Epidermal Growth Factor-mediated Transient Phosphorylation and Membrane Localization of Myosin II-B Are Required for Efficient Chemotaxis*

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Epidermal growth factor (EGF) stimulation of prostate metastatic tumor cells results in transient phosphorylation and cellular localization of non-muscle myosin heavy chain II-B (NMHC II-B) with kinetics similar to those seen in chemotaxis. We demonstrate that expression of 18- and 72-kDa fragments derived from the NMHC II-B C terminus that contain EGF-dependent NMHC II-B phosphorylation sites serve as dominant-negative mutations for EGF-dependent NMHC II-B phosphorylation and localization. Both fragments inhibited the EGF-dependent phosphorylation by competing with NMHC II-B on the myosin heavy chain kinase. However, only expression of the 72-kDa fragment resulted in cells with abnormalities in cell shape, focal adhesions, and chemotaxis. We found that the 72-kDa (but not 18-kDa) fragment is capable of self-assembly. To our knowledge, these results provide the first strong evidence that EGF-dependent NMHC II-B phosphorylation is required for the cellular localization of NMHC II-B and that NMHC II-B is required for normal cell attachment and for chemotactic response.

Cell motility and chemotaxis play a critical role in numerous physiological and pathological processes. Two cellular systems are involved in mammalian cell chemotaxis: the signaling system that binds the extracellular signal to a receptor and to intracellular signaling proteins. These latter proteins transfer the signal to the cytoskeletal system (actin and myosin II) that serves as a motor for moving the cell toward the chemotactic signal (for reviews, see Refs. 1 and 2). Very little is known about the involvement and regulation of myosin II in these processes.

Myosin II is a hexamer composed of two heavy chains of ~200 kDa and two pairs of light chains of 20 and 17 kDa. Vertebrates have at least two genes for non-muscle myosin II heavy chains, and these encode separate isoforms of the heavy chain (A and B) (3, 4). Recent studies indicate that myosins II-A and II-B have different enzymatic activity and are differently localized within the cell (5–10).

Recently, we demonstrated that epidermal growth factor (EGF) stimulation of prostate metastatic tumor cells (TSU-pr1) results in transient phosphorylation and alteration in the cellular localization of non-muscle myosin heavy chain (NMHC) II-A and NMHC II-B with different kinetics (10). We further showed that the kinetics of subcellular localization correlate with the in vivo kinetics of NMHC II-B (but not NMHC II-A) phosphorylation. Thus, NMHC II-B may be involved in the chemotaxis of prostate tumor cells toward EGF (10). Here, we further investigate the role of NMHC II-B and its regulation by phosphorylation in the chemotaxis of TSU-pr1 cells toward EGF. We expressed NMHC II-B rod fragments containing the in vivo EGF-dependent phosphorylation sites and found that they served as dominant-negative mutations abolishing EGF-dependent NMHC II-B phosphorylation. In addition, the two fragments differ in their ability to interact with NMHC II-B in vivo. This difference allowed us to determine the role of phosphorylation of NMHC II-B in its cellular localization and cell adhesion, as well as the role of NMHC II-B in chemotaxis. We present evidence that phosphorylation of NMHC II-B in response to EGF is required for its localization within the cell and that NMHC II-B plays a crucial role in chemotaxis.

EXPERIMENTAL PROCEDURES

Cell Line and Culture Conditions—This study used a prostate carcinoma cell line, TSU-pr1 (11), derived from human prostatic adenocarcinoma metastasized to lymph nodes (kindly provided by Dr. Antonnino Passanitti, Department of Pathology, University of Maryland School of Medicine, Baltimore, MD). Cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Cloning of NMHC II-B Rod Domains—All DNA manipulations were carried out using standard methods (12). We used the expression vector pEGFP (Clontech), which allows expression of proteins fused to green fluorescent protein (GFP). The pGFP-Tail expression vector was constructed as follows. The 502-bp reverse transcription-PCR product obtained from the 3′-end of NMHC II-B (10) was restricted with HindIII and rendered blunt, followed by restriction with EcoRI and ligation into EcoRI-Smal sites in plasmid pEGFP-C1 (hereafter termed GFP-tail). To generate the pGFP-Rod expression vector, full-length NMHC II-B (13) was restricted with BamHI to give a 1971-bp fragment from the 3′-end of NMHC II-B. The BamHI fragment was cloned into pEGFP-C2 restricted with BamHI (hereafter termed GFP-Rod). Expression of the NMHC II-B tail fragment in Escherichia coli was described previously (10). For expression of the rod fragment in E. coli, we constructed pET21-Rod. The BamHI fragment containing the rod sequences described above was cloned into pET21 restricted with BamHI. Tail and rod fragments were purified as described previously (10).

Cell Transfection—Cells were grown to 60–80% confluence in a 6-well plate and then transfected with 7 μg of plasmid DNA in N1/2,3-dioleoyloxypropryl-N,N,N-trimethylammonium transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. After 14 days of selection with 400 μg/ml G418, antibiotic-resistant cell colonies were transferred to a 24-well plate. Stable transfected clones were isolated after careful examination under a fluorescent microscope. The most fluorescent clones

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The abbreviations used are: EGF, epidermal growth factor; NMHC, non-muscle myosin heavy chain; GFP, green fluorescent protein; PBS, phosphate-buffered saline.

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were collected and tested by Western blot analysis for expression of GFP, GFP-tail, and GFP-rod.

**Gel Electrophoresis and Western Blot Analyses**—Gel electrophoresis was carried out following the method of Laemmli (14). As described above, cells were washed twice with ice-cold PBS and scraped off the plate. They were counted and lysed in SDS gel loading buffer (50 mm Tris (pH 6.8), 100 mm dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol) and resolved by SDS-PAGE. Western blots were blocked with 5% milk-Tris-buffered saline/Tween 20 and probed with an affinity-purified polyclonal antibody against the C terminus of NMHC II-B (1:2000) or with anti-GFP antibody (1:3000) (Rockland Inc.). The blots were developed using horseradish peroxidase coupled to a secondary antibody (1:4000; Jackson Immunoresearch Laboratories, West Grove, PA). Immunoreactive bands were detected using ECL as previously described (10).

**In Vivo Phosphorylation and Immunoprecipitation**—1 × 10^5 cells were grown on 100-mm plates as described above. Cells were washed once with 5 ml of prewarmed starvation medium without phosphate (RPMI-H = RPMI 1640 medium containing 12 mm HEPES (pH 7.4)) and incubated for 2 h in RPMI-H containing 100 µCi of ^32P. After starvation, cells were stimulated with 7 ng/ml EGF. At different times after stimulation, cells were lysed with 1.5 ml of ice-cold 2× modified radioimmunoprecipitation assay buffer (80 mm Tris (pH 7.5), 2% Nonidet P-40, 1% deoxycholic acid, 50 mm sodium pyrophosphate, 100 mm sodium fluoride, and protease inhibitor mixture (Sigma)). The extracts were incubated on ice for 10 min and spun at 30,000 × g for 15 min at 4°C. The supernatants were incubated with an affinity-purified polyclonal antibody against the C terminus of NMHC II-B or with anti-GFP antibody for 2 h on a rotator at 4°C. Protein A-agarose beads (Invitrogen) were added, and the mixture was incubated for an additional 2 h. The immunoprecipitates were washed twice with ice-cold 1× modified radioimmunoprecipitation assay buffer, subjected to Western blot analysis, and analyzed by autoradiography. The amounts of NMHC II-B and rod and tail fragments immunoprecipitated and the ^32P incorporated into the proteins were determined by scanning the Western blot and autoradiogram, respectively, with a scanning laser densitometer. Band areas were quantified using the Quantity One software program (Bio-Rad). Relative phosphorylation of the proteins was determined by dividing the values obtained from autoradiography by the values obtained from Western blotting.

For co-immunoprecipitation of the rod fragment and NMHC II-B, the rod fragment was immunoprecipitated with anti-GFP antibody (Rockland Inc.) as described above. The immunoprecipitates were resolved by 7% SDS-PAGE and analyzed by Western blotting with anti-NMHC II-B antibody.

**Triton-insoluble NMHC II-B Assay**—3 × 10^5 TSU-pr1 cells were plated on 30-mm plates 1 day prior to the experiment. The cells were starved in RPMI-H for 2 h and stimulated with 7 ng/ml EGF. At the indicated times, the cells were washed on ice, washed once with ice-cold PBS, and then lysed on ice with 100 µl of Triton solubility lysis buffer (50 mm Tris-HCl (pH 7.4), 50 mm NaCl, 1% Triton X-100, 5 mm MgCl₂, 1 mm phenylmethylsulfonyl fluoride, and 5 µl protease inhibitor mixture). The Triton-soluble proteins were collected from the plates into fresh tubes to which 20 µl of 5× SDS gel loading buffer was added. To collect the Triton-insoluble proteins, 100 µl of 2× SDS-PAGE sample buffer was added to the plates; the plates were scraped; and the proteins were collected into fresh tubes. Equal samples from the Triton-soluble and -insoluble proteins were analyzed by 7% SDS-PAGE and immunoblotted with anti-NMHC II-B antibody (1:10000). To determine the relative amounts of NMHC II-B in the different fractions, the Western blots were scanned with a scanning laser densitometer as described above.

**Microscopy and Image Analysis**—For indirect immunofluorescent staining, cells were grown to 60% confluence in 6-well dishes, in which coverslips coated with 27 µg/ml rat tail collagen I were placed at the bottom of the wells. Cells were washed twice with RPMI-H and incubated in serum-free RPMI-H containing 10% FCS for 2.5 h at 37°C. To stimulate with EGF, 30 µg/ml EGF was placed at a prelabeled corner of the coverslip for 4 min and the cells were fixed in 3.7% paraformaldehyde and incubated at room temperature for 10 min, followed by three washes with PBS. A solution of 0.5% bovine serum albumin and 0.2% Triton X-100 in PBS was added to the coverslips, and they were incubated for 30 min at room temperature, followed by three washes with PBS. A monoclonal antibody against vinculin (Sigma) at 1:100 dilution, or a monoclonal antibody against phosphotyrosine clone PT66 (Sigma) was added to the coverslips and incubated for 45 min at 37°C. Cy5-conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) at 1:150 dilution was added to the coverslips and incubated for 30 min at 37°C, followed by three washes with PBS. 8 µl of mounting solution (Vector Laboratories, Inc.) was added to each slide before mounting the coverslips. These were then examined using a ×40 objective under a Zeiss LSM 410 inverted confocal laser scanning system. Confocal images were converted to TIFF format and transferred to a Zeiss imaging workstation for pseudo-color representation.

The relative intensity ratios of NMHC II-B in the cortex and cytoplasm were measured using Zeiss Imaging Software Version 3.80. Two random fields from each cell were determined for analysis (Cy5 recordings of the cortex and the cytoplasm (Fig. 3B). The intensity of the NMHC II-B fluorescence in each region was determined per µm² using the above program.

To visualize live cells, cells were grown in a chambered cover glass (Nunc) and observed using the above microscope with Nomarski optics. To visualize actin, cells were fixed and permeabilized as described above, followed by staining with rhodamine-labeled phalloidin (1 units/ml). Cells were then examined using a ×82 objective under a Zeiss Axiosvert 200 microscope equipped with a Sensicam cooled imaging camera (Pco Inc.).

**In Vitro Solubility Assay of E. coli Expressed Rod Fragments**—An E. coli expressed rod fragment (30 µg/ml) was dialyzed into 10 mm Tris-HCl (pH 7.5), 0.1 mm EDTA (pH 7.5), 50 mm NaF, 10 mm MgCl₂, and 60 min in a humidified incubator as described above. Unattached cells were removed by replacing the medium. The number of adherent cells was determined by counting the number of fluorescent cells in 10 random fields at ×10 magnification.

**Chemosensitive Assay**—Blind-well Boyden chambers (NeuroProbe, Cabin John, MD) were used with 13-mm diameter polycarbonate filters (12-µm pore size; Osmonics, Inc., Livermore, CA) coated with 5 µg/filter collagen IV (Sigma). 3 × 10^5 cells were resuspended in serum-free RPMI 1640 medium containing 0.1% bovine serum albumin and were placed in the upper compartment of the chamber. 5 ng/ml EGF in the above medium was added to the lower compartment, and the chambers were incubated for 1–4 h in a humidified incubator as described above. At the times indicated, the filters were removed, fixed, and stained using the Diff-Quik stain set (Dade Behring AG, Duderberg, Switzerland). Non-motachic cells were removed from the top surface of the filter with a wet cotton swab. The filters were mounted on glass slides with the bottom part facing up. 10 random fields from each filter were counted at ×40 magnification.

**RESULTS**

**EGF-dependent NMHC II-B Phosphorylation Is Inhibited by Expression of Rod and Tail Fragments**—EGF stimulation of TSU-pr1 cells results in a transient increase in NMHC II-B phosphorylation that correlates with the EGF-dependent cellular localization of NMHC II-B (10). Thus, EGF-dependent NMHC II-B phosphorylation may play an important role in regulating NMHC II-B localization within the cell. To begin analyzing the role of phosphorylation of NMHC II-B in its regulation, we expressed, in TSU-pr1 cells, fragments of 18- or 72-kDa proteins that derived from the C terminus of NMHC II-B tagged with GFP. The resulting fusion proteins were the rod (102 kDa) and tail (48 kDa) fragments (Fig. 1). We previously showed that EGF stimulation of TSU-pr1 cells results in phosphorylation of NMHC II-B mediated by a kinase from the protein kinase C family (10), and both the rod and tail fragments contain the previously mapped protein kinase C phosphorylation sites (15–17). Therefore, expression of these fragments may function as dominant-negative mutations and compete with endogenous NMHC II-B for the EGF-dependent
myosin heavy chain kinase. In this case, NMHC II-B would not be phosphorylated in response to EGF stimulation, allowing the examination of the role of EGF-dependent NMHC II-B phosphorylation in the regulation of NMHC II-B.

To study whether expression of the rod and tail fragments inhibits EGF-dependent NMHC II-B phosphorylation, we assayed NMHC II-B phosphorylation in cells expressing GFP only (control cells) and in cells expressing the tail or rod fragment (tail and rod cells) in response to EGF stimulation as described under “Experimental Procedures.” A representative experiment is shown in Fig. 2A. EGF stimulation of control cells resulted in a transient increase in NMHC II-B phosphorylation, as previously shown for TSU-pr1 cells (10), whereas expression of rod or tail fragments abolished this phosphorylation. Quantification of several experiments such as that in Fig. 2A further established that expression of both rod and tail fragments inhibited EGF-dependent NMHC II-B phosphorylation (Fig. 2B). Both rod and tail fragments appeared to function as dominant-negative mutations for EGF-dependent NMHC II-B phosphorylation. In addition, these results indicate that the sites for in vivo EGF-dependent phosphorylation reside within the tail fragment. Furthermore, EGF stimulation of control cells resulted in a transient increase in NMHC II-B phosphorylation compared with unstimulated cells. These findings indicate that EGF stimulation of control cells leads to the phosphorylation of at least three to four sites on NMHC II-B.

To confirm that EGF-dependent NMHC II-B phosphorylation is inhibited by rod or tail fragments due to competition on the myosin heavy chain kinase, we measured the in vivo phosphorylation levels of the rod or tail fragments in response to EGF stimulation. Fig. 2B shows that stimulation of rod and tail cell lines resulted in a transient increase in rod and tail phosphorylation with kinetics similar to those of NMHC II-B in control cells. The rod and tail fragments thus undergo phosphorylation in response to EGF stimulation, and cells expressing these fragments are unable to phosphorylate NMHC II-B. Therefore, these fragments function as dominant-negative mutations by competing with endogenous NMHC II-B for the EGF-dependent myosin heavy chain kinase.

In simpler eukaryotes such as Acanthamoeba or Dictyostelium, in vitro and in vivo studies indicate that myosin heavy chain phosphorylation affects the assembly properties of myosin II (18–20), and phosphorylation of sites on the tail of mammalian myosin II has also been observed, but its biological significance is not clear (17, 21). To begin analyzing the role of NMHC II-B phosphorylation in the regulation of NMHC II-B filament assembly, we tested the effect of tail and rod fragment expression on the assembly properties of NMHC II-B in response to EGF stimulation. For this purpose, we subjected these cells to Triton solubility assay; control, tail, and rod cells stimulated with EGF were lysed in Triton X-100, and the amounts of Triton-insoluble MJHC II-B in the Triton-insoluble and -soluble fractions were determined by Western blot analysis with anti-NMHC II-B antibody.

As shown in Fig. 2B, EGF stimulation of control cells resulted in a transient increase in Triton-insoluble NMHC II-B. In contrast, this increase was not observed in tail and rod cells. These results indicate that the inhibition of EGF-dependent NMHC II-B phosphorylation by expression of tail or rod fragments also affects the assembly properties of NMHC II-B. EGF stimulation of control cells resulted in a peak of Triton-insoluble NMHC II-B at 2 min and in a peak of phosphorylation at 6 min (Fig. 2B). NMHC II-B was unphosphorylated at the peak of NMHC II-B Triton insolubility, whereas there was a very small amount of Triton-insoluble NMHC II-B when NMHC II-B was at its peak of phosphorylation. These results suggest that phosphorylation of NMHC II-B makes the previously insoluble protein Triton-soluble.

This hypothesis gains support from observations on rod and tail cells. Measurement of Triton-insoluble NMHC II-B in these cells revealed ~20% more than in control cells. Most importantly, this amount of Triton-insoluble NMHC II-B in cells expressing rod or tail fragments did not change regardless of EGF stimulation. These results suggest that most of the NMHC II-B protein is Triton-insoluble in its unphosphorylated form, possibly because most of the protein is in the filament form. Taken together, these results suggest that heavy chain phosphorylation is biologically significant and plays a role in the regulation of NMHC II-B function through an effect on NMHC II-B filament formation.

**Fig. 1.** Schematic presentation and Western blot analysis of endogenous NMHC II-B and expressed rod and tail domains tagged with GFP. *A,* numbers indicate amino acid residues; *N-ab* is the N-terminal site for specific antibodies used for staining of NMHC II-B (see Fig. 3); *C-ab* is the C-terminal site for specific antibodies used for Western blotting and immunoprecipitations; and PKC is the protein kinase C phosphorylation site (17, 30). *B,* extracts of stable TSU-pr1 cell lines expressing GFP (control cells; lanes 1 and 4), the rod fragment (lanes 2 and 5), and the tail fragment (lanes 3 and 6) were resolved by 7% (lanes 1–3) or 10% (lanes 4–6) SDS-PAGE. The gels were blotted onto nitrocellulose membrane and probed with anti-NMHC II-B and anti-GFP antibodies, respectively. See “Experimental Procedures” for details in this and all other figures.
EGF-dependent NMHC II-B Localization Is Disrupted by Expression of Rod or Tail Fragments—As mentioned above, EGF stimulation of TSU-pr1 cells results in NMHC II-B phosphorylation that correlates with NMHC II-B achieving a specific cellular localization (10). Because expression of rod and tail fragments abolished EGF-dependent NMHC II-B phosphorylation (Fig. 2), we next determined the localization properties of NMHC II-B in these cell lines. To do this, we stimulated control, rod, and tail cell lines with EGF and stained them for indirect immunofluorescence using an antibody directed against the N-terminal domain of NMHC II-B (Fig. 1A, N-ab). Because the rod and tail fragments lack this domain, only endogenous NMHC II-B was stained (Fig. 3A).

EGF stimulation of control cells caused NMHC II-B to associate with the cell cortex (Fig. 3A) (10). Expression of tail and rod fragments disrupted EGF-dependent NMHC II-B localization such that EGF stimulation of these cells did not result in NMHC II-B translocation to the cell cortex. NMHC II-B was localized in the cytoplasm in rod cells and in the cell cortex in tail cells regardless of EGF stimulation (Fig. 3A). To further establish that expression of rod and tail fragments disrupts EGF-dependent NMHC II-B translocation to the cortex, we determined the ratio of NMHC II-B fluorescence in the cell cortex to that in the cytoplasm before and after EGF stimulation as described under "Experimental Procedures." First, we defined the cortex and the cytoplasm regions for each cell (Fig. 3B) and then determined the fluorescence intensity of NMHC II-B per μm² for each region. As shown in Fig. 3C, the cortex/cytoplasm fluorescence ratio of NMHC II-B in unstimulated GFP cells was 1.21; after EGF stimulation, the ratio increased to 1.82, an increase of ~49%. In contrast, the cortex/cytoplasm ratio of NMHC II-B in rod and tail cells did not increase following EGF stimulation. The amount of NMHC II-B in rod cells before and after EGF stimulation was similar to that in unstimulated control cells, whereas the amount of NMHC II-B in tail cells was similar to that in stimulated control cells.
These results clearly indicate that expression of rod and tail fragments disrupts EGF-dependent NMHC II-B translocation to the cell cortex.

Expression of rod fragments also resulted in aberrant cell morphology (Fig. 3A) as well as in long protrusions of an average length of 38 μm (data not shown). Furthermore, the rod fragments co-localized with endogenous NMHC II-B (Fig. 3A, yellow spots), implying that rod fragments interact with endogenous NMHC II-B. Tail fragments did not show co-localization with NMHC II-B, indicating that, in contrast to rod fragments, tail fragments did not interact with NMHC II-B. This difference between rod and tail fragments with respect to their interaction with endogenous NMHC II-B was confirmed by immuno-precipitating the rod and tail fragments from rod and tail cell lines, respectively, using anti-GFP antibody, followed by Western blot analysis with anti-NMHC II-B antibody. Fig. 3D shows that NMHC II-B co-immunoprecipitated with rod fragments; similar experiments performed using tail cells indicated that tail fragments did not co-immunoprecipitate with NMHC II-B (data not shown). Quantification of several experiments such as that in Fig. 3D indicated that 11.5 ± 3.4% (S.D.) of total NMHC II-B co-immunoprecipitated with rod fragments. These results indicate that the rod (but not tail) fragment interacts with NMHC II-B. It is plausible that the rod fragment and NMHC II-B co-assembled to form heterodimers.

To begin exploring the mechanism by which rod fragments co-assemble with NMHC II-B, we studied the assembly properties of rod and tail fragments in vitro. The rod and tail fragments were expressed in E. coli and assayed for solubility as described under “Experimental Procedures.” As shown in Fig. 4, in 25 mM NaCl, ~75% of the rod fragment was soluble; increasing the NaCl concentration to 125 mM resulted in an increase in rod insolubility such that only 30% of the rod fragment was soluble. Increasing the salt concentration resulted in solubility of the rod fragment; so at 275 mM NaCl, 75% of the rod fragment was soluble. Similar results were reported by Murakami et al. (16). In contrast, the tail fragment was soluble in buffer containing NaCl up to 275 mM. These differences in the solubility properties between rod and tail fragments may result from the difference in lengths of the two fragments.

Assuming that the observations shown in Fig. 4 reflect the in vivo situation of rod and tail fragments, then the rod fragment is most likely to be in the insoluble form and the tail fragment in the soluble form at physiological ionic strength. Furthermore, the ability of the rod fragment to assemble may
result in the formation of rod and NMHC II-B heterodimers. This hypothesis is consistent with the results in Fig. 3A, in which the rod fragment appeared to be in aggregates and co-localized with endogenous NMHC II-B.

As shown in Fig. 3A, the expressed rod fragment formed aggregates in different regions throughout the cell. The tail fragment accumulated in the perinuclear region (Fig. 3A). Using confocal microscopy sections, we found that these accumulations of the tail fragment were located in the perinuclear region and not in the nucleus (data not shown). GFP fusion proteins, especially the truncated form, have frequently been shown to aggregate at the cell perinuclear region (22, 23). As mentioned above, both rod and tail fragments abolished EGF-dependent NMHC II-B phosphorylation by competing with the EGF-dependent myosin heavy chain kinase. These results indicate that the formation of aggregates does not affect the ability of these NMHC II-B fragments to serve as substrates for the EGF-dependent myosin heavy chain kinase. These results suggest that, although the rod and tail fragments aggregate in the cell, they are functional as efficient substrates for the EGF-dependent myosin heavy chain kinase.

To gain greater insight into the effect of rod and tail fragment expression on the morphology of TSU-pr1 cells, the above cell lines were grown in chambered cover glasses and visualized with Nomarski optics. Control and tail cells showed a very similar morphology; but rod cells were very flat with long extensions, and the cell-surface bore ruffle-like structures (Fig. 5).

These results indicate that EGF-dependent myosin heavy chain II-B phosphorylation plays an important role in the cellular localization of NMHC II-B. Recruitment of NMHC II-B by the rod fragment not only disrupts NMHC II-B localization, but also leads to aberrant cell morphology, suggesting that NMHC II-B is also essential for maintaining normal cell morphology.

Expression of Rod Fragments Affect the Actin Cytoskeleton—Focal adhesions are thought to be anchored to the cell matrix by stress fibers that are a complex of actin and myosin II. As mentioned above, expression of the rod (but not tail) fragment affects focal adhesion morphology; it is therefore possible that expression of the rod fragment also affects the actin cytoskeleton. To examine this possibility, we stained F-actin fibers with rhodamine-labeled phalloidin. Indeed, expression of the rod (but not tail) fragment resulted in disruption of the F-actin cytoskeleton. As shown in Fig. 6, in control cells as well as in tail cells, the stress fibers were abundant; in contrast, in rod cells, the stress fibers disappeared. This disruption was seen regardless of the absence or presence of EGF (data not shown). These results strongly indicate that expression of the rod fragment disrupts the actomyosin cytoskeleton.

Expression of Rod Fragments Affects the Distribution of Focal Adhesions—Most cells adhere to the extracellular matrix by focal adhesions, which are complexes of structural and signaling proteins. Focal adhesions are mediated by integrins and interact with the interior part of the cell via actomyosin (for review, see Ref. 24). Because expression of the rod domain resulted in aberrant NMHC II-B localization and cell morphology (Fig. 5), we wished to determine whether the altered NMHC II-B localization also affected the focal adhesion properties of the cell and whether this was the cause of the aberrant cell morphology. Control, rod, and tail cell lines were stained for vinculin and for phosphotyrosine, which are markers of focal adhesions (25). Fig. 7 shows that control cells displayed typical focal adhesions, and these were distributed in punctate form all over the cell surface, as previously reported (25). The focal adhesion morphology and distribution of tail cells were similar to those exhibited by control cells (Fig. 7). In contrast, vinculin staining in rod cells revealed dot-like structures, which formed an almost continuous shell around the cell periphery (Fig. 7). Staining of control, tail, and rod cells with anti-phosphotyrosine antibody gave similar results (data not shown). These results strongly indicate that expression of rod (but not tail) fragments resulted in abnormal focal adhesion structure and localization and that this may cause the aberrant cell morphology.

**FIG. 4.** The *E. coli* expressed rod (but not tail) fragment assembles in a salt-dependent manner. The proteins were adjusted to a concentration of 30 μg/ml and dialyzed against various NaCl concentrations. The mixture was then centrifuged at 30,000 × g for 30 min. The proteins in the supernatants and pellets were analyzed by 10% SDS-PAGE, and their relative amounts were determined by scanning densitometry as described under “Experimental Procedures.” The percentage of NMHC II-B in the supernatant was plotted.

**FIG. 5.** Expression of the rod fragment affects cell morphology. Cells were grown in a chambered cover glass and visualized with Nomarski optics. Bars = 10 μm.
**Expression of Rod Fragments Affects Cell Attachment**—Because focal adhesion complexes play an important role in cell attachment to the extracellular matrix and because expression of rod fragments disrupts the appearance and localization of focal adhesion complexes, we next assayed the attachment properties of rod and tail cells to determine whether they differ from those of control cells. As shown in Fig. 8, 30 and 60 min after replating, the numbers of rod cells that adhered to the substratum were 86 and 233% higher than those of control and tail cells. Expression of the rod fragment, which disrupted cell morphology and focal adhesions, also affected cell attachment properties. Thus, disruption of NMHC II-B phosphorylation and localization affects cell attachment. The results also provide strong evidence that NMHC II-B plays a major role in maintaining intact focal adhesions that are required for normal cell attachment and cell morphology. Furthermore, EGF-dependent NMHC II-B phosphorylation regulates cell attachment; disruption of this process by expression of the rod fragment affects the attachment properties of these cells.

**Expression of Rod Fragments Affects Chemotaxis toward EGF**—Cells stimulated with chemoattractant respond by sending wide lamellipodia toward the source of the chemoattractant, followed by detaching the posterior part of the cell from the substratum, resulting in forward movement (1). Because expression of the rod fragment caused an abnormal appearance and distribution of focal adhesions, we compared the chemotactic ability of this cell line to that of control and tail cell lines (Fig. 9). Chemotaxis was assayed using a Boyden chamber as described under “Experimental Procedures.” Control and tail cell lines exhibited similar chemotactic ability toward EGF, and the number of cells chemotaxing toward EGF increased linearly for up to 4 h of incubation. In contrast, there was a dramatic reduction in the ability of rod cells to chemotax toward EGF. 4 h after incubation of the different cell lines in the Boyden chamber, 50% fewer rod cells than control cells chemotaxed toward EGF (Fig. 9). The inefficient chemotaxis of the rod cells may result from their stronger attachment to the substratum, leading to slower movement.

**DISCUSSION**

Two cellular systems, the signaling and cytoskeletal systems, are involved in chemotaxis. Although much is known about these two systems, very little is known about the cross-talk between them that allows a cell to sense the chemoattractant and to transfer this information to the cytoskeletal system, which functions as a motor to move the cell toward the chemoattractant.

NMHC II-B appears to be the myosin II isoform that plays a role in cell motility and chemotaxis, and it is localized to the leading lamellipodium in cells migrating quickly (9). We have previously shown that NMHC II-B undergoes phosphorylation and cellular localization in response to EGF stimulation and that the kinetics of these processes are similar (10). Here, we further explored the role and regulation of NMHC II-B in chemotaxis; we expressed, in TSU-pr1 cells, two fragments of NMHC II-B that functioned as dominant-negative mutations for EGF-dependent NMHC II-B phosphorylation and cellular localization. Expression of both rod and tail fragments abolished the EGF-dependent phosphorylation, indicating that the EGF-dependent phosphorylation sites are located within the tail fragment. It also disrupted the EGF-dependent NMHC II-B localization within the cell, indicating that NMHC II-B phosphorylation and localization are connected. These observations are consistent with other studies showing that phosphorylation of myosin II regulates its filament assembly and cellular localization (20, 26–28).

The important outcome of the studies described here is that heavy chain phosphorylation is biologically significant and plays a role in the regulation of NMHC II-B function plausibly through an effect on NMHC II-B filament formation. EGF...
stabilization of control cells resulted in a 3–4-fold increase in NMHC II-B phosphorylation compared with unstimulated cells, and this phosphorylation is carried out by a member of the protein kinase C family (10). It is therefore plausible that EGF activates protein kinase C, which phosphorylates NMHC II-B on at least three to four sites, leading to its disassembly, which is reflected by its Triton solubility. This process is necessary for the cellular reorganization of NMHC II-B in response to EGF stimulation that is required for proper cell morphology, attachment, and chemotaxis.

Support for a physiological role of myosin heavy chain phosphorylation in filament formation comes from studies on the effects of heavy chain phosphorylation by protein kinase C and casein kinase II using in vitro assays designed to evaluate the assembly of fragments of the myosin II tail (17). Phosphorylation of NMHC II-B by protein kinase C results in disassembly of this myosin II isoform, but casein kinase II phosphorylation has only minimal effects on its solubility. Together, these results strongly indicate that heavy chain phosphorylation is biologically significant and plays a role in the regulation of NMHC II-B function through effects on filament formation.

Although the tail and rod fragments inhibited endogenous NMHC II-B phosphorylation and localization, only expression of the rod fragment dramatically affected cell morphology, attachment, and chemotaxis. The differences in the cellular effects of the rod and tail fragments may result from the differences in their solubility properties. Although the rod fragment is capable of assembling under low salt concentrations, the tail fragment is soluble under a wide range of salt concentrations.

These findings may indicate that, in vivo, the rod (but not tail) fragment forms heterodimers with endogenous NMHC II-B. Indeed, the rod (but not tail) fragment co-localized and co-immunoprecipitated with endogenous NMHC II-B. The differences in the cellular effects of the rod and tail fragments allowed us to distinguish between the effect of inhibition of EGF-dependent phosphorylation and localization of NMHC II-B and the effect of these plus the sequestering of NMHC II-B by the rod fragment. Expression of the rod fragment not only inhibited EGF-dependent NMHC II-B phosphorylation, but also sequestered this myosin II isoform, plausibly diminishing its cellular amount. In addition, the rod fragment may form heterodimers with endogenous NMHC II-B that are possibly nonfunctional because the rod fragment is missing the head domains. The interaction between NMHC II-B and the rod fragment disrupts its function, which may provide the contractile shell required to maintain cell shape. Indeed, recruitment of NMHC II-B and/or formation of heterodimers resulted not only in aberrant cell morphology, but also in cells with long cell extensions (Figs. 3 and 5). These extensions may result from a decrease in the amount of functional NMHC II-B around the cell cortex, a certain amount of which may be required to inhibit their development.

This hypothesis gains support from the finding that rod cells also have abnormal focal adhesions. Stress fibers (a complex of actin and myosin II) are thought to attach to focal adhesions, thus providing the force required for anchoring them to the cell matrix. Disrupting the NMHC II-B network decreases the force potential of this myosin II, so the focal adhesions are not properly anchored and are instead distributed as a shell around the cell periphery. The changed distribution of focal adhesions in rod cells may also explain the flat appearance of these cells (Fig. 6). Normal cells in which the focal adhesions are distributed in punctuate form around the cell periphery are not so flat, and more of them are polarized. Our results are consistent with the finding that expression of truncated fragments of NMHC II-A alters cell shape through a rearrangement of actin filaments and disappearance of focal adhesions (13).

Only 11% of the total NMHC II-B was sequestered into aggregates with the rod fragment (Fig. 4), yet this small change caused extreme morphological effects. This finding is consistent with a recent report that the myosin II in the furrow cortex represents only 10% of the total cellular myosin II, and it is this myosin II that produces the force required for cell cleavage (29). Accordingly, this seemingly small reduction in NMHC II-B is
sufficient to disrupt the overall balance of NMHC II-B, leading to the morphological changes observed in rod cells. The changed cell morphology and focal adhesion due to rod fragment expression also altered the ability of the cells to attach to a substratum; rod cells attached faster than control cells (Fig. 8).

Our study thus indicates that an intact and regulated NMHC II-B network is necessary for normal cell morphology, focal adhesion, and cell attachment. Strikingly, the abnormalities described above also affect chemotaxis toward EGF. It appears that a very precisely regulated NMHC II-B is required for a cell to chemotax. The results presented here indicate that myosin II rod fragments serve as a useful general tool for studying myosin II regulation and function.

We propose the following model for the regulation of NMHC II-B in the chemotaxis of TSU-pr1 cells based on our previous (10) and present studies. In unstimulated cells, NMHC II-B forms a shell consisting of a contractile network in the cortex. This network presumably inhibits events necessary for pseudopodial projection. EGF stimulation of TSU-pr1 cells activates protein kinase C, which phosphorylates NMHC II-B, leading to its translocation to the cytoplasm (10). The removal of NMHC II-B from the cell cortex allows the cell to send pseudopodia in the direction of the attractant. This leads to reorientation of the cell and net movement toward EGF. Recruitment of NMHC II-B by the rod fragment may disrupt the NMHC II-B shell in the cell cortex and thus allow random cell extensions. These random cellular movements decrease the efficiency of the cells in polarizing and chemotaxing. These results thus highlight the importance not only of EGF-dependent phosphorylation of NMHC II-B, but also of conserving intact NMHC II-B in the contractile shell.

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