the cells contained numerous densely packed stress fibers (Fig. 4, left).

Focal adhesions are subcellular structures which are formed at regions of close contact between cells and their underlying substratum. Several proteins are specifically localized in focal adhesions including vinculin, paxillin, talin, and α-actinin (52). Here we demonstrate that addition of rPMT to quiescent Swiss 3T3 cells induced localization of vinculin into focal adhesions, first visible after 1 h and reaching a maximum after 8 h (Fig. 4, right). Addition of 25 μM cycloheximide did not prevent the localization of vinculin into focal contacts induced by rPMT (results not shown). The relative amount of vinculin in focal adhesions, as judged by the intensity of immunofluorescent staining, increased in parallel with the association of stress fibers at these sites of the plasma membrane. Thus, rPMT induced actin stress fiber formation and focal adhesion assembly in Swiss 3T3 cells with kinetics that closely parallels rPMT induced tyrosine phosphorylation of p125FAK and paxillin.

Cytochalasin D Inhibits rPMT-Stimulated Tyrosine Phosphorylation of Multiple Bands Including p125FAK—The striking effects of PMT on stress fiber formation and focal adhesion assembly shown in Fig. 4 prompted us to examine whether the integrity of the actin filament network is necessary for rPMT stimulated tyrosine phosphorylation. Quiescent Swiss 3T3 cells were pretreated for 1 h with increasing concentrations of cytochalasin D and then stimulated with 20 ng/ml rPMT for 6 h. Cytochalasin D blocked rPMT-induced tyrosine phosphorylation of all phosphorylated bands including tyrosine phosphorylation of p125FAK in a similar, concentration-dependent manner (Fig. 5A). A complete inhibition of p125FAK tyrosine phosphorylation was achieved at the concentration of 1.2 μM cytochalasin D.

The inhibitory effect of cytochalasin D may, in theory, have resulted from interference with the entry and activation of PMT. To examine this possibility we tested the ability of rPMT to activate PKC and phosphorylate 80K/MARCKS in cells treated in the absence or presence of cytochalasin D. Quiescent Swiss 3T3 cells labeled with 32P, for 12 h, were pretreated with 1.2 μM cytochalasin D for 1 h and then challenged with 20 ng/ml rPMT for further 6 h. Fig. 5B shows that treatment with cytochalasin D at a concentration that completely inhibited rPMT-induced tyrosine phosphorylation did not prevent rPMT mediated stimulation of 80K/MARCKS phosphorylation.

Effect of High Concentrations of PDGF on rPMT-induced Actin Stress Fiber Formation and p125FAK Tyrosine Phosphorylation—Recent data from our laboratory have shown that PDGF at high concentrations (30 ng/ml) abolishes bombesin- and LPA-induced actin stress fiber and focal contacts (31, 38). This prompted us to investigate the effect of PDGF on rPMT induced stress fiber formation. Quiescent Swiss 3T3 cells were treated with 20 ng/ml PDGF for 4 h, and then 30 ng/ml PDGF was added for 10 min. Cells were then fixed and stained with TRITC-conjugated phallolidin. As shown in Fig. 6B, rPMT caused a marked increase in stress fiber formation, whereas 30 ng/ml PDGF caused disruption of the actin stress fibers and retraction of cell bodies (Fig. 6C). Interestingly, addition of 30 ng/ml PDGF to rPMT-treated cells reduced the number of actin stress fibers (Fig. 6D) and focal contacts (results not shown) induced by rPMT. The remaining stress fibers in rPMT- and PDGF-treated cells lost their unidirectional arrangement as tightly packed bundles. In contrast, PDGF at 5 ng/ml did not disrupt the actin reorganization induced by rPMT (results not shown).

As shown previously in Fig. 5, rPMT-induced tyrosine phosphorylation requires the integrity of the actin cytoskeleton. Given that 30 ng/ml PDGF also destabilized the actin stress fibers we decided to investigate the effects of PDGF on tyrosine phosphorylation induced by rPMT. Quiescent Swiss 3T3 cells were treated with 20 ng/ml rPMT for 4 h and then 5 ng/ml or 30 ng/ml PDGF were added to the cells for further 10 min. As shown in Fig. 7, rPMT induced tyrosine phosphorylation of either p125FAK or paxillin was markedly inhibited by addition of 30 ng/ml PDGF but not by 5 ng/ml PDGF.

Microinjection of C. botulinum C3 Exoenzyme Inhibits rPMT-induced Tyrosine Phosphorylation at Focal Contacts—The rho gene product p21 rho has been implicated in the neuropeptide-stimulated formation of focal adhesions, actin stress fibers, and tyrosine phosphorylation of p125FAK and paxillin (40, 53–55). To investigate the role of p21rho in the rPMT-stimulated tyrosine phosphorylation of focal adhesion-associated proteins we utilized the C. botulinum C3 exoenzyme which ADP-ribo-

sylates Asn41 of p21rho and thereby prevents its function (56). Recombinant C3 exoenzyme was microinjected at a concentration of 100 μg/ml into confluent and quiescent Swiss 3T3 cells, and the cultures were further treated with 20 ng/ml rPMT for

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**Fig. 3. Effect of GF109203X and thapsigargin on the sti-
lumination of tyrosine phosphorylation of p125FAK by rPMT.**

A, quiescent Swiss 3T3 cells were preincubated for 1 h with GF109203X (+) or an equivalent amount of solvent (−) and then stimulated with 20 ng/ml rPMT for a further 5 h. Cells were lysed and the lysates immunoprecipitated with mAb 2A7 directed against p125FAK and further analyzed by Western blotting with anti-Tyr(P) mAbs. B, quiescent Swiss 3T3 cells were pretreated with 30 nM thapsigargin (+) or an equivalent amount of solvent (−) for 30 min. Cells were then stimulated with 20 ng/ml rPMT, incubated for a further 6 h and subsequently lysed. The lysates were immunoprecipitated with mAb 2A7 against p125FAK and Western blotted with anti-Tyr(P) mAbs. Anti-Tyr(P) immunoreactivity of the p125FAK band was quantified by scanning densitometry. A and B show the values of the mean of three independent experiments and are expressed as the percentage of the maximum stimulation by 20 ng/ml rPMT.
Cells were then fixed, permeabilized and stained for tyrosine phosphorylated proteins which are predominantly localized at the focal contacts in rPMT treated cells (Fig. 8). The tyrosine phosphorylation of focal adhesion proteins in response to rPMT was profoundly inhibited in cells microinjected with C3 exoenzyme (indicated by the arrows in Fig. 8, right). In parallel experiments, Swiss 3T3 cells were microinjected only with immunoglobulin and then stimulated with 20 ng/ml rPMT. These cells display the typical pattern of tyrosine phosphorylated proteins demonstrating that microinjection itself did not interfere with rPMT-induced tyrosine phosphorylation of focal adhesion proteins (Fig. 8, left).

**DISCUSSION**

The results presented here show for the first time that rPMT, an intracellularly acting bacterial toxin, induces tyrosine phosphorylation of multiple substrates in Swiss 3T3 cells. In this study, we identified two substrates, p125\(^{FAK}\) and paxillin, which were tyrosine-phosphorylated in response to rPMT. p125\(^{FAK}\) is a cytosolic tyrosine protein kinase localized in focal adhesions that lacks SH2 and SH3 domains but associates with other proteins including v-Src and paxillin (23, 24, 46). Paxillin, a M, 70,000 protein, is a major phosphotyrosyl protein in chicken embryo and like p125\(^{FAK}\), is localized to focal adhe-
nprecipitated with 2A7 mAb against p125 FAK (cells, for an additional 10 min. Cells were lysed and the lysates immu-
dications (41, 42). Recent molecular cloning revealed that paxillin is a multidomain protein that may function as an adaptor capable of associating with p125 FAK, Crk, and Src (25, 26, 46). A coordinate increase in tyrosine phosphorylation of p125 FAK and paxillin is induced by a variety of molecules that regulate cell growth and differentiation (27–38). Our results suggest that p125 FAK and paxillin tyrosine phosphorylation could also play a role in the signaling pathways stimulated by rPMT.

rPMT increases the accumulation of the second messengers diacylglycerol and inositol 1,4,5-trisphosphate, which activate PKC and mobilize Ca2+ from intracellular stores, respectively (49), and direct PKC activation is known to stimulate tyrosine phosphorylation of p125 FAK and paxillin (27, 29). However, neither a selective inhibitor of PKC nor depletions of intracellular Ca2+ stores blocked the rPMT-stimulated tyrosine phosphorylation of p125 FAK. Thus, rPMT induces tyrosine phosphorylation of p125 FAK by a pathway largely independent of PKC activation and Ca2+ mobilization.

The effects of many bacterial toxins require them to bind and enter cells via endocytic pathways (1, 57, 58). Activation of some bacterial toxins is believed to occur during their transit through the endosomal/lysosomal compartments. In these acidic compartments, the toxin may be activated by proteolysis or by conformational changes. Interestingly, rPMT becomes susceptible to proteolysis at lysosomal pH values (59). Membrane-permeant weak bases that increase the pH of the acidic intracellular compartments block the process of activation. The characteristic lag period in the action of many toxins is a manifestation of the complex events of entry and activation. Several lines of evidence indicate that the stimulation of protein tyrosine phosphorylation by rPMT also requires cell entry and activation of the toxin. For example, rPMT induced tyrosine phosphorylation of multiple substrates including p125 FAK and paxillin after a pronounced (1 h), cycloheximide-insensitive, lag period and its effect was selectively blocked by the lysosomotropic agent methylamine or by reducing the temperature of incubation to 22 °C, which prevents vesicular trafficking (48). Other responses induced by rPMT, including polyphosphoinositide hydrolysis, Ca2+ mobilization, PKC activation, and commitment to DNA synthesis exhibit similar characteristics (5, 16, 18). Recently, colloidal gold-labeled rPMT has been shown to be rapidly internalized into endocytic vesicles in toxin-sensitive cell lines (60). Together, these findings suggest that rPMT enters the cell via endosomal/lysosomal compartments and then initiates events leading to the activation of signal transduction pathways, including p125 FAK and paxillin tyrosine phosphorylation.

Tyrosine phosphorylation of p125 FAK and paxillin stimulated by bombesin, LPA, sphingosine, and PDGF is closely related to changes in the organization of the actin microfilament network induced by these ligands in Swiss 3T3 cells (29, 31, 38). The cytoskeletal changes induced by LPA and bombesin require functional p21rho protein (40). In addition, bombesin and LPA stimulate tyrosine phosphorylation by a pathway critically dependent on the integrity of the actin cytoskeleton (28, 29, 31). These findings raised the possibility that tyrosine phosphorylation of p125 FAK and paxillin, actin stress fiber formation, focal adhesion assembly and p21rho function may lie in a novel signal transduction pathway. Having established that rPMT induces p125 FAK and paxillin tyrosine phosphorylation, it was, therefore, of interest to determine whether this toxin can also induce changes in the organization of the actin cytoskeleton. In addition, the polymerization of the actin cytoskeleton has been postulated to play an important role in the endocytosis of many bacteria by animal cells (61).

Here we report that rPMT elicits dramatic cytoskeletal responses in quiescent Swiss 3T3 cells. Specifically, rPMT induces striking formation of actin stress fibers and focal adhesion plaques in these cells. This is the first time that rPMT has been shown to induce accumulation of actin stress fibers and to promote focal adhesion assembly in any cell type. rPMT shows amino acid sequence homology with the NH2-terminal region of CNF1 and CNF2 produced by pathogenic E. coli strains (14, 15). Interestingly, the E. coli toxins have been shown to induce reorganization of actin cytoskeleton which has been postulated to block cytokinesis leading to multinucleated cells. In contrast, rPMT induces actin reorganization that does not interfere with cell division since rPMT promotes striking proliferation in fibroblast cell lines (4, 5). It would be interesting, therefore, to compare the cytoskeletal responses induced by these toxins in the same cell type.

The kinetics of the cytoskeletal responses induced by rPMT closely parallel the time course of rPMT stimulated tyrosine phosphorylation. Pretreatment of quiescent Swiss 3T3 with cytochalasin D completely disrupted the actin cytoskeleton and blocked the tyrosine phosphorylation of p125 FAK stimulated by rPMT. Thus, the integrity of the actin cytoskeleton is essential for rPMT induced tyrosine phosphorylation.

Recent results from our laboratory revealed that PDGF at high concentrations caused disruption of the actin cytoskeleton (31, 38). As tyrosine phosphorylation and actin stress fiber formation induced by rPMT appear to be closely linked in Swiss
3T3 cells, we examined a possible cross-talk between PDGF and rPMT on actin stress fiber organization and p125*FAK tyrosine phosphorylation. PDGF at a high concentration (30 ng/ml) profoundly inhibited rPMT induced tyrosine phosphorylation of p125*FAK, establishing a novel cross-talk between PDGF and rPMT on tyrosine phosphorylation. This can be explained by the ability of PDGF to interfere with rPMT induced actin stress fiber fiber organization and focal adhesion assembly in rPMT-treated cells.

In view of these results it was plausible, that p21* was also be involved in rPMT induced cytoskeletal changes and tyrosine phosphorylation. Microinjection of C3 also be involved in rPMT induced cytoskeletal changes and focal adhesion assembly in rPMT-treated cells.

The integrity of the actin stress fibers appears to be driven by an increase in the formation of actin stress fiber and focal adhesion proteins in response to rPMT. Thus, p21* is upstream of tyrosine phosphorylation in response to rPMT.

Most normal cells require contact with an adhesive substratum to proliferate and oncogenic transformation removes this requirement for anchorage-dependent growth (4). A salient feature of the results presented here is that the striking increase in p125*FAK and paxillin tyrosine phosphorylation, actin stress fiber organization and focal adhesion assembly in rPMT treated cells.

Tyrosine phosphorylation. This can be explained by integrin-mediated signals. Interestingly, we have previously reported that rPMT is a potent inducer of anchorage-independent colony formation in certain target cells (4). A salient feature of the results presented here is that the striking increase in p125*FAK and paxillin tyrosine phosphorylation, actin stress fiber formation and focal adhesion assembly induced by rPMT remain undiminished even after 24 h of incubation. It is tempting to speculate that rPMT circularly in vivo and suggests that we have not yet been shown to induce protein tyrosine phosphorylation in animal cells.

In conclusion, our results demonstrate, for the first time, that rPMT stimulates tyrosine phosphorylation of multiple bands including p125*FAK and paxillin. Furthermore, rPMT induces striking increase in the formation of actin stress fiber and focal adhesion assembly in Swiss 3T3 cells. The integrity of the polymerized actin network and functional p21* are essential for rPMT-induced tyrosine phosphorylation. To our knowledge, this is the first time, that an intracellularly acting bacterial toxin has been shown to induce protein tyrosine phosphorylation in animal cells.

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