Nerve Growth Factor Triggers Microfilament Assembly and Paxillin Phosphorylation in Human B Lymphocytes

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Summary

Increasing evidence suggests that the nervous system is involved in allergic inflammation. One of the potential regulatory molecules of the neuroimmune system is nerve growth factor (NGF). Recent studies from our group demonstrated the presence of a functional NGF receptor (NGFR) on human B lymphocytes. Moreover, we showed that $gp140^{trk}$ tyrosine kinase, which serves as an NGFR, was involved in transduction of early signaling events in human B lymphocytes. The mechanisms by which NGF initiates the signaling cascade and the link between the neuroimmune systems are unknown. We have focused on the role of the cytoskeleton as a possible mediator for transduction of signals induced by NGF. Polymerized actin (F-actin) content was determined by fluorescent staining and immunoblotting with antiactin antibody. Addition of NGF caused a time- and concentration-dependent increase in F-actin content, and maximum effects were noted after 1 min. These increases in F-actin content and NGF-induced thymidine incorporation could be blocked by incubating the cells with cytochalasin D and botulinum C_2 toxin before the addition of NGF. Incubation of human B lymphocytes with 10 nM K252a, an inhibitor of Trk kinase, decreased NGF-induced microfilament assembly by 75%. In immunoprecipitation experiments, addition of NGF to B cells induced a rapid increase in the tyrosine phosphorylation of paxillin, one of a group of focal adhesion proteins involved in linking actin filaments to the plasma membrane. Coimmunoprecipitation studies demonstrated the association between $gp140^{trk}$ kinase and paxillin. Together, these observations suggest that actin assembly is involved in NGF signaling in human B cells, and that paxillin may be essential in this pathway after phosphorylation by $gp140^{trk}$ kinase.

Increasing evidence suggests that the nervous system is involved in allergic inflammation (1). One of the potential regulatory molecules in the neuroimmune axis is nerve growth factor (NGF)[1] (2), and recent studies from our laboratory have demonstrated a functional NGF receptor (NGFR) on human B lymphocytes (3). Moreover, we showed that Trk tyrosine kinase, which serves as an NGFR, was involved in the transduction of early signaling events in human B lymphocytes (Melamed, I., C. A. Kelleher, R. A. Franklin, C. Brodie, J. B. Hempstead, D. Kaplan, and E. W. Gelfand,

manuscript submitted for publication). The mechanisms linking the early metabolic changes induced by NGF to later events in these cells remain to be defined. Systems must exist for the transmission and regulation of such information from the cell surface to the nucleus, and the cytoskeleton may be one candidate. The cytoskeleton is a complex composed of microfilaments, microtubules, and intermediate filaments (4). Immunocytochemical studies in lymphocytes demonstrated that microfilaments, composed primarily of actin, are associated with the inner surface of the plasma membrane and may link the plasma membrane to the nucleus (5). In neuronal cells, a rapid redistribution of filamentous (F) actin is induced within a few minutes after addition of NGF. The appearance of ruffling with rapid redistribution of F-actin has also been shown to be closely related to the subsequent outgrowth of

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[1] Abbreviations used in this paper: F, filamentous; G, globular; NBD, 7-nitrobenz-2-oxa-1, 3-diazole; NGF, nerve growth factor; NGFR, NGF receptor; PLCγ1, phospholipase Cγ1; RFI, relative fluorescence index; SH, Src homology domain.

neurites (6) and, as a consequence of the redistribution of F-actin, might be one of the earliest steps in this process (7).

The cytoskeleton plays an active role in propagation of signals in B lymphocytes. One of the earliest changes in cytoskeleton assembly in human B lymphocytes after ligand binding to its receptor is the conversion of monomeric globular (G) actin to F-actin (8). This process is apparently linked to the activation of the *src* family of protein tyrosine kinases (PTKs) and precedes B cell proliferation (9). However, the role of the microfilament system in transducing signals initiated by NGF in B lymphocytes and the involvement of Trk in microfilament assembly has not been studied.

The propagation of intracellular signals emanating from a tyrosine-phosphorylated growth factor receptor like Trk depends on interactions between the receptor and different substrates including phospholipase Cγ1 (PLCγ1), phosphatidylinositol 3- kinase (PI3-kinase) (10), and SHC (11). Knowledge of the roles of the Src homology domains of these kinases in this process has expanded markedly over the past several years, and newer evidence implicates interactions of these domains with different cytoskeletal proteins (SH3) and signaling molecules (SH2) (11). One of the cytoskeletal proteins with a potential role in interactions with the signaling molecules triggered by NGFR activation is paxillin (12, 13). Paxillin belongs to a group of focal adhesion molecules that link actin filament bundles to the plasma membrane at sites of cell adhesion (13). In fibroblasts, tyrosine phosphorylation of paxillin increases as cells bind to fibronectin during cell spreading (14), concurrent with the assembly of focal adhesions (13). In PC-12 cells, tyrosine phosphorylation of paxillin occurs during neuritic outgrowth (14). In this report, we have characterized NGF-induced microfilament assembly in human B lymphocytes and present evidence that tyrosine phosphorylation of paxillin by gp140^{trk} kinase is associated with microfilament reorganization.

Materials and Methods

Cells. Tonsillar B cells were obtained after Ficoll-Hypaque gradient centrifugation and E-rosette depletion as previously described (15). All cells were maintained in culture in RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Hyclone Laboratories, Inc., Logan, UT), 10 μg/ml L-glutamine, 50 U/ml penicillin, 50 ng/ml streptomycin, and 5 μg/ml sodium pyruvate (Gibco Laboratories).

Reagents. Anti-NGF antibody, NaF, AlCl$_3$, and cytochalasin D were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]thymidine and ^{125}I-protein A and γ-[^{32}P]ATP were obtained from Amersham Corp. (Arlington Heights, IL) and protein A-Sepharose CL4B from Pharmacia (Uppsala, Sweden). NGF (2.5S) and affinity-purified monoclonal antiphosphotyrosine antibody [anti-Tyr(P)] were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Trk A antibody was generated to the unique extracellular sequences of human Trk-A (10); antipaxillin antibody was prepared as described (12), and antibody to actin was kindly provided by Dr. J. Lessard (University of Cincinnati, Cincinnati, OH); K252a was obtained from Kyowa Medex (Tokyo, Japan), 7-nitrobenz-2-oxa-1, 3-diazole (NBD)-phallacidin from Molecular Probes, Inc. (Eugene, OR), and lysophosphatidylcholine from

Avanti Polar Lipids, Inc. (Pelham, AL). Botulinum C$_2$ toxin was isolated and purified as described previously (16).

Western Blot. Cells (4 $\times$ 10^6) were incubated in the presence or absence of various ligands as indicated. Control cells were incubated with the appropriate excipient buffer control. Western blotting was performed essentially as described (15). Briefly, cells were pelleted and the reaction stopped by lysing cells in 5 $\times$ SDS-gel sample buffer, followed immediately by boiling for 5 min. Lysates were frozen, thawed, centrifuged at 12,000 g for 5 min, and supernatants analyzed by SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose or immobilon (Millipore Corp., Bedford, MA), and the unreacted sites on nitrocellulose or immobilon were blocked overnight with 5% BSA (essentially fatty acid and globulin free, Calbiochem Novabiochem. Corp., San Diego, CA) in 10 mM Tris, 150 mM NaCl, and 0.01% NaN$_3$, pH 8.0 (blocking solution). The blot was incubated with the specific antibody in 4 ml of blocking solution and washed twice for 10 min in blocking solution. This was followed by a 10-min wash with blocking solution containing 0.5% NP-40 and twice more with blocking buffer. The filters were then incubated with 1 μCi of ^{125}I-labeled protein A in 5 ml of blocking solution for 1 h and washed as before. The filters were then subjected to autoradiography.

Immunoprecipitation. Cells (2 $\times$ 10^7) were incubated in RPMI-1640 for 2 min in the absence or presence of NGF at the indicated concentrations. Cells were subsequently washed and resuspended in lysis buffer containing 20 mM Hepes (pH 7.4), 125 mM NaCl, 1% Triton X-100, 10 mM EDTA, 2 mM EGTA, 2 mM Na$_3$VO$_4$, 50 mM sodium fluoride, 20 mM ZnCl$_2$, 10 mM sodium pyrophosphate, 1 mM PMSF, and 5 μg/ml leupeptin, and centrifuged for 15 min at 12,000 g. The supernatants were used as cell lysates. The lysates were incubated with 3 μg of antipaxillin antibody per 0.5 ml of cell lysate for 1 h on ice, and then with protein A-Sepharose CL4B for 1 h. The Sepharose beads containing the immunoprecipitates were washed three times with washing buffer (as above except for PMSF and leupeptin), once with 0.5 M LiCl-0.1 M Tris hydrochloride (pH 7.5) and once with 0.1 M NaCl-1 mM EDTA-2 mM Tris hydrochloride (pH 7.5). SDS-sample buffer was added to the washed immunoprecipitates, and the samples were electrophoretically separated on a 10% SDS- polyacrylamide gel.

Coimmunoprecipitation. B cells (20 $\times$ 10^6) were stimulated for 5 min with NGF (500 ng/ml). The cells were then lysed in 1% NP-40 lysis buffer, clarified, and then precleared with protein A-Sepharose beads for 4 h at 4°C. Antipaxillin antibody was added to the cells and allowed to bind for 2 h at 4°C. Immune complexes were then precipitated by adding protein A beads. The beads were washed three times in lysis buffer and twice in in vitro kinase assay buffer (150 nM NaCl, 10 nM Hepes, 1 mM PMSF, and 2 mM Na$_3$VO$_4$), resuspended in 20 μl of kinase buffer, and then incubated for 15 min at 30°C in the presence of 10 μCi of γ[^{32}P]ATP. Beads were then washed twice in lysis buffer to remove free ATP and then boiled in 1 vol of 20 mM Tris-HCl (pH 8.0) containing 0.5% SDS and 1 mM dithiothreitol to disrupt protein–protein interactions. Secondary immunoprecipitations were performed by adding anti-Trk antibody and protein A beads to samples after diluting SDS to 0.1% with 4 vol of lysis buffer. Beads were then washed in lysis buffer and boiled in sample buffer, and proteins were resolved by SDS-10% PAGE and analyzed by autoradiography.

Antibody Blocking of NGF-induced Responses. NGF (500 ng/ml) was incubated with various concentrations of anti-NGF antibody (1–10 μg/ml) for 30 min at room temperature in a volume of 30 μl. This mixture was then used to stimulate the B cells. These conditions were previously shown to abolish the activity of NGF

in assays of B cell proliferation (17). As a control, we used the same concentrations of anti-BSA antibody.

F-Actin Determination. The content of polymerized actin (F-actin) in human B lymphocytes was monitored by staining fixed and permeabilized cells with NBD-phallacidin by use of the methanol extraction method described by Howard and Oresajo (18). This fluorescence method has been shown to correlate with biochemical measurements of F-actin (17). Cells were excited with an argon laser at 488 nm and emission recorded at 520 nm with band pass and short pass filters. Gating was done on the forward angle and right angle light scatter only to exclude debris and cell clumps. A minimum of 10,000 cells was measured per condition. The results were quantified by fluorescence spectrophotometry. The relative fluorescence index (RFI) was calculated as the ratio of the fluorescence of stimulated to control cell populations by use of the ratio of the linearized mean fluorescence of the cell populations in question as provided by the data processing software. Selected experiments were also analyzed on (Epics Profile; Coulter Corp., Hialeah, FL).

Triton X-100 Extraction. For Triton X-100 extraction (9), 2 × 10^6 cells were sedimented and resuspended in Triton-PHEM buffer (0.75% Triton X-100, 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$) containing 1 mM PMSF, 20 μg/ml leupeptin, 80 μg/ml aprotinin, and 156 μg/ml benzamidine at 4°C and allowed to incubate on ice for 20 min. Next, the insoluble fraction was sedimented in a microfuge by spinning at 12,000 g for 5 min. The supernatant (soluble fraction) was removed and the pellet resuspended in boiling sample buffer.

Results

NGF Induces Rapid Assembly of F-Actin in B Lymphocytes. Cross-linking the surface antigen receptor on B cells triggers a variety of membrane changes, including patching, capping, and endocytosis of the receptor (19). Recent studies from our laboratory have demonstrated the presence of functional NGF receptors on human B lymphocytes (17). To determine if microfilament assembly is involved in the early activation events triggered by NGF, we first determined the effect of NGF on microfilament assembly in human B lymphocytes, monitoring changes in fluorescence of NBD-phallacidin–stained cells, which detect F-actin. As shown in Fig. 1, *A* and *B*, NGF induces a concentration- and time-dependent increase in F-actin content. F-actin assembly was observed in the presence of 100 ng/ml NGF, with maximum increases observed with 500 ng/ml (Fig. 1 *A*). The microfilament assembly was detected within 1 min, with maximal increases detected at ∼2 min after addition of NGF (Fig. 1 *B*).

The NGF-induced conversion of G-actin to F-actin was also demonstrated by immunoblotting with antiactin antibody (Fig. 2). Under stimulated conditions, total actin is distributed between the cytoplasmic Triton X-100–soluble fraction and the cytoskeletal Triton X-100–insoluble fraction. When B cells were stimulated with NGF (250 and 500 ng/ml) for 2 min, the amount of actin associated with the cytoskeleton (insoluble fraction, F-actin) increased as detected in immunoblots with antiactin antibody (Fig. 2). The fluorescence (F-actin) distribution histograms confirmed the large increases in anti-F-actin after NGF stimulation (500 ng/ml) (Fig. 3).
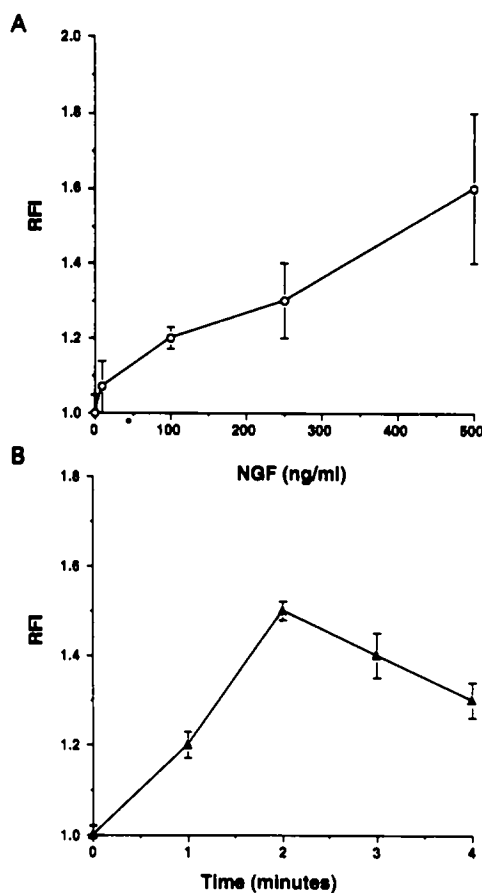
Figure 1. F-actin content in B cells stimulated with NGF antibody. B cells (10^6) were stimulated for 2 min with the indicated concentrations of NGF (*A*). The kinetics of the response were determined by measuring F-actin content in B cells cultured with NGF (500 ng/ml) for various periods of time as indicated (*B*). RFI represents the relative F-actin content assayed by NBD-phallacidin staining and was calculated as described in Materials and Methods. RFI data represent the mean ± SEM of six separate experiments conducted with different preparations of B lymphocytes.
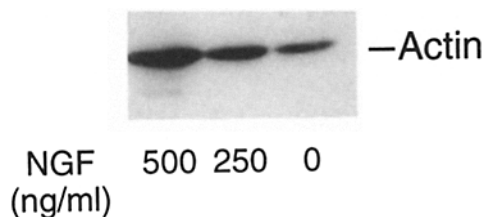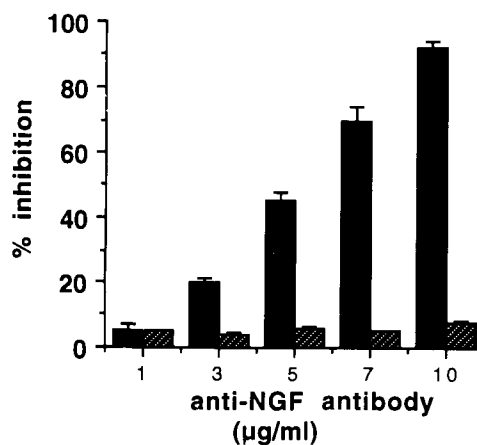

Figure 2. F-actin content in cytoskeletal fractions of stimulated B cells. Antiactin immunoblots of Triton X-100–insoluble fractions of lysates from unstimulated (control) or stimulated human B cells were prepared. The incubation was terminated after 2 min by pelleting the cells and solubilizing them in SDS sample buffer, as described in Materials and Methods. After electrophoresis and blotting, F-actin was detected by use of antiactin antibody and ^{125}I-protein A beads and the mixture subjected to autoradiography. Densitometric analysis indicated that in this experiment, treatment with NGF resulted in five- (500 ng/ml) and twofold (250 ng/ml) increases in F-actin in the Triton X-100–insoluble fraction.
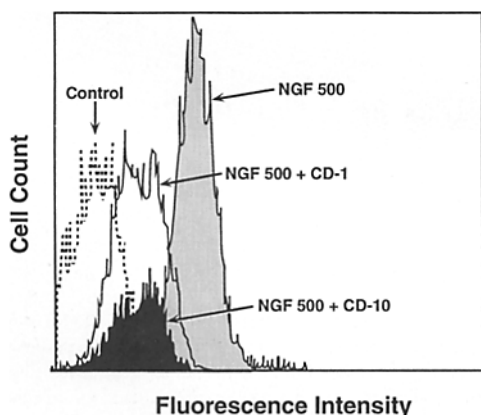
These effects of NGF were specific since preincubation of NGF with anti-NGF antibody eliminated the increases in F-actin content (Fig. 4).

Cytochalasin D and Botulinum C_2 toxin Block NGF-induced Actin Assembly. Since actin polymerization appears to be associated with NGF transduction of signals in B lymphocytes, we determined if there is a direct link between the cytoskeleton reorganization and the signaling events induced by NGF. To this end, we treated cells with compounds that disrupt microfilament assembly and monitored their effects on a late activation event induced by NGF in B lymphocytes, namely, cell proliferation. If actin polymerization was essential for the transduction of NGF mitogenic signals, then prevention of assembly should inhibit cell proliferation induced by NGF. NGF-dependent cell proliferation, assessed by [^{3}H]thymidine incorporation, was blocked by cytochalasin D in a concentration-dependent manner; 10 μM was shown to be an optimal inhibiting concentration (Fig. 5 A). Delaying the addition of cytochalasin D by 1 h after addition of NGF did not affect [^{3}H]thymidine incorporation, thus eliminating drug toxicity as an explanation of these and subsequent results (Fig. 5 A). A 30-min pretreatment of the cells with this concentration of cytochalasin D (10 μM) also prevented NGF-induced microfilament assembly (Fig. 5 B).

Similar results were obtained with a second microfilament-disrupting agent, botulinum C_2 toxin. Botulinum C_2 toxin belongs to a new class of bacterial ADP-ribosyl transferases that modify nonmuscle G-actin (16). The toxin is binary in structure and consists of two different components. Compound I (50,000 mol wt) possesses ADP-ribosyl transferase activity (16), whereas compound II is involved in the binding of the toxin to the cell membrane. Botulinum C_2 toxin ADP ribosylates nonmuscle G-actin at Arg-177 (16). Moreover, modified G-actin binds to barbed ends of F-actin in a capping proteinlike manner to inhibit polymerization of actin. Consistent with our previous observations in human B lym-
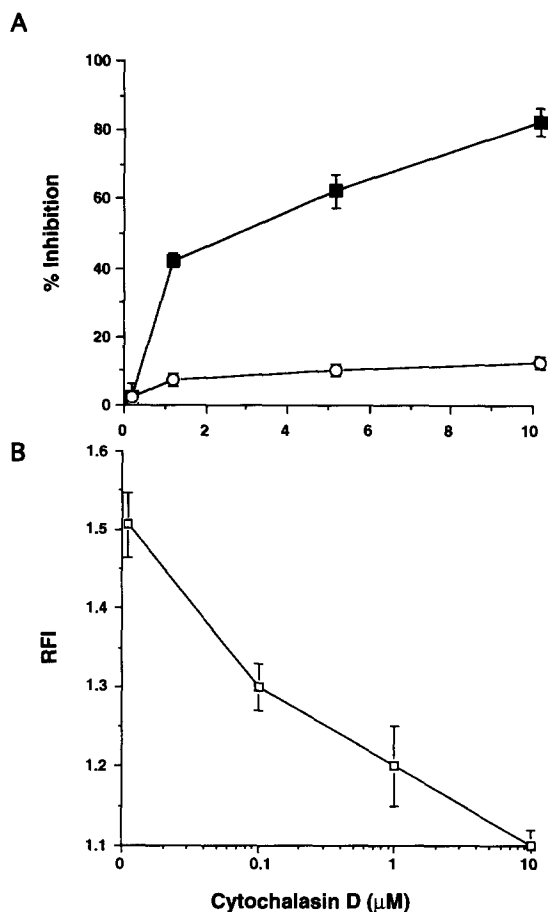

Figure 4. Effect of anti-NGF antibody on F-actin content induced by NGF. NGF (500 ng/ml) was preincubated with different concentrations of anti-NGF antibody (*solid columns*) or anti- BSA antibody (*hatched columns*) for 30 min, and the mixture was added to B cells (10^6/ml) for 2 min. RFI was measured as described in Materials and Methods, and the results (% inhibition) represent mean ± SEM of six separate experiments. Control RFI was 1.8 ± 0.3.

phocytes (8), botulinum C_2 toxin reduced B cell proliferation (Fig. 6 A) and actin assembly (Fig. 6 B) induced by NGF. As with cytochalasin D, delaying the addition of the toxin for 1 h after NGF failed to affect B cell proliferation (Fig. 6 A). The inhibitory effect of botulinum C_2 toxin was dose dependent, and the maximum inhibitory effect was obtained with a dose of 2,000 ng/ml (Fig. 6 B). These results indicate that cytochalasin D and botulinum C_2 toxin, which inhibit NGF-induced B cell proliferation, also block polymerization of actin, suggesting a functional link between NGF induction of cell proliferation and cytoskeleton reorganization.
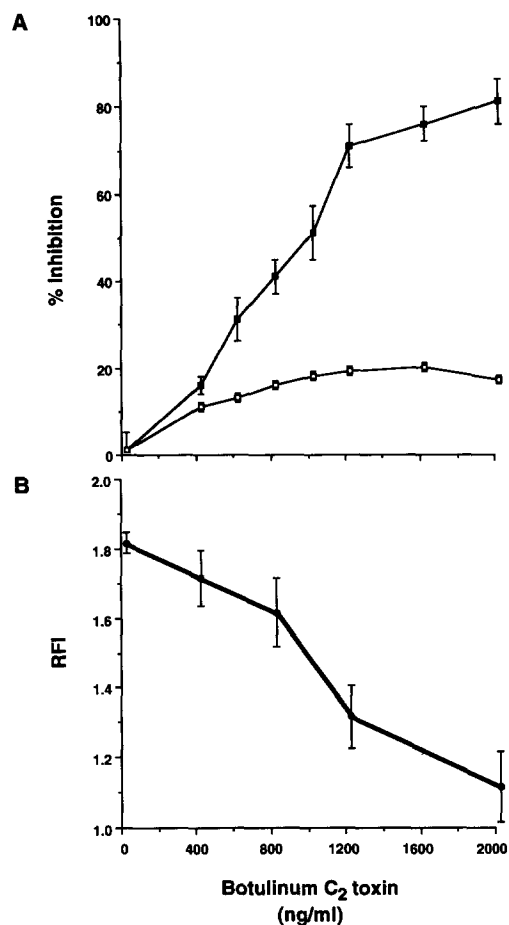
Trk as a Mediator of Microfilament Assembly Induced by NGF. The multiple effects of NGF on B lymphocytes are dependent on binding of NGF to a specific cell surface receptor, where it forms a receptor–ligand complex on the cell surface followed by its translocation to the cytoplasm (2). At the cell surface, NGF interacts with at least two components, gp140^{trk}, a member of the tyrosine kinase transmembrane receptor family, and p75^{NGFR} (20; Melamed, I., et al., manuscript submitted for publication). In neuronal cells, Trk is required for the transduction of the neutrophic signal (21). In certain cells, p75^{NGFR} performs an accessory role, modifying the ligand-binding affinity, neurotrophin specificity, dose responsiveness to NGF, and tyrosine kinase activity of Trk (22, 23). If actin polymerization induced by NGF is dependent on NGF-Trk–mediated increases in tyrosine kinase activity, then inhibition of the tyrosine kinase activity should interfere with actin assembly. We tested this possibility by measuring F-actin content in NGF-stimulated human B cells that were pretreated with K252a, a drug shown to be an inhibitor of Trk (24). As indicated in Fig. 7, a 10-min preincubation with 10 nM K252a decreased the content of F-actin induced by 500 ng/ml NGF by 70%. As a control, anti-IgM antibody induced increases in F-actin that were unaffected

Figure 3. F-actin fluorescence histogram. Human B cells were incubated in the absence or presence of 1 μm (*CD-1*) or 10 μm (*CD-10*) cytochalasin D for 30 min and then stimulated with NGF (500 ng/ml) for 2 min. The cells were stained with NBD- phallacidin and analyzed by flow cytometry as described. Computer traces of one (of three) representative histograms are illustrated.

A

B

Cytochalasin D (μM)

A

B

Botulinum C₂ toxin
(ng/ml)

Figure 5. Effect of cytochalasin D on F-actin content and B cell proliferation induced by NGF. B cells (10⁶/ml) were treated for 30 min with various concentrations of cytochalasin D and then stimulated with NGF (500 ng/ml) (*A*) for a period of 72 h, and B cell proliferation was determined by thymidine incorporation, or (*B*) for 2 min for F-actin content. Delay in addition of cytochalasin D for 1 h after NGF is shown in *A* (O-O) and was without effect. The data are shown as the percentage of inhibition compared with cells not pretreated with cytochalasin D and represent the mean ± SEM of three experiments. Control cpm were 28,000 ± 1,400.

Figure 6. Effect of botulinum C₂ toxin on F-actin content and B cell proliferation induced by NGF. B cells (10⁶/ml) were treated for 1 h with various concentrations of botulinum C₂ toxin and then stimulated with NGF (500 ng/ml) for a period of 72 h. B cell proliferation was determined by thymidine incorporation (*A*) or the cells were analyzed after 2 min for F-actin content (*B*). Delay in addition of the toxin for 1 h after NGF is shown in *A* (□-□) and was without effect. The data represent the percentage of maximum stimulation compared with cells not pretreated with botulinum C₂ toxin and represent the mean ± SEM of three experiments. Control cpm were 25,000 ± 1,400.

by K252a (data not shown). This concentration of K252a inhibits Trk kinase phosphorylation in B cells by 80% (Melamed, I., et al., manuscript submitted for publication) and B cell proliferation by 65% (data not shown), suggesting that Trk does indeed mediate the microfilament assembly induced by NGF.

NGF Leads to Phosphorylation of Paxillin. The vinculin-binding, focal adhesion protein paxillin is tyrosine phosphorylated in response to several mitogenic factors including bombesin, vasopressin (25), and also during cell adhesion and transformation (26). These events are also associated with major rearrangements of the actin cytoskeleton. Therefore, tyrosine phosphorylation of paxillin may accompany the actin filament reorganization observed in NGF-stimulated B lymphocytes. Antiphosphotyrosine immunoprecipitates, prepared from lysates of NGF-stimulated B lymphocytes, were assayed for paxillin by immunoblot analysis with an antipaxillin an-

tibody. As shown in Fig. 8, paxillin protein (68 kD) was phosphorylated on tyrosine residues in human B lymphocytes treated with NGF (500 ng/ml) for 2 min.

Paxillin Coimmunoprecipitates with Trk. Since we identified gp140trk as an NGFR in human B lymphocytes and showed that the protein contains tyrosine kinase activity, which is activated by NGF binding (Melamed, I., et al., manuscript submitted for publication), we tested the hypothesis that gp140trk transduces microfilament assembly and the tyrosine phosphorylation of paxillin. Different tyrosine-phosphorylated growth factor receptors can interact with their substrates, resulting in the propagation of intracellular signals emanating from these growth factor receptors (11). First, we evaluated the ability of Trk to coimmunoprecipitate with paxillin. Trk was immunoprecipitated from NGF (500 ng/ml)-stimulated B lymphocytes, and then the products were labeled in an in vitro kinase reaction and boiled in 0.5% SDS buffer.
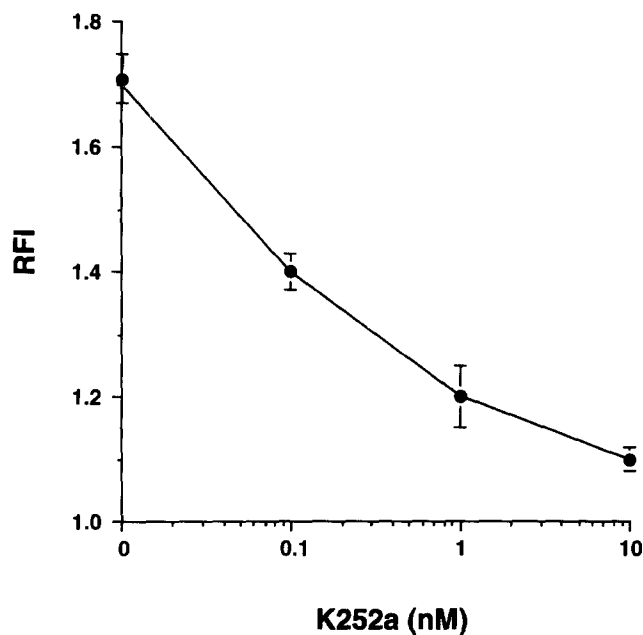
Figure 7. Effect of K252a on F-actin content induced by NGF. B cells (10^6/ml) were treated for 1 h with various concentrations of K252a and then stimulated for 2 min for F-actin content. The data represent the mean ± SEM of three separate experiments.

The mixture was diluted in 1% NP-40 lysis buffer (to a final concentration of 0.1% SDS), and antipaxillin antibody and protein A beads were then added to detect paxillin, which had coimmunoprecipitated with Trk. Immune complexes were then washed, run on SDS-10% PAGE gels, and subjected to autoradiography. The results shown in Fig. 9 illustrate that paxillin associates with Trk in NGF-stimulated B lymphocytes.
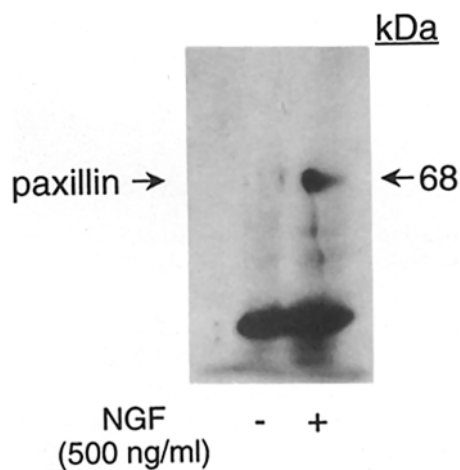
Figure 8. Immunoprecipitation of paxillin in B cells treated with NGF antibody. Paxillin was immunoprecipitated from human B cells (2 × 10^7) stimulated with NGF (500 ng/ml). After the separation of immunoprecipitated proteins on SDS–polyacrylamide gels, proteins were transferred to a nitrocellulose sheet and immunoblotted with monoclonal antiphosphotyrosine antibodies. Immune complexes were detected with ^{125}I-labeled protein A and autoradiography. The results represent one of three similar experiments. Molecular weight marker (kDa) is shown on the right.
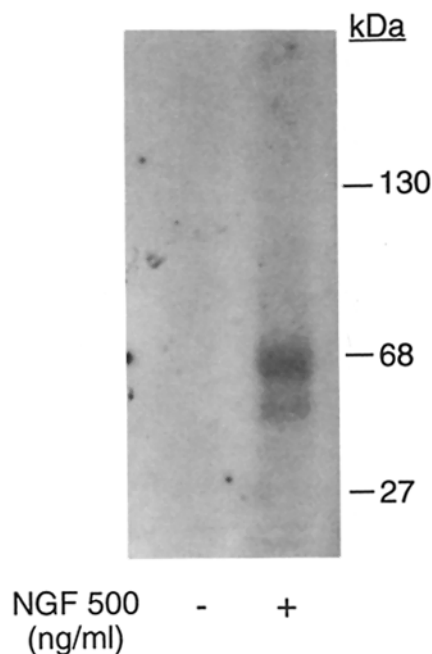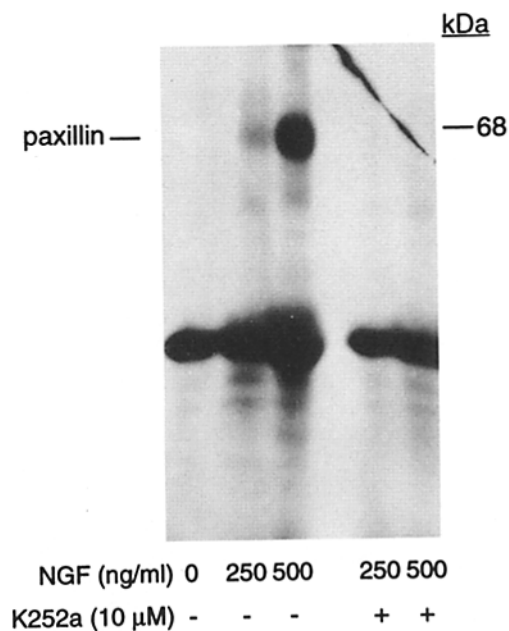

Figure 9. Coimmunoprecipitation of paxillin with Trk. Trk A was immunoprecipitated from control or NGF (500 ng/ml)-stimulated B cells (5 × 10^7/ml) and subjected to in vitro kinase assays. Samples were then thoroughly washed and boiled in a 0.5% SDS buffer to disrupt complexes. Secondary paxillin immunoprecipitations were then performed after samples were diluted to adjust SDS to 0.1%. Reaction products were separated by 10% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (kDa) are shown on the right. The results represent one of four separate experiments.

K252a Inhibits Paxillin Phosphorylation. To delineate the role of Trk tyrosine kinase in tyrosine phosphorylation of paxillin, we used the inhibitor K252a and examined paxillin phosphorylation by NGF. Before the addition of NGF (250 and 500 ng/ml), we preincubated B lymphocytes with the inhibitor (10 nM) for 10 min. As can be seen in Fig. 10, the tyrosine phosphorylation of paxillin was inhibited after pretreatment with K252a.

Discussion

Several pathways of signal transduction are described for converting a ligand–receptor–mediated signal received at the extracellular surface of the plasma membrane into an intracellular signal that ultimately alters gene expression in the nucleus. These pathways consist of a series of chemical reactions that alter enzyme function. One of the central issues we approached in this study was to define one element of the cascade initiated by the binding of NGF to its receptor. Specifically, we examined the role of the microfilament system and paxillin, a focal adhesion protein linked to the microfilament system, in the propagation of signals initiated by NGF binding to its receptor on human B cells.

The microfilament components of the cytoskeleton are composed primarily of actin, which undergoes a rapid and dramatic alteration in conformation and arrangement in human

Figure 10. K252a inhibits paxillin phosphorylation. B lymphocytes were stimulated for 2 min with the indicated concentrations of NGF. In the last two lanes, K252a (10 μM) was added 10 min before the addition of NGF. Paxillin was immunoprecipitated from human B cells (2 × 10^7/cells). After the separation of immunoprecipitated proteins on an SDS–polyacrylamide gel, proteins were transferred to a nitrocellulose sheet and immunoblotted with monoclonal antiphosphotyrosine antibodies. Immune complexes were detected with ^{125}I-labeled protein A and autoradiography. Molecular weight marker (kDa) is shown on the right. The results represent one of three similar experiments.

B lymphocytes in response to NGF stimulation. In addition to causing obvious membrane changes (ruffling and capping), these alterations in the cytoskeleton may be involved in the actual transduction of signals from the surface receptor, leading to cell activation and proliferation. Here, we provide evidence that the conversion of G- to F-actin may be a prerequisite for NGF signaling, based on the findings that disruption of these processes by cytochalasin D and botulinum C_2 toxin resulted in inhibition of B cell proliferation induced by NGF.

The multiple effects of NGF on cell signaling are dependent on binding of NGF to specific cell surface receptors. p75^{NGFR} and Trk are required for transduction of the neurotrophic signals and proliferation events induced by NGF (27). The relationship between these two molecules and their specific contributions to NGF-induced signaling is still controversial. Moreover, the interaction of these receptors with the cytoskeleton in B lymphocytes is not clear. Previous studies have shown that changes in cytoskeletal organization modulate the distribution of NGFR (7), and that NGF is associated with cytoskeletal elements after its binding to receptors on PC-12 cells (28). In this study, we attempted to clarify the role of Trk tyrosine kinase in actin assembly. We show that Trk phosphorylation is an essential process in microfilament assembly. By using a drug that abolishes Trk tyrosine kinase activity (24), we could inhibit microfilament assembly induced by NGF. These data establish the involvement of Trk

kinase in the process of microfilament assembly induced by NGF and confirm previous data that different tyrosine kinases are involved in microfilament assembly (9). The Trk tyrosine kinase is thought to be activated by the formation of NGF-induced dimers or oligomers of Trk followed by transphosphorylation on tyrosine residues (29). Trk tyrosine kinase activity is maximal 2–10 min after NGF binding and is rapidly attenuated (30). The reduction in Trk kinase activity is most likely due to internalization of Trk receptors (31). Microfilament reorganization may play a role in this internalization process.

Since actin polymerization is linked to Src and Trk kinase activation, there are potential interactions between these two different families of kinases. Several of the proteins that form complexes with Trk also form stable, independent complexes with other signal-transducing proteins (32). Complexes of multiple signaling proteins may preexist in the cytoplasm and rapidly translocate to the membrane, thereby localizing Trk to the membrane after addition of NGF to the cells. Actin reorganization may be important for this translocation. A common feature of these signaling molecules is the presence of common motifs, the SH2 and SH3 regions (11). While the physiological role of the SH2 domains of the signaling molecule is slowly being unraveled, little is known about the function of the SH3 domains. SH3-like domains have been identified not only in SH2-containing proteins but also in many molecules related to the cytoskeleton and may serve as actin-binding sites (11). Recent studies indicate that SH3 domains are responsible for the targeting of signaling molecules to specific subcellular locations, where they can interact with their downstream target molecules (33). This linkage was recently observed by Bar-Sagi et al. (33), who demonstrated that SH3 domains of PLCγ_1 by themselves can localize to the actin cytoskeleton. They proposed that the SH3 domain may have a specific role in directing PLCγ_1 to a subcellular location where it could function to regulate actin polymerization and interact with other substrates. Our data, demonstrating that Trk kinase is involved in the propagation of signals in B cells, phosphorylation of downstream signaling molecules (Melamed, I., et al., manuscript submitted for publication), and in microfilament assembly, support the suggested linkage between SH2 domains of the potential signaling molecules and SH3 domains of the cytoskeletal proteins.

In PC-12 cells, the redistribution of F-actin occurs within 10 min of NGF addition, marking the first step in the formation of focal contacts, the sites that colocalize with vinculin by immunofluorescence staining (13). Paxillin is a vinculin-binding protein and was originally identified as a component of focal adhesion contact where the actin cytoskeleton is linked to the extracellular matrix via integrin receptors (13). Interest in paxillin as a cytoskeletal protein with an important role in NGF signaling is based on the following: (*a*) paxillin is one of the SH3-binding proteins (34), and (*b*) paxillin is also a major substrate of pp60^{src} in v-Src–transformed cells, as well as a major tyrosine kinase substrate in developing tissue (35). Localization of Src via its SH3 domain to focal adhesions through paxillin may be

one mechanism for linking tyrosine phosphorylation and focal adhesion formation (36, 37). We demonstrated that paxillin is involved in the signaling initiated by NGF binding to human B lymphocytes. NGF leads to tyrosine phosphorylation of paxillin, and paxillin can coimmunoprecipitate with Trk, suggesting that gp140^trk can interact with the cytoskeletal proteins and the microfilament system through its activation of paxillin. Moreover, we have shown that Trk kinase has an active role in paxillin phosphorylation. By using a Trk kinase inhibitor, we could delineate the role of Trk in microfilament assembly and B cell proliferation. In addition, we have shown an association between Trk and paxillin, indicating an important link between the Trk kinase and the microfilament system through the association with paxillin. Similar associations have been shown in the past between a different focal adhesion molecule, FAK, and a different tyrosine kinase family, the Src family of kinases (38). Such interactions between different cytoskeletal proteins and specific tyrosine kinases may determine how different growth factors and ligands, sharing the same signaling molecules, can achieve specificity in the signaling cascade.

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