Cytotoxic Necrotizing Factor 1 from *Escherichia coli* and Dermonecrotic Toxin from *Bordetella bronchiseptica* Induce p21<sup>rho</sup>-dependent Tyrosine Phosphorylation of Focal Adhesion Kinase and Paxillin in Swiss 3T3 Cells*

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Hadriano M. Lacerda‡‡, Gill D. Pullinger‡, Alistair J. Lax‡, and Enrique Rozengurt‡**

*From the ‡Imperial Cancer Research Fund, P. O. Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom, the §UMDS Guy's and St. Thomas's Medical and Dental School, Floor 28 Guy's Tower, Guy's Hospital, London SE1 9RT, United Kingdom, and the ¶Department of Chemical Endocrinology, St. Bartholomew's Hospital Medical College, 51-53 Bartholomew Close, London EC1A 7BE, United Kingdom*

Treatment of Swiss 3T3 cells with cytotoxic necrotizing factor 1 (CNF1) from *Escherichia coli* and dermonecrotic toxin (DNT) from *Bordetella bronchiseptica*, which directly target and activate p21<sup>rho</sup>, stimulated tyrosine phosphorylation of focal adhesion kinase (p125<sub>fak</sub>) and paxillin. Tyrosine phosphorylation induced by CNF1 and DNT occurred after a pronounced lag period (2 h), and was blocked by either lysosomotropic agents or incubation at 22 °C. CNF1 and DNT stimulated tyrosine phosphorylation of p125<sub>fak</sub> and paxillin, actin stress fiber formation, and focal adhesion assembly with similar kinetics. Cytchalasin D and high concentrations of platelet-derived growth factor disrupted the actin cytoskeleton and completely inhibited CNF1 and DNT induced tyrosine phosphorylation. Microinjection of *Clostridium botulinum* C3 exoenzyme which ADP-ribosylates and inactivates p21<sup>rho</sup> function, prevented tyrosine phosphorylation of focal adhesion proteins in response to either CNF1 or DNT. In addition, our results demonstrated that CNF1 and DNT do not induce protein kinase C activation, inositol phosphate formation, and Ca<sup>2+</sup> mobilization. Moreover, CNF1 and DNT stimulated DNA synthesis without activation of p42<sup>mapk</sup> and p44<sup>mapk</sup> providing additional evidence for a novel p21<sup>rho</sup>-dependent signaling pathway that leads to entry into the S phase of the cell cycle in Swiss 3T3.

An increase in the tyrosine phosphorylation of the non-receptor protein tyrosine kinase p125<sub>fak</sub> (1, 2) and the cytoskeletal associated protein paxillin (3, 4) has recently been identified as an early event in the action of diverse signaling molecules that mediate cell growth and differentiation (5), including mitogenic neuropeptides (6–8), growth factors such as PDGF<sup>1</sup> (9, 10), bioactive lipids (11–15), extracellular matrix proteins (16–20), transforming variants of pp60<sup>src</sup> (17, 21), and the potent mitogenic toxin PMT (22). p125<sub>fak</sub> lacks SH2 and SH3 domains but associates with other proteins including Src, paxillin, and p130<sup>crk</sup> (1, 2, 23). Paxillin is a multidomain protein that may function as an adaptor capable of complex formation with p125<sub>fak</sub>, Crk, Src, and vinculin (3, 4, 23). Gene disruption experiments indicate a critical role of p125<sub>fak</sub> in embryonic development and cell locomotion (24). The increases in p125<sub>fak</sub> and paxillin tyrosine phosphorylation are accompanied by profound alterations in the organization of the actin cytoskeleton and in the assembly of focal adhesions (9, 13, 15, 26), the distinct areas of the plasma membrane where p125<sub>fak</sub> and paxillin are localized (1, 2, 27). The small G protein p21<sup>rho</sup>, a member of the Ras superfamily of small GTP-binding proteins, has been implicated in the mitogen-stimulated formation of focal adhesions and actin stress fibers as well as in the tyrosine phosphorylation of p125<sub>fak</sub> and paxillin (22, 25, 28–30). These findings suggest the existence of a distinct signal transduction pathway in which p21<sup>rho</sup> is upstream of cytoskeletal reorganization and tyrosine phosphorylation of focal adhesion proteins (5).

The mechanism of action of bacterial toxins has provided novel insights into the control of cellular regulatory processes, including signal transduction and cell proliferation. For example, the *Clostridium botulinum* C3 exoenzyme and the enterotoxins A and B from *Clostridium difficile* which selectively inactivate members of the Rho subfamily, have provided useful tools to evaluate the role of these small G proteins in signal transduction and cytoskeletal organization (31–33). In contrast to these clostridial toxins, CNF toxins produced by some pathogenic strains of *Escherichia coli* (34) and DNT from *Bordetella bronchiseptica* (35) directly target and activate p21<sup>rho</sup> (36, 37). CNF and DNT induce actin reorganization and multinucleation in several cell types (37–40) but their effects on signal transduction pathways, specially on p125<sub>fak</sub> and paxillin tyrosine phosphorylation have not been examined.

Here we report that CNF1 and DNT stimulate tyrosine phosphorylation of p125<sub>fak</sub> and paxillin and induce a concomitant increase in the formation of actin stress fibers and in the assembly of focal adhesion plaques in Swiss 3T3 cells. Microinjection of C3 exoenzyme prevented both the cytoskeletal responses and the increase in tyrosine phosphorylation of focal adhesion proteins. In contrast to most other stimuli that promote tyrosine phosphorylation of p125<sub>fak</sub> and paxillin (6–15, 25, 26), the distinct areas of the plasma membrane where p125<sub>fak</sub> and paxillin are localized (1, 2, 27). The small G protein p21<sup>rho</sup>, a member of the Ras superfamily of small GTP-binding proteins, has been implicated in the mitogen-stimulated formation of focal adhesions and actin stress fibers as well as in the tyrosine phosphorylation of p125<sub>fak</sub> and paxillin (22, 25, 28–30). These findings suggest the existence of a distinct signal transduction pathway in which p21<sup>rho</sup> is upstream of cytoskeletal reorganization and tyrosine phosphorylation of focal adhesion proteins (5).

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22), CNF1 and DNT do not activate phospholipase C-mediated events including inositol phosphate production, Ca$^{2+}$ mobilization, and PKC activation. In addition, CNF1 and DNT both stimulated reinitiation of DNA synthesis but neither induced activation of p42$^{mapk}$ (ERK2). Thus, our results demonstrating that CNF1 and DNT induce a specific subset of molecular and cytoskeletal responses in Swiss 3T3 cells provide novel evidence for the existence of a signal transduction pathway that links p21$^{ras}$ to tyrosine phosphorylation of focal adhesion proteins.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Stock cultures of Swiss 3T3 fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO$_2$ and 90% air at 37°C. For experimental purposes, cells were plated either in 30-mm Nunc Petri dishes or 90-mm dishes at 6 × 10$^5$ cells/dish, in DMEM containing 10% fetal bovine serum and used after 6–8 days when the cells were confluent and quiescent (41).

**DNA Synthesis Measurements**—Quiescent cultures of Swiss 3T3 cells were washed twice with DMEM and incubated with DMEM/Waymouth's medium 1:1 (v/v) containing 10$^{-5}$M thymidine (44). The cultures were then gently removed from the dishes by scraping with a sterile glass or plastic pipette. The cell suspension was transferred to a 1-cm$^2$ quartz cuvette and stirred at 37°C for 4 min prior to any additions. Fluorescence was measured in a Perkin-Elmer LS-5 fluorometer with an excitation wavelength of 510 nm. [Ca$^{2+}$]$_i$ was calculated from the maximum and minimum fluorescence of the fura-2, as described (46, 49).

**Immunostaining of Cells**—Quiescent Swiss 3T3 cells were washed twice with DMEM and incubated for the indicated times in DMEM/Waymouth's medium 1:1 (v/v) at 37°C with CNF1, DNT, or PFT. 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 FITC-conjugated phallolidin (0.25 μg/ml) for 30 min at room temperature and visualized using a Zeiss Axiophot microscope. In experiments in which quiescent Swiss 3T3 cells were labeled with both FITC-conjugated phallolidin and anti-vinculin antibody, the cells were fixed and permeabilized as described above and then stained with a mixture of FITC-conjugated phallolidin (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 then incubated with Cy3-labeled rabbit anti-mouse IgG as a secondary antibody at a dilution of 1:100 for another 30 min at room temperature.

**Microinjection of C3 Exoenzyme**—For microinjection experiments Swiss 3T3 cells were plated in 33-mm Nunc Petri dishes at 10$^5$ cells/dish in DMEM containing 10% fetal bovine serum and used after 6–8 days when the cells were confluent and quiescent. Approximately 50 cells were microinjected with 100 pg/ml recombinant C3 exoenzyme. The efficiency of injection was determined by co-injecting guinea pig immunoglobulin at 0.5 mg/ml followed by staining with FITC-linked goat anti-guinea pig IgG. A single batch of C3 transferase was used in all experiments described.

**Confocal Microscopy**—Confocal imaging was performed using a Bio-Rad MRC 1000 laser scanning confocal microscope fitted with an Nikon Optistep 60X N.A./1.4 planachromat oil immersion lens (Nikon) used for all imaging. FITC-conjugated phallolidin and Cy3 anti-mouse IgG fluorochromes were excited at 488 and 568 nm, respectively, using a krypton/argon 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 486 and 569 nm, whereas the K2 filter is a 560-nm dichroic 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 FITC-conjugated phallolidin channel was sufficiently bright relative to the fluorescein 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 problems was carried out using COMOS and Somo programs (Bio-Rad) run on a Compaq Deskpro 66M 486 computer (66 MHz). Data are presented as projections of sequential optical sections. For Z-series, optical sections were recorded at 0.5-μm intervals. Final images were photographed directly from the VDU screen.

**Shift Assay for MAP Kinase Activation**—Activation of p42$^{mapk}$ and p44$^{mapk}$ was determined by the appearance of slower migrating forms in gel electrophoresis due to phosphorylation of specific threonine and tyrosine residues (50). Lysates from quiescent cells in 33-mm dishes were prepared as above subjected to SDS-PAGE and transferred to Immobilon membranes. Membranes were blocked using 5% nonfat dried milk in PBS, pH 7.2, and incubated for 3–5 h at 22°C with antibodies to p42$^{mapk}$ and p44$^{mapk}$ which were labeled with horseradish peroxidase and detected using ECL detection reagents. Western Blotting—Treatment of quiescent cultures of cells with factors, cells lysis, and immunoprecipitations were performed as described above. After separation by SDS-PAGE, proteins were transferred to Immobilon membranes (43). Membranes were blocked using 5% nonfat dried milk in PBS, pH 7.2, and incubated for 3–5 h at 22°C with antibodies to p42$^{mapk}$ and p44$^{mapk}$ which were labeled with horseradish peroxidase and detected using ECL detection reagents. Western Blotting—Treatment of quiescent cultures of cells with factors, cells lysis, and immunoprecipitations were performed as described above. After separation by SDS-PAGE, proteins were transferred to Immobilon membranes (43). Membranes were blocked using 5% nonfat dried milk in PBS, pH 7.2, and incubated for 3–5 h at 22°C with antibodies to p42$^{mapk}$ and p44$^{mapk}$ which were labeled with horseradish peroxidase and detected using ECL detection reagents.
plasmid pDT1 which consists of a 5-kilobase fragment encoding the DNT gene (B. bronchiseptica 858) inserted into the vector pBluescript. Control strain pB20 is E. coli XL1-Blu with plasmid pBluescript. Strain pTOX2 is E. coli XL1-Blu with plasmid pTOX2 which consists of the vector pBluescript with an approximately 4-kilobase pair insert encoding PMT. Control strain pB38 is E. coli XL1-Blu with plasmid pBluescript here nominated PC12.

Preparation of Samples of CNF1, DNT, and PMT—The E. coli strains were grown overnight from a fresh colony. They were lysed in the lysis buffer (50 mM l-lysine, 5 mM EDTA, 0.1% tolune, 0.05 mM Tris–Cl, pH 7.2, 0.1% NaCl) treated with RNase and DNase at 10 μg/ml each to remove nucleic acids and filtered through a 0.2-μm nitrocellulose filter.

Materials—Bombein, cytchalasin D, FITC-conjugated phalloidin, monoclonal anti-velin mAb, Cy3-linked anti-mouse IgG, guinea pig IgG, and FITC-conjugated goat anti-guinea pig IgG were obtained from Sigma. Recombinant PDGF (BB homodimer), 125I-sheep anti-mouse IgG, and FITC-conjugated goat anti-guinea pig IgG were obtained from Amersham Corp., United Kingdom. Agarose-linked anti-Tyr(P) mAb was purchased from Oncogene Science Inc., New York. 4G10 anti-Tyr(P) mAb was from UBI, Lake Placid, NY. mAb 2A7 directed against p125 fak was from TCS Biologicals Ltd., Buckingham, United Kingdom. p125 fak immunoblotting was performed with mAb from Transduction Laboratories, Lexington, KY. The anti-Tyr(P) mAb PY72 was obtained from the hybridoma development unit, Imperial Cancer Research Fund. Clone pISS392 was kindly supplied by Dr. A. Caprioli, Istituto Sperimenale di Sanita, Rome, Italy. All other reagents were of the highest grade commercially available. The C3 C. botulinum exoenzyme was a gift from Dr. N. Morii and Professor S. Narumiya, Department of Pharmacology, Kyoto University Faculty of Medicine, Sakyoku 606, Japan.

RESULTS

CNF1 Stimulates Tyrosine Phosphorylation of p125 fak and Paxillin in Swiss 3T3 Cells—To examine the effects of CNF1 on tyrosine phosphorylation, quiescent cultures of Swiss 3T3 cells were treated with the control bacterial lysate (pGEM3) or with lysate containing CNF1 for 6 h. Cell extracts were immunoprecipitated with anti-Tyr(P) mAb followed by Western blotting with an anti-p125 fak mAb. As shown in Fig. 1A, CNF1 markedly stimulated the tyrosine phosphorylation of a group of bands migrating with an apparent Mr of 110,000–130,000 and 70,000–80,000. The pattern of tyrosine-phosphorylated proteins induced by CNF1 is similar to that stimulated by bombesin, bioactive lipids, and PMT in Swiss 3T3 cells (8, 13, 15, 22). The cytosolic tyrosine kinase p125 fak and the adaptor protein paxillin have been identified as prominent tyrosine-phosphorylated proteins in Swiss 3T3 cells treated with these agents. To determine whether these cellular proteins were also substrates for CNF1-induced tyrosine phosphorylation, extracts from Swiss 3T3 cells, incubated with the control bacterial lysate (pGEM3) or CNF1 for 6 h, were immunoprecipitated with mAbs that recognize either p125 fak or paxillin, and the immunoprecipitates were analyzed by Western blotting with anti-Tyr(P) mAb. As shown in Fig. 1A, CNF1 markedly stimulated tyrosine phosphorylation of p125 fak and paxillin in Swiss 3T3 cells. The control bacterial lysate (pGEM3) did not stimulate tyrosine phosphorylation of these bands or of any other protein (data not shown).

CNF1 Enters the Cell to Transiently Stimulate Tyrosine Phosphorylation of p125 fak and Paxillin—Neuropeptides and lysophosphatidic acid induce tyrosine phosphorylation of p125 fak and paxillin within minutes of treatment (6, 8, 19), whereas there is a lag period of 1 h between the addition of PMT and a detectable increase in tyrosine phosphorylation of these proteins (22). As shown in Fig. 1B, CNF1-stimulated tyrosine phosphorylation of p125 fak and paxillin occurred after a lag period of 2 h, reached a maximum after 8 h of treatment and declined to 56±9.5 (mean ± S.E.) of the maximum after 24 h. This is in contrast to the tyrosine phosphorylation of p125 fak and paxillin stimulated by PMT which remains maximal after 24 h of treatment (data not shown). Immunoblotting with p125 fak mAb of anti-p125 fak immunoprecipitates prepared in parallel with those used for the assays of tyrosine phosphorylation verified that similar amounts of p125 fak were recovered after different times of CNF1 treatment (Fig. 1B). Next we examined whether the lag period in CNF1 action reflects a requirement for internalization and activation of the toxin during transit through endosomal/lysosomal compartiments. The lysosomotropic agent methylamine, a membrane-permeant weak base known to inhibit lysosomal processing, completely blocked tyrosine phosphorylation of the Mr 110,000–130,000 and Mr 70,000–80,000 bands in response to CNF1 (Fig. 1C). Methylamine also prevented the increase in tyrosine phosphorylation of p125 fak in response to CNF1 (Fig. 1C). The inhibitory effect of methylamine was selective because it did not prevent the increase in tyrosine phosphorylation induced by bombesin in parallel cultures (data not shown). It is also known that the entry of many bacterial toxins into the cell endoplasmin is temperature-dependent. Treatment of Swiss 3T3 cells with CNF1 for 6 h at 22 °C failed to induce tyrosine phosphorylation (Fig. 1C). In contrast, bombesin stimulated tyrosine phosphorylation of Mr 110,000–130,000 and Mr 70,000–80,000 bands in parallel cultures incubated at 22 °C (data not shown). Therefore, CNF1 appears to enter cells via an endosomal/lysosomal pathway where it is processed and re-

FIG. 1. CNF1 induces tyrosine phosphorylation of multiple bands including p125 fak and paxillin in Swiss 3T3 cells. A, quiescent cultures of Swiss 3T3 cells were treated in DMEM/Maymouth's medium (1:1, v/v) with (−)5 μg/ml control bacterial lysate (pGEM3) or with (+)5 μg/ml bacterial lysate containing CNF1 for 6 h. Cells were lysed and the lysates were immunoprecipitated with agaerose-linked anti-Tyr(P) mAb, anti-p125 fak mAb 2A7, or anti-paxillin mAb 165. The immunoprecipitates were fractionated by SDS-PAGE and further analyzed by immunoblotting with a mixture of anti-Tyr(P) mAbs. B, quiescent cultures of Swiss 3T3 cells were treated in DMEM/Maymouth's medium (1:1, v/v) with 5 μg/ml bacterial lysate containing CNF1 for various times (0–24 h) as indicated. Cells were lysed and the lysates were immunoprecipitated using anti-p125 fak mAb or anti-paxillin mAb and analyzed by immunoblotting with a mixture of anti-Tyr(P) mAbs (upper and lower autoradiograms). Immunoblotting with p125 fak mAb of anti-p125 fak immunoprecipitates verified that similar amounts of p125 fak were recovered after different times of CNF1 treatment (middle autoradiogram). The positions of p125 fak and paxillin are indicated by arrowheads. The plot shows the values of the mean of three independent experiments which are expressed as the percentage of the maximum stimulation of tyrosine phosphorylation of p125 fak band at the indicated times quantified by scanning densitometry. C, quiescent cultures of Swiss 3T3 cells were incubated for 6 h in DMEM/Maymouth's (1:1, v/v) medium containing 5 μg/ml bacterial lysate containing CNF1 with or without 10 mM CH3NH2 or incubated at 22 °C. Cells were lysed and the lysates were immunoprecipitated with anti-Tyr(P) mAb or anti-p125 fak mAb 2A7. The immunoprecipitates were fractionated by SDS-PAGE and further analyzed by anti-Tyr(P) mAb immunoblotting. The results shown in this and subsequent figures are representative autoradiograms of at least three independent experiments.
leased into the cytosol in an active form to stimulate tyrosine phosphorylation of p125\textsubscript{fak} and paxillin.

**CNF1 Transiently Induces Actin Stress Fiber Formation and Focal Contact Assembly**—Given the localization of p125\textsubscript{fak} and paxillin to focal contacts which form at the end of actin stress fibers we examined the kinetics of actin cytoskeleton reorganization and focal adhesion assembly induced by CNF1. As shown in Fig. 2, CNF1 treatment of Swiss 3T3 cells induced the formation of new actin stress fibers after a 2-h lag period. Actin reorganization reached a maximum after 8 h of exposure to
Interestingly, after 24 h of CNF1 treatment, the number of actin stress fibers decreased and actin appeared to accumulate in a cortical fashion. Vinculin staining showed the recruitment of this protein to focal adhesions formed at the ends of actin stress fibers. The number of focal adhesions also reached a maximum after 8 h and decreased after 24 h treatment (Fig.)
FIG. 4. CNF1 does not induce PKC activation, inositol phosphate production, Ca\(^{2+}\) mobilization, or p42\(^{\text{mapk}}\)/p44\(^{\text{mapk}}\) activation. A, quiescent cells, labeled with 50 \(\mu\)Ci/ml \([^{32}\text{P}]\)Pi, for 18 h, were treated with 5 \(\mu\)g/ml control bacterial lysate (pGEM3), 5 \(\mu\)g/ml bacterial lysate containing CNF1, 2 \(\mu\)g/ml control bacterial lysate (PC12), or 2 \(\mu\)g/ml bacterial lysate containing PMT and incubated for further 6 h. Cells were subsequently lysed and the lysates were immunoprecipitated with anti-80K/MARCKS antibody and further analyzed by SDS-PAGE. B, cells were prelabeled with 10 \(\mu\)Ci/ml \([^{2}\text{H}]\)inositol for 16 h treated with the same conditions as described above adding directly to the dishes and the cultures were incubated at 37 °C for 6 h. LiCl (20 mM) was then added, and after a further 30 min the cellular inositol phosphate content was determined. C, [Ca\(^{2+}\)], was measured as described under "Experimental Procedures." Quiescent cells were pretreated with 5 \(\mu\)g/ml control bacterial lysate (pGEM3), 5 \(\mu\)g/ml bacterial lysate containing CNF1, 2 \(\mu\)g/ml control bacterial lysate (PC12), or 2 \(\mu\)g/ml bacterial lysate containing PMT for 4 h, and the increase in [Ca\(^{2+}\)], induced by bombesin (10 nm) was then determined. D, quiescent cells were treated for 4 h as described above and lysed. The cell extracts were subjected to SDS-PAGE, followed by Western blotting with anti-p42\(^{\text{mapk}}\)/p44\(^{\text{mapk}}\) antibody.

The control bacterial lysate did not induce actin stress fiber formation or focal adhesion assembly (2–24 h, data not shown).

CNF1-stimulated Tyrosine Phosphorylation Requires Integrity of the Actin Cytoskeleton and Functional p21\(^{\text{rho}}\)—The time-dependent effects of CNF1 on actin stress fiber formation and focal adhesion assembly depicted in Fig. 2 prompted us to examine whether the integrity of the actin cytoskeleton was necessary for CNF1-induced tyrosine phosphorylation. Quiescent cultures of Swiss 3T3 cells were pretreated with 1.2 \(\mu\)g cytochalasin D for 1 h and then stimulated with CNF1 for 6 h. As shown in Fig. 3, cytochalasin D prevented the assembly of focal adhesions (upper panel C) and completely blocked tyrosine phosphorylation of p125\(^{40k}\) (middle panel) and paxillin (data not shown) in response to CNF1.

Recent data from our laboratory have shown that PDGF, at a high concentration (30 ng/ml), completely abolishes bombesin-, lysophosphatidic acid-, and PMT-induced actin stress fiber formation and focal adhesions assembly. Similarly, the marked increase in focal adhesion assembly and actin stress fibers induced by CNF1 was prevented by the addition of PDGF at 30 ng/ml (Fig. 3, upper panel D, and results not shown). Since CNF1-stimulated actin stress fiber formation and phosphorylation by a mechanism dependent on the integrity of the actin cytoskeleton, we examined whether CNF1-stimulated tyrosine phosphorylation of p125\(^{40k}\) could be affected by high concentrations of PDGF. As shown in Fig. 3 (middle panel), PDGF (30 ng/ml) markedly reduced CNF1-stimulated tyrosine phosphorylation of p125\(^{40k}\).

The rho gene product, p21\(^{\text{rho}}\), has been implicated in the assembly of focal adhesions and in tyrosine phosphorylation of p125\(^{40k}\) and paxillin (9, 25, 29, 30). It has been suggested that CNF1 directly targets and activates p21\(^{\text{rho}}\) (36, 52). To investigate the role of p21\(^{\text{rho}}\) in the CNF1-stimulated tyrosine phosphorylation of focal adhesion-associated proteins we utilized the C. botulinum C3 exoenzyme which ADP-ribosylates Asn 41 of p21\(^{\text{rho}}\) and thereby prevents its function (53–55). Recombinant C3 exoenzyme and pure guinea pig IgG were coinjected into confluent and quiescent Swiss 3T3 cells, and the cultures were further treated with CNF1 for 4 h. Cells were then fixed, permeabilized, and stained for tyrosine-phosphorylated proteins which are predominantly localized at the focal contacts in CNF1-treated cells (Fig. 3, lower panel B). The tyrosine phosphorylation of focal adhesion proteins in response to CNF1 was profoundly inhibited in cells coinjected with C3 exoenzyme (Fig. 3, lower panel D). Microinjection itself did not interfere with CNF1-induced tyrosine phosphorylation of focal adhesion proteins (Fig. 3, lower panels A and B).

CNF1, Unlike PMT, Does Not Induce PKC Activation, Inositol Phosphate Formation, Ca\(^{2+}\) Mobilization, or MAPK Activation—PKC activation is a potential signal transduction pathway leading to increased tyrosine phosphorylation of p125\(^{40k}\) and paxillin (8). As the effect of CNF1 on PKC activation was unknown, we...
The immunoprecipitates were fractionated by SDS-PAGE and further and the lysates were immunoprecipitated with anti-Tyr(P) 4G10 mAb. Material lysate containing DNT for various times (0–24 h). Cells were lysed using DNT for the times indicated, and then washed with PBS, fixed in 3.7% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Immunostaining with monoclonal anti-vinculin antibody demonstrates focal adhesions. A Cy3-conjugated rabbit anti-mouse IgG antibody was used to perform confocal imaging. Control bacterial lysate at 10^3, quiescent cultures of Swiss 3T3 cells were washed with DMEM and incubated in DMEM/Waymouth’s medium (1:1, v/v) containing 10 μg/ml bacterial lystate containing DNT for various times (0–24 h). Cells were lysed and the lysates were immunoprecipitated with anti-Tyr(P) 4G10 mAb. The immunoprecipitates were fractionated by SDS-PAGE and further analyzed by immunoblotting with p125^fak (2A7 mAb) or paxillin (165 mAb). The results shown are representative autoradiographs of at least three independent experiments. Bottom, quiescent cultures of Swiss 3T3 cells were washed with DMEM and incubated in DMEM/Waymouth’s medium (1:1, v/v) containing 10 μg/ml bacterial lystate containing DNT for the times indicated, and then washed with PBS, fixed in 3.7% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Immunostaining with monoclonal anti-vinculin antibody demonstrates focal adhesions. A Cy3-conjugated rabbit anti-mouse IgG antibody was used to perform confocal imaging. Control bacterial lystate at 10 μg/ml neither induced tyrosine phosphorylation of p125^fak and paxillin nor focal adhesion plaques at any of the time points examined (0–24 h, results not shown).

To substantiate that CNF1 does not activate a phospholipase C-dependent pathway that leads to the production of the intracellular second messengers diacylglycerol and Ins(1,4,5)P_3, we determined the effect of CNF1 on the production of total inositol phosphates. Quiescent cultures of Swiss 3T3 cells labeled with [β-32P]Pi were treated with CNF1 for 6 h. Fig. 4B shows that CNF1 did not stimulate a detectable increase in total inositol phosphate production. In contrast, PMT potently induced the formation of inositol phosphates in parallel cultures of Swiss 3T3 cells.

Prolonged incubation of cells with PMT depletes Ca^{2+} from intracellular stores and thereby prevents Ca^{2+} release by subsequent addition of bombesin (57). To examine whether CNF1 reduces the Ins(1,4,5)P_3-sensitive Ca^{2+} pool in Swiss 3T3 cells, quiescent cultures of these cells were treated with CNF1 or PMT for 4 h, loaded with fura-2-tetraacetoxymethyl ester, and stimulated with 10 nM bombesin. As shown in Fig. 4C, treatment with CNF1 did not reduce the Ca^{2+} mobilization induced by subsequent addition of bombesin. In contrast, PMT in parallel cultures caused a dramatic reduction of bombesin-mediated Ca^{2+} mobilization. We conclude that CNF1 stimulates tyrosine phosphorylation through a phospholipase C- and PKC-independent pathway.

Several mitogens stimulate rapid and transient activation of p45^{mapk} and p44^{mapk} through various signal transduction pathways including Ca^{2+} mobilization and PKC activation (58, 59). A significant property of MAPKs activation is the requirement for both Thr and Tyr phosphorylation within its protein kinase subdomain VIII, resulting in a mobility shift in SDS-PAGE gels. To determine if CNF1 induces p45^{mapk} and p44^{mapk} activation, lysates of Swiss 3T3 stimulated for various times with control bacterial lystate (pGEM3), CNF1, or PMT were analyzed by Western blotting with anti-p45^{mapk}/p44^{mapk} antibody. Only PMT induced p45^{mapk} and p44^{mapk} activation as judged by the appearance of slower migrating forms of p45^{mapk} and p44^{mapk} (Fig. 4D). We verified that CNF1 failed to stimulate p45^{mapk} and p44^{mapk} activation after various times of incubation (2–6 h).

The striking differences between the early events induced by CNF1 and PMT in Swiss 3T3 cells prompted us to examine the effect of CNF1 on DNA synthesis in these cells. Quiescent cultures of Swiss 3T3 cells were transferred to serum-free medium containing various concentrations of the control bacterial lystate (pGEM3) or CNF1. Cumulative [3H]thymidine incorporation was measured after 40 h. As shown in Fig. 5A, CNF1 induced DNA synthesis in a dose-dependent manner whereas the control bacterial lystate (pGEM3) did not induce any measurable increase in [3H]thymidine incorporation in parallel cell cultures. Insulin potentiated the mitogenic effect of CNF1. The maximum levels of DNA synthesis induced by the combination of CNF1 and insulin were similar to those stimulated by medium containing 10% (v/v) fetal bovine serum. Methylamine (10 mM) profoundly inhibited the stimulation of DNA synthesis induced by CNF1 and insulin. Fluorescence-activated cell sorter analysis confirmed that CNF1 stimulated DNA synthesis and induced multinucleation in Swiss 3T3 cells, as described in other cell types (data not shown).

CNF1 and DNT Stimulation of Tyrosine Phosphorylation

To substantiate that CNF1 does not activate a phospholipase C-dependent pathway that leads to the production of the intracellular second messengers diacylglycerol and Ins(1,4,5)P_3, we determined the effect of CNF1 on the production of total inositol phosphates. Quiescent cultures of Swiss 3T3 cells labeled with [β-32P]Pi were treated with CNF1 for 6 h. Fig. 4B shows that CNF1 did not stimulate a detectable increase in total inositol phosphate production. In contrast, PMT potently induced the formation of inositol phosphates in parallel cultures of Swiss 3T3 cells.

Prolonged incubation of cells with PMT depletes Ca^{2+} from intracellular stores and thereby prevents Ca^{2+} release by subsequent addition of bombesin (57). To examine whether CNF1 reduces the Ins(1,4,5)P_3-sensitive Ca^{2+} pool in Swiss 3T3 cells, quiescent cultures of these cells were treated with CNF1 or PMT for 4 h, loaded with fura-2-tetraacetoxymethyl ester, and stimulated with 10 nM bombesin. As shown in Fig. 4C, treatment with CNF1 did not reduce the Ca^{2+} mobilization induced by subsequent addition of bombesin. In contrast, PMT in parallel cultures caused a dramatic reduction of bombesin-mediated Ca^{2+} mobilization. We conclude that CNF1 stimulates tyrosine phosphorylation through a phospholipase C- and PKC-independent pathway.

Several mitogens stimulate rapid and transient activation of p45^{mapk} and p44^{mapk} through various signal transduction pathways including Ca^{2+} mobilization and PKC activation (58, 59). A significant property of MAPKs activation is the requirement for both Thr and Tyr phosphorylation within its protein kinase subdomain VIII, resulting in a mobility shift in SDS-PAGE gels. To determine if CNF1 induces p45^{mapk} and p44^{mapk} activation, lysates of Swiss 3T3 stimulated for various times with control bacterial lystate (pGEM3), CNF1, or PMT were analyzed by Western blotting with anti-p45^{mapk}/p44^{mapk} antibody. Only PMT induced p45^{mapk} and p44^{mapk} activation as judged by the appearance of slower migrating forms of p45^{mapk} and p44^{mapk} (Fig. 4D). We verified that CNF1 failed to stimulate p45^{mapk} and p44^{mapk} activation after various times of incubation (2–6 h).

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**CNF1 and DNT Stimulation of Tyrosine Phosphorylation**

**FIG. 6.** DNT induces tyrosine phosphorylation of multiple bands including p125^{fak} and paxillin, and focal adhesion assembly in Swiss 3T3 cells. **Top**, quiescent cultures of Swiss 3T3 cells were treated in DMEM/Waymouth’s medium (1:1, v/v) with 10 μg/ml bacterial lystate containing DNT for various times (0–24 h). Cells were lysed and the lysates were immunoprecipitated with anti-Tyr(P) 4G10 mAb. The immunoprecipitates were fractionated by SDS-PAGE and further analyzed by immunoblotting with p125^{fak} (2A7 mAb) or paxillin (165 mAb). The results shown are representative autoradiographs of at least three independent experiments. **Bottom**, quiescent cultures of Swiss 3T3 cells were washed with DMEM and incubated in DMEM/Waymouth’s medium (1:1, v/v) containing 10 μg/ml bacterial lystate containing DNT for the times indicated, and then washed with PBS, fixed in 3.7% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Immunostaining with monoclonal anti-vinculin antibody demonstrates focal adhesions. A Cy3-conjugated rabbit anti-mouse IgG antibody was used to perform confocal imaging. Control bacterial lystate at 10 μg/ml neither induced tyrosine phosphorylation of p125^{fak} and paxillin nor focal adhesion plaques at any of the time points examined (0–24 h, results not shown).

**DNT Stimulates p125^{fak} and Paxillin Tyrosine Phosphorylation in a p21^{rho}-dependent Fashion**—Recently, DNT from *B. bronchiseptica* has been shown to directly target and activate p21^{rho} (37). As shown in Fig. 5B, DNT also promotes DNA synthesis in Swiss 3T3 cells. Consequently, we examined whether DNT stimulates a pattern of early events comparable to that induced by CNF1. As shown in Fig. 6, treatment of Swiss 3T3 cells with a bacterial lystate containing DNT stimulated tyrosine phosphorylation of p125^{fak} and paxillin (upper...
panel) with similar kinetics to stress fiber formation (data not shown) and focal adhesion assembly (lower panel). The observed lag period suggests that internalization and processing of the toxin in the endosomal/lysosomal compartment is required. Indeed, addition of 10 mM methylamine to the culture medium or reduction of the temperature to 22 °C inhibited...
DNT-stimulated tyrosine phosphorylation (data not shown).

Next, we tested whether the integrity of the cytoskeleton was necessary for DNT-stimulated tyrosine phosphorylation. As shown in Fig. 7, cytochalasin D or PDGF blocked DNT-stimulated stress fiber formation (data not shown), focal adhesion assembly (upper panel), and tyrosine phosphorylation of p125<sub>fak</sub> (middle panel). In addition, microinjection of C3 exoenzyme profoundly inhibited tyrosine phosphorylation of focal adhesion proteins in response to DNT (lower panel).

CNF1 stimulates p125<sub>fak</sub> and paxillin tyrosine phosphorylation and stimulate DNA synthesis without promoting phospholipase C-mediated hydrolysis of PtdIns(4,5)<sub>2</sub> or PKC activation. As shown in Fig. 8, DNT failed to stimulate an increase in the phosphorylation of 80K/MARCKS or to promote a detectable increase in total inositol phosphate production. In addition, DNT, like CNF1, did not induce p42<sup>mapk</sup> activation. All these responses were induced by PMT in parallel cultures of Swiss 3T3 cells.

**DISCUSSION**

The results presented here show for the first time that CNF1 and DNT, both intracellularly acting bacterial toxins, induce tyrosine phosphorylation of multiple substrates in Swiss 3T3 cells. In this study, we identified two substrates, p125<sub>fak</sub> and paxillin, which were tyrosine phosphorylated in response to CNF1 and DNT. A coordinate increase in tyrosine phosphorylation of p125<sub>fak</sub> and paxillin is induced by a variety of molecules that regulate cell growth and differentiation (6–9, 11–20, 22). Our results suggest that p125<sub>fak</sub> and paxillin tyrosine phosphorylation could also play a role in the signaling pathways stimulated by CNF1 and DNT.

Recent findings have raised the possibility that tyrosine phosphorylation of p125<sub>fak</sub> and paxillin, actin stress fiber formation, focal adhesion assembly, and p21<sup>rho</sup> function may lie in a novel signal transduction pathway (5, 22, 28, 30) that regulates cell motility and cell proliferation (24, 60). Here we used CNF1 and DNT to examine further the connections predicted by this pathway. We found that CNF1 and DNT stimulated tyrosine phosphorylation of p125<sub>fak</sub> and paxillin, actin stress fiber formation, and focal adhesion assembly with similar kinetics. Furthermore, pretreatment of quiescent Swiss 3T3 cells with cytochalasin D, at concentrations that completely disrupted the actin cytoskeleton and the focal adhesion plaques, abolished tyrosine phosphorylation of p125<sub>fak</sub> stimulated by both toxins. These results indicate that integrity of the cytoskeleton is essential for CNF1- and DNT-induced tyrosine phosphorylation. This conclusion was substantiated by experiments using PDGF at a high concentration (30 ng/ml), which disrupted actin stress fibers and focal adhesion assembly in response to CNF1 and DNT. At this concentration PDGF also profoundly decreased CNF1- and DNT-induced tyrosine phosphorylation of p125<sub>fak</sub>, revealing a novel cross-talk between these toxins and PDGF. Microinjection of C. botulinum C exoenzyme, which ADP-ribosylates and inactivates p21<sup>rho</sup> function, prevented tyrosine phosphorylation of focal adhesion proteins in response to either CNF1 or DNT. Thus, our results with CNF1 and DNT provide a novel line of evidence for the existence of a pathway in which p21<sup>rho</sup> is upstream of cytoskeletal reorganization and tyrosine phosphorylation of p125 and paxillin.

Most extracellular stimuli that promote actin reorganization and tyrosine phosphorylation of p125<sub>fak</sub> and paxillin also stimulate phospholipase C-mediated hydrolysis of PtdIns(4,5)<sub>2</sub> leading to Ins(1,4,5)P<sub>3</sub>-mediated Ca<sup>2+</sup> mobilization and to the activation of diacylglycerol-dependent isoforms of PKC. Previous studies indicated that neither Ca<sup>2+</sup> mobilization nor PKC activation mediate tyrosine phosphorylation of p125<sub>fak</sub> and paxillin in response to bombesin, lysophosphatidic acid, or PMT (61, 13, 22). However, activated phospholipase C not only generates Ins(1,4,5)P<sub>3</sub> and diacylglycerol but also decreases PtdIns(4,5)<sub>2</sub> in the plasma membrane. PtdIns(4,5)<sub>2</sub> binds to several actin-binding proteins and vinculin (61–64) and thereby could modulate the organization of the actin cytoskeleton (65). In addition, p21<sup>rho</sup> activates phosphatidylinositol-4-phosphate-5-OH kinase and elevates PtdIns(4,5)<sub>2</sub> synthesis (66). These results suggest that the Ins(1,4,5)P<sub>3</sub>-sensitive pool of Ca<sup>2+</sup> and by the failure of these toxins to stimulate the phosphorylation of 80K/MARCKS, a prominent substrate of diacylglycerol-activated PKC isoforms expressed in Swiss 3T3 cells. In contrast, all these responses were induced by PMT in parallel cultures. Thus, our results demonstrate that p21<sup>rho</sup>-dependent actin cytoskeleton reorganization, focal adhesion assembly, and tyrosine phosphorylation...
tion of p125<br>phosphorylation of p125 and paxillin can be dissociated from hydrolysis of PtdIns(4,5)P₂ in CNF1- and DNT-treated Swiss 3T3 cells.

The findings presented here have another interesting implication. A wide range of extracellular signals including growth factors and mitogenic neuropetides activate one or more members of the family of the highly conserved serine/threonine mitogen-activated protein (MAP) kinases (ERKs) (58, 59, 67). MAP kinases, important intermediates in signal transduction pathways leading to mitogenesis or differentiation, are activated predominantly via PKC in Swiss 3T3 cells stimulated by pathways leading to mitogenesis or differentiation, are activated predominantly via PKC in Swiss 3T3 cells stimulated by bombesin. Once activated p42<sup>mapk</sup> (ERK2) and p44<sup>mapk</sup> (ERK1) phosphorylate an array of cellular proteins including protein kinases, transcription factors, and proteins involved in the regulation of cell growth (68–71). Because CNF1 and DNT stimulate DNA synthesis in Swiss 3T3 cells, we investigated the ability of these toxins to induce activation of p42<sup>mapk</sup> and p44<sup>mapk</sup>. Our results demonstrated that unlike PMT, CNF1 and DNT did not stimulate p42<sup>mapk</sup> and p44<sup>mapk</sup> in these cells. These results suggest that CNF1 and DNT can signal entry into S phase without activating p42<sup>mapk</sup> and p44<sup>mapk</sup> in Swiss 3T3 cells.

The signaling events triggered by CNF and DNT appear to be very similar which suggest that both toxins trigger the same initial event, and indeed there is evidence that CNF and DNT each directly target and modify Rho (36, 37). CNF and DNT are known to activate a variety of cellular pathways involved in the signaling of tyrosine phosphorylation of p125.<br>

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Hadriano M. Lacerda, Gill D. Pullinger, Alistair J. Lax and Enrique Rozengurt  

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