Myosin II heavy chain isoforms are phosphorylated in an EGF-dependent manner: involvement of protein kinase C

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SUMMARY

To explore the involvement and regulation of the nonmuscle myosin II heavy chains isoforms, MHC-A and MHC-B in the chemotaxis of metastatic tumor cells, we analyzed the changes in phosphorylation and cellular localization of these isoforms upon stimulation of prostate tumor cells with epidermal growth factor (EGF). EGF stimulation of prostate tumor cells resulted in transient increases in MHC-A and MHC-B phosphorylation and subcellular localization with quite different kinetics. Furthermore, the kinetics of subcellular localization correlated with the in vivo kinetics of MHC-B phosphorylation but not of MHC-A phosphorylation, suggesting different modes of regulation for these myosin II isoforms. We further showed that protein kinase C (PKC) is involved in the EGF-dependent phosphorylation of MHC-A and MHC-B. To our knowledge, this is the first report demonstrating that MHC phosphorylation might regulate its subcellular localization and that the EGF signal is transmitted to MHC-A and MHC-B via PKC. The correlation between MHC-B phosphorylation and localization in response to EGF stimulation might suggest that MHC-B is the myosin II isoform that is involved in chemotaxis.

Key words: Myosin II, MHC localization, MHC phosphorylation, Protein kinase C

INTRODUCTION

Cell motility and chemotaxis are mediated by changes in the organization and function of actin- and myosin-II-containing cytoskeletal structures (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Myosin II is a hexamer composed of two heavy chains of ~200 kDa and two pairs of light chains of 20 kDa and 17 kDa. In vertebrates, there are at least two nonmuscle myosin II heavy chains (MHC) genes that encode separate isoforms of the heavy chain, MHC-A and MHC-B (Katsuragawa et al., 1989; Kawamoto and Adelstein, 1991). These isoforms are 87% identical at the amino acid level in the head region of the molecule and only 72% identical in the rod (Takahashi et al., 1992). Recent studies have indicated that MHC-A and MHC-B have different enzymatic activity and subcellular localization (Cheng et al., 1992; Kelley et al., 1996; Maupin et al., 1994; Miller et al., 1992; Rochlin et al., 1995). Kelley et al. have shown that the movement of actin filaments by MHC-A was 3.3 times faster than that by MHC-B. Likewise, the V_{max} of the actin-activated Mg^{2+}-ATPase activity of MHC-A was 2.6 times greater than that of MHC-B (Kelley et al., 1996). Immunolocalization of MHC-A and MHC-B in culture cells indicates that the two isoforms have different subcellular distributions (Kelley et al., 1996; Kolega, 1998). MHC-A was absent from the cell periphery and was arranged in a fibrillar pattern in the cytoplasm. MHC-B was present in the cell cortex and diffusely arranged in the cytoplasm. In highly polarized, rapidly migrating cells, the lamellipodium was dramatically enriched for MHC-B, suggesting a possible involvement of MHC-B-based contraction in leading extension and/or retraction (Kelley et al., 1996).

Myosin II light chain and MHC phosphorylation in vivo has been demonstrated in a wide variety of organisms and cell types (Tan et al., 1992). It has been shown in *Dictyostelium* cells that phosphorylation of MHC plays a crucial role in myosin II function by regulating its filament assembly state (Kuczmarski and Spudich, 1980; Pasternak et al., 1989; Ravid and Spudich, 1992; Abu-Elneel et al., 1996; Egelhoff et al., 1993; Kolman et al., 1996). Furthermore, our laboratory has shown that a PKC specific to MHC plays a critical role in the in vivo regulation of myosin II filament formation and is important for proper chemotaxis of *Dictyostelium* cells (Abu-Elneel et al., 1996; Ravid and Spudich, 1992).

Recent cloning, sequencing and biochemistry studies suggest that heavy chains of all non-muscle myosin II have a PKC phosphorylation site within their tail regions (Moussavi et al., 1993). PKC phosphorylates MHC from human platelets and rat basophil leukemic cells both in vivo and in vitro (Kawamoto et al., 1989; Ludowyke et al., 1989). Later, this myosin II was identified as MHC-A and the PKC phosphorylation site is the serine residue at position 1917 (Moussavi et al., 1993). An analogous site has been shown to be present in MHC-B but, in this case, the phosphorylatable residue is a threonine, not a serine, residue (Moussavi et al., 1993; Takahashi et al., 1992). Both sites are located within the rod region of these MHCs. Using C-terminal fragment of

MHC-B, Murakami et al. found that a mixture of several PKC isoforms phosphorylate this MHC-B fragment on serine residues in vitro (Murakami et al., 1998; Murakami et al., 2000; Murakami et al., 1995). They further showed that the phosphorylation of MHC-A and MHC-B by PKC have different effects on the assembly properties of these myosin II isoforms. Phosphorylation of MHC-B by PKC resulted in inhibition of MHC-B assembly in vitro; by contrast, the assembly properties of MHC-A were not affected by PKC phosphorylation (Murakami et al., 1995). These results indicate that assembly of myosin II isoforms can be regulated via phosphorylation of the heavy chain by PKC in an isoform-specific manner, which might cause a rearrangement of myosin II, resulting in cell shape changes.

Cell motility and chemotaxis play a crucial role in physiological as well as in pathological processes, but we know very little about the involvement and the regulation of myosin II in these processes. To begin understanding the role and regulation of myosin II in chemotaxis of mammalian cells, we used prostate metastatic tumor cells (TSU-pr1) (Iizumi et al., 1987). These cells preferentially metastasize to bony sites and lymph nodes at a frequency in excess than would be predicted by random tumor cell dissemination (Saitoh et al., 1984). The prostate tumor cells spread to these sites owing to the presence of epidermal growth factor (EGF) (Rajan et al., 1996). Furthermore, inhibition of EGF receptors (EGFRs) using anti-EGFR monoclonal antibody inhibited the chemomigration of TSU-pr1 cells towards EGF (Zolfaghari and Djakiew, 1996).

In this study, we report that MHC-A and MHC-B in TSU-pr1 cells respond to EGF stimulation by different kinetics of MHC phosphorylation and cellular rearrangement. These results might indicate the involvement of myosin II in chemotaxis of prostate tumor cells towards EGF. Furthermore, we demonstrate that PKC is involved in the phosphorylation of MHC-A and MHC-B in response to EGF stimulation of TSU-pr1 cells.

MATERIALS AND METHODS

Cell line and culture conditions

The cell line used in this study is the prostate carcinoma cell line TSU-pr1 (Iizumi et al., 1987). This cell line was derived from human prostatic adenocarcinoma metastatic to lymph node (kindly provided by A. Passaniti, University of Maryland National Institute of Health, Baltimore). Cells were maintained in RPMI 1640 (Sigma), supplemented with 10% fetal calf serum and antibiotics (100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Gel electrophoresis and western blot analyses

Gel electrophoresis was performed either by the method described by Kelley et al. (Kelley et al., 1996) or using the system of Laemmli (Laemmli, 1970). Cells were washed twice in ice-cold PBS, scraped off the plate and transferred to tubes. Cells were counted and lysed in ice-cold 20 mM Tris (pH 7.5), 0.1% NP40, 1 mM DTT, 10 mM EDTA and a mixture of protease inhibitors (Sigma). Western blots were blocked with 5% milk-TBS and probed with affinity-purified specific polyclonal antibodies against MHC-A and MHC-B (kindly provided by R. S. Adelstein, Laboratory of Molecular Cardiology NIH/NHLBI, Bethesda, MD). Antibodies specific for different PKC isoforms were purchased from Transduction Laboratories and Santa Cruz Biotechnology. The blots were developed using a horseradish peroxidase coupled to a secondary antibody (Jackson Immunoresearch Laboratories). Electrochemiluminescence (ECL) was performed using a kit from Amersham.

In vivo phosphorylation of MHC

3×10^5 TSU-pr1 cells were grown on 60 mm plates for about 14 hours. Cells were washed twice in 2 ml prewarmed RPMI-H (RPMI 1640 medium containing 12 mM HEPES (pH 7.4)) without phosphate. 1 ml of RPMI-H containing 50 μCi ^{32}P orthophosphate was added to each plate and the plates were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 2.5 hours. The plates were washed three times with 1 ml prewarmed RPMI-H without phosphate. 7 ng ml^{-1} EGF or 200 nM TPA or 100 nM calphostin C (Kobayashi et al., 1989) were then added to each plate. The medium was removed at different times after incubation and the cells were lysed in lysis buffer (LB) (60 mM Tris (pH 7.4), 200 mM NaCl, 100 mM sodium pyrophosphate, 200 mM sodium floride, 1 mM sodium vanadate, 20 mM EGTA, 1.5% NP-40 and protease inhibitor mix (Sigma)). The cell extracts were incubated on ice for 10 minutes and spun for 10 minutes at 4°C at 30,000 ***g***. 300 μl H_2O were added to the cell extracts, which were transferred to tubes containing 200 μl of the complex *Staphylococcus* A cells-MHC-A or MHC-B antibodies (prewashed in LB). The mixture was incubated for 2 hours on a rotator at 4°C. The immunoprecipitates were washed four times in LB and analyzed on 7% SDS-PAGE gels. To determine the relative phosphorylation of the immunoprecipitated MHC-A and MHC-B, the gels were scanned with a scanning laser densitometer and the peak areas were evaluated using the Gelscan XL software program, which integrates the area under the peak. The amounts of ^{32}P incorporated into MHC-A and MHC-B were determined using phosphorimaging with a Fujix Bas2000 bioimage analyzer. Relative phosphorylation of MHC-A and MHC-B was determined by dividing the values obtained with the PhosphorImager by the values obtained by scanning of the Coomassie-Blue-stained gels.

Co-immunoprecipitation

For detection of the co-immunoprecipitation of MHC-A and MHC-B with the different PKC isoforms, TSU-pr1 cells were grown, stimulated with EGF for 0 minutes, 1 minutes, 2 minutes, 4 minutes, 6 minutes and 10 minutes, after which the cells were lysed, and MHC-A or MHC-B were immunoprecipitated as described above. The immunoprecipitates were analyzed on western blots using the different PKC isoforms antibodies (as listed below). In the reciprocal experiments, the different PKC isoforms were immunoprecipitated as described above and the immunoprecipitates were analyzed on western blots using the polyclonal antibodies against MHC-A and MHC-B as described above.

Immunomicroscopy

For indirect immunofluorescent staining using MHC-A and MHC-B antibodies, cells were grown to 30% confluence in six-well dishes in which cover slips coated with 27 μg ml^{-1} rat tail collagen I were placed at the bottom of the wells. Cells were washed twice in RPMI-H and cells were incubated at 37°C for 2.5 hours. EGF stimulation was carried out by placing 5 μl of 7 ng ml^{-1} EGF at a prelabeled corner of the cover slip and incubating for 0 minutes, 1 minutes, 2 minutes, 4 minutes, 6 minutes and 10 minutes at 37°C. The cells were fixed in 3.7% paraformaldehyde and incubated at RT for 10 minutes followed by three washes with PBS. Triton solution (0.5% BSA and 0.2% Triton X-100 in PBS) was then added to the cover slips and they were incubated at room temperature for 3 minutes followed by three washes with PBS. Polyclonal antibodies specific for MHC-A and MHC-B were applied at 1:500 dilution and incubated for 45 minutes at 37°C. Cy5-conjugated, affinity-purified goat anti-rabbit IgG (Jackson) at 1:150 dilution was added to each cover slip and incubated for 45 minutes at 37°C. 8 μl of mounting solution (Vector Laboratories) was added to each slide before mounting the cover slips.

The labeled corner of each cover slip was examined using a 40× objective under a Zeiss LSM 410 confocal laser scanning system attached to the Zeiss Axiovert 135 M inverted microscope with 40× Apochromat oil immersion lens (Carl Zeiss, Thornwood, NY). The system was equipped with a 25-mW air-cooled argon laser (488 nm excitation line with 515 nm long pass barrier filter for the excitation of green fluorescence). Red fluorescence was excited with the 633 nm internal helium neon laser. Confocal images were converted to TIF format and transferred to a Zeiss imaging workstation for pseudocolor representation.

Cloning and site-directed mutagenesis of MHC-A and MHC-B tail domains

All DNA manipulations were carried out using standard methods (Sambrook et al., 1989). We used the expression vector pET21 (Novagene), which allows the production of proteins carrying an N-terminal His tag. pET21-MHC-A and pET21-MHC-B were constructed as follows. First, mRNA was isolated from TSU-pr1 cells using an mRNA capture kit (Boehringer Mannheim). The mRNA was used for reverse transcriptase (RT) PCR (Boehringer Mannheim) as described by the supplier. Primers for MHC-A were: 29-mer, upstream sequence containing an *Eco*RI site (underlined) 5′-GGAATTCCATGGACCAGATCAACGCCGAC-3′; 24-mer, downstream sequence containing *Hin*dIII site (underlined), 5′-AAGCTTTTCGGCAGGTTTGGCCTC-3′ corresponding to the human MHC-A (accession no. M31013) sequences 3140-3157 and 3725-3742, respectively. Primers for MHC-B: 29-mer, upstream sequence containing *Eco*RI site (underlined) 5′-GGAATTCCATGAAGTCTAAGTTCAAGGCC-3′; 24-mer, downstream sequence containing *Hin*dIII site (underlined), 5′-CCCAAGCTTCTCTGACTGGGGTGG-3′ corresponding to the human MHC-B (accession no. M69181) sequences 5504-5522 and 5993-6006, respectively. The RT-PCR products (MHC-A 602 bp and MHC-B 502 bp) were subcloned into pGEM kit (Promega) and sequenced. The RT-PCR products were digested with *Eco*RI and *Hin*dIII, isolated, and ligated into *Eco*RI and *Hin*dIII sites in the plasmid pET21.

The MHC-A and MHC-B RT-PCR products containing the previously mapped PKC phosphorylation sites MHC-A Ser^{1917} and MHC-B Thr^{1923} (Moussavi et al., 1993) were replaced with alanine residues (MHC-A S/A and MHC-B T/A, respectively) using site-directed mutagenesis as described (Deng and Nickoloff, 1992). The mutagenesis primers used were: 5′-TTGGCGCTTCAGTCGgCgGATTTCTTGTTCGAG-3′ for MHC-A S/A and 5′-TCGGCGCTCCAGTCTgCgCTTTTCTTGGCCGAC-3′ for MHC-B T/A. The mutagenesis was verified by sequencing. The mutagenized RT-PCR products were subcloned into pET21 as described above.

Bacterial expression and purification of recombinant MHC-A and MHC-B tail domains

Expression and purification of the MHC-A, MHC-B, MHC-A S/A and MHC-B T/A were carried out essentially as described (O'Halloran et al., 1990). Briefly, bacteria containing the expression vectors were grown to an optical density of 0.5, then 2 mM IPTG was added and the cells were grown for an additional 3 hours. The bacteria were pelleted, lysed using sonication and the cell debris removed by centrifugation. These supernatants were boiled and cleared using centrifugation. These supernatants contained the different MHC tail domains with about 90% purity and were used for myosin heavy chain kinase (MHCK) assays.

Partial purification of MHC-A and MHC-B kinases

2×10^6 TUS-pr1 cells were grown in 100 mm Petri dishes for 14-18 hours. Cells were washed twice in RPMI-H and incubated in RPMI-H at 37°C for 2 hours. 7 ng ml^{-1} EGF was added to the cells that were incubated for 1 minute or 4 minute, after which 1 ml lysis buffer (50 mM Tris (pH 7.4), 50 mM NaCl, 5 mM EDTA, 0.2% Triton X-100 and protease inhibitor mix (Sigma)) was added to the plate. The cell extracts were transferred to fresh tubes, incubated on ice for 10 minutes and spun for 15 minutes at 30,000 ***g*** at 4°C. To purify the MHC-A kinase, the supernatants obtained from cells that were stimulated for 1 minute with EGF were loaded on MHC-A tail fragment affinity columns. To purify the MHC-B kinase, the supernatants obtained from cells that were stimulated for 4 minutes with EGF were loaded on MHC-B tail fragment affinity column. The columns were prepared as described (Ravid and Spudich, 1989). After several washes with lysis buffer the kinases were eluted in a step wise manner with a lysis buffer containing 100 mM, 150 mM, 200 mM or 250 mM NaCl. These fractions were assayed for MHC-A and MHC-B kinase activities as described below.

MHC-A and MHC-B kinase assay

4×10^5 TUS-pr1 cells were grown in 60 mm Petri dishes for 14-18 hours. Cells were washed twice in RPMI-H and incubated in RPMI-H at 37°C for 2 hours. 7 ng ml^{-1} EGF or 200 nM TPA or 7 ng ml^{-1} EGF in the presence of 100 nM calphostin C (Kobayashi et al., 1989) were added to the cells, and 400 μl of lysis buffer (50 mM Tris (pH 7.4), 50 mM NaCl, 5 mM EDTA, 0.2% Triton X-100 and protease inhibitor mix (Sigma)) were added to the plate at intervals indicated below. The cell extracts were transferred to fresh tubes, incubated on ice for 10 minutes and spun for 15 minutes at 30,000 ***g*** at 4°C. The supernatants or fractions obtained from the MHC-A and MHC-B tail fragment columns described above were used for MHCK assay described below.

For MHCK assay, 10 μl of the solubilized kinase was incubated with 15 μl of 2× MHCK assay mix (50 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$ and 2 mM DTT, and the expressed MHC tail domain (0.5-1 mg ml^{-1})). The reaction was initiated by adding 5 μl ATP mix (50 mM Tris (pH 7.4), 1.2 mM γ^{32}P-ATP), after which tubes were incubated for 20 minutes at 37°C (the reaction was linear up to 40 minutes). The reactions were stopped by the addition of an equal volume of ice-cold 10% trichloroacetic acid (TCA). To determine the amount of phosphate incorporated into the MHC tail domains, we used one of the following techniques. In the first, the TCA-treated samples were pelleted in a microcentrifuge after incubation for 15 minutes on ice, washed twice with 5% TCA, resuspended in 25 μl SDS-PAGE sample buffer and electrophoresed on 12% SDS-PAGE gels. To determine the amount of ^{32}P incorporated into the MHC tail domains, the gels were stained and destained, the bands were cut out of the gels and counted in a scintillation counter in 5 ml of scintillation fluid. In the second technique, the TCA-treated samples were filtered through nitrocellulose filters, washed three times with 5% TCA and the filters were counted in scintillation counter as described above. Protein concentration was determined using the Bradford assay (Bradford, 1976) to calculate the MHCK specific activity.

RESULTS

TSU-pr1 cells expressed MHC-A and MHC-B isoforms

There are at least two vertebrate nonmuscle myosin II heavy chains (MHC) genes (Katsuragawa et al., 1989; Kawamoto and Adelstein, 1991), which encode separate isoforms of myosin II heavy chain, MHC-A and MHC-B. To study the regulation of myosin II during chemotaxis to EGF in the prostate carcinoma TSU-pr1 cell line, it was first necessary to identify the MHC isoforms expressed by this cell line. We therefore performed the following experiments. First, we used a gel system described by Kelley et al. (Kelley et al., 1996) that allows the separation of MHC-A and MHC-B. Cell extract derived from TSU-pr1 cells was subjected to western blot

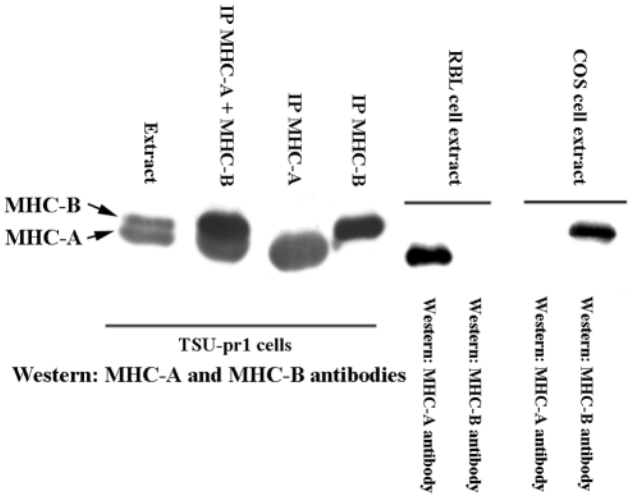

Fig. 1. Analysis of MHC-A and MHC-B expression in TSU-pr1. Immunoblots of TSU-pr1 cell extracts immunoprecipitated with antibodies against either MHC-A or MHC-B, or both, were electrophoresed as described (Kelley et al., 1996). Extracts of RBL and COS cells electrophoresed in 7% SDS-PAGE. The proteins were transferred to nitrocellulose membranes and were probed with an antibody specific to either MHC-A or MHC-B, or with both.

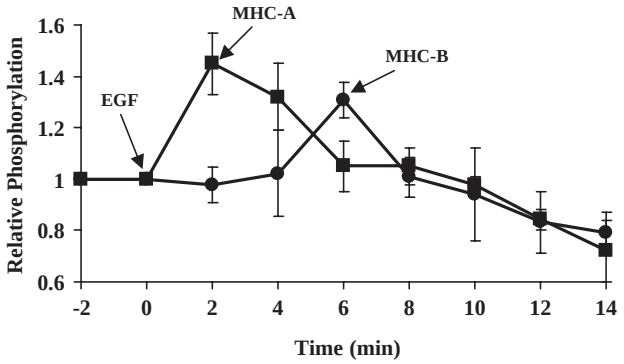

Fig. 2. EGF-induced increases in MHC-A and MHC-B phosphorylation of TSU-pr1 cells. Cells were incubated in the presence of ^{32}P orthophosphate and stimulated with 7 ng ml^{-1} EGF. Aliquots of TSU-pr1 were removed at different times before and after stimulation with EGF and subjected to immunoprecipitation as described in Materials and Methods. The immunoprecipitated MHC-A and MHC-B were analyzed on 7% SDS-PAGE gels and using a PhosphorImager (Fuji). Relative phosphorylation of MHC-A and MHC-B was determined by dividing the values obtained with the PhosphorImager by the values obtained by scanning of the Coomassie-Blue-stained gels, as described in Materials and Methods. The results are the average of three to six experiments.

analysis using a mixture of human MHC-A and MHC-B specific antibodies. This yielded two bands with the expected molecular weight (Fig. 1). To confirm that these two bands correspond to MHC-A and MHC-B isoforms, TSU-pr1 cell extract was immunoprecipitated either with a mixture of MHC-A and MHC-B antibodies or with each of the antibodies separately (Fig. 1). Subjecting TSU-pr1 cell extract to immunoprecipitation using both antibodies yielded two bands similar to the cell extract (Fig. 1). However, using of each of the antibodies separately yielded single bands with the expected molecular weights of MHC-A and MHC-B (Fig. 1). These results indicate that TSU-pr1 cells express both MHC-A and MHC-B isoforms. Furthermore, the isoform-specific antibodies distinguish between the two isoforms. To investigate the specificity of the MHC-A and MHC-B antibodies further, we used RBL-2H3 cells, which produce only MHC-A (Choi et al., 1996) and COS cells that produce only MHC-B (R. S. Adelstein et al., unpublished). As shown in Fig. 1, each of the antibodies recognized a single band in these extracts, further indicating the specificity of the MHC-A and MHC-B antibodies.

Response to EGF: a transient increase in in vivo phosphorylation

It has been shown in lower eukaryotes that, during chemotaxis, myosin II undergoes reorganization that is regulated by MHC phosphorylation (Abu-Elneel et al., 1996; Berlot et al., 1987; Berlot et al., 1985; Egelhoff et al., 1993; Kolman et al., 1996; Nachmias et al., 1989; Soll et al., 1990; Yumura and Fukui, 1985). However, this phenomenon had not been described for mammalian cells. It was therefore of interest to find out whether MHC-A and MHC-B undergo phosphorylation in response to EGF stimulation. For this purpose, TSU-pr1 cells were incubated in the presence of ^{32}P orthophosphate and then stimulated with EGF. MHC-A and MHC-B were immunoprecipitated using MHC-A- and MHC-B-specific antibodies (see Materials and Methods). The phosphorylation levels of MHC-A and MHC-B were analyzed using SDS-PAGE and PhosphorImager (see Materials and Methods). We first determined the EGF concentration that resulted in maximal phosphorylation of MHC-A and MHC-B. This was 7 ng ml^{-1} for both MHC-A and MHC-B (data not shown). We therefore used this concentration to study the EGF-dependent phosphorylation of MHC-A and MHC-B in TSU-pr1 cells.

The EGF stimulation of the TSU-pr1 cells resulted in transient increases of both MHC-A and MHC-B phosphorylation with quite different kinetics and to different extents (Fig. 2). Quantification of the EGF-dependent relative phosphorylation showed that the peak of MHC-A phosphorylation occurred 2 minutes after EGF stimulation, whereas the peak of MHC-B phosphorylation occurred 6 minutes after stimulation. Furthermore, EGF stimulation of TSU-pr1 cells resulted in an ~45% increase in MHC-A phosphorylation and an ~30% increase in MHC-B phosphorylation (Fig. 2). Interestingly, the increase in phosphorylation of both MHC-A and MHC-B was followed by a decrease in their phosphorylation, which might indicate that a MHC phosphatase(s) was activated.

EGF-dependent translocation to the cell cortex

To gain an insight into the EGF-dependent localization of MHC-A and MHC-B in TSU-pr1 cells, and to begin identifying the myosin II isoform involved in TSU-pr1 chemotaxis, we subjected these cells to EGF stimulation, followed at various times after stimulation by indirect immunofluorescence analysis using the MHC-A and MHC-B specific antibodies (Figs 3, 4). In unstimulated TSU-pr1 cells (Fig. 3; 0 minutes), MHC-A was localized mainly in the cytoplasm. By 1 minute after EGF stimulation, most of MHC-A translocated to the cell cortex (Fig. 3). The amount of MHC-A in the cell cortex continued to increase with the time after EGF stimulation (Fig. 3) until, 6 minutes after EGF

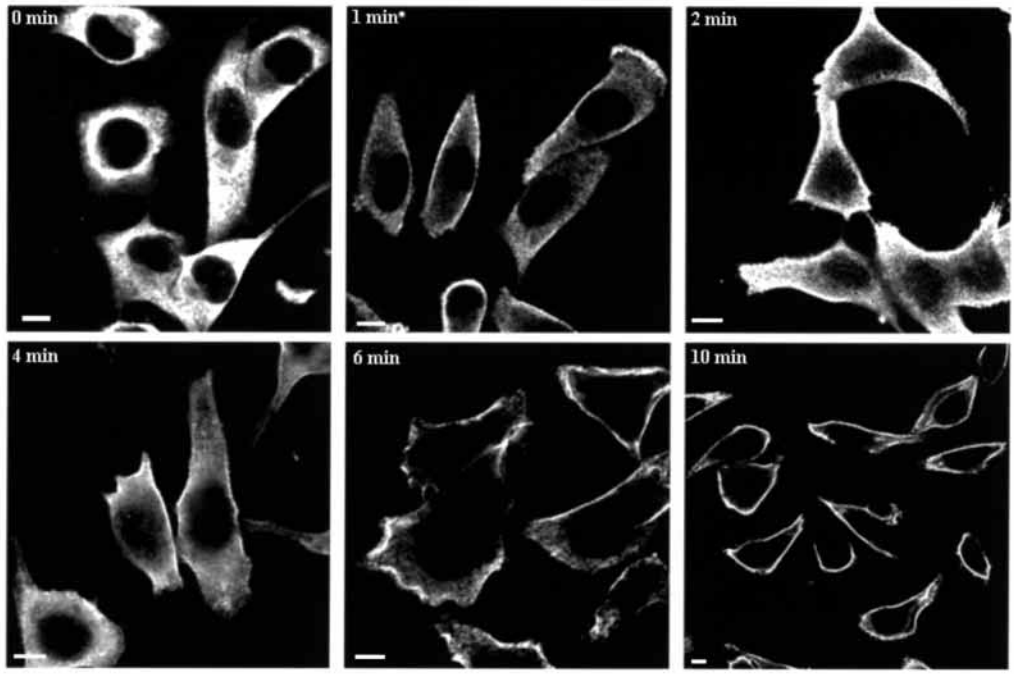

Fig. 3. EGF-dependent MHC-A localization in TSU-pr1 cells. Cells were grown on cover slips coated with collagen I, stimulated with EGF and samples taken at different time points, fixed, and prepared for indirect immunofluorescence using the MHC-A affinity-purified antibodies as described in Materials and Methods. In unstimulated TSU-pr1 cells (0 minutes), MHC-A was localized mainly in the cytoplasm. 1 minute after EGF stimulation, most of the MHC-A translocated to the cell cortex. The amount of MHC-A increased in the cortex with the time of EGF stimulation (2-10 minutes). Bar, 10 μm.

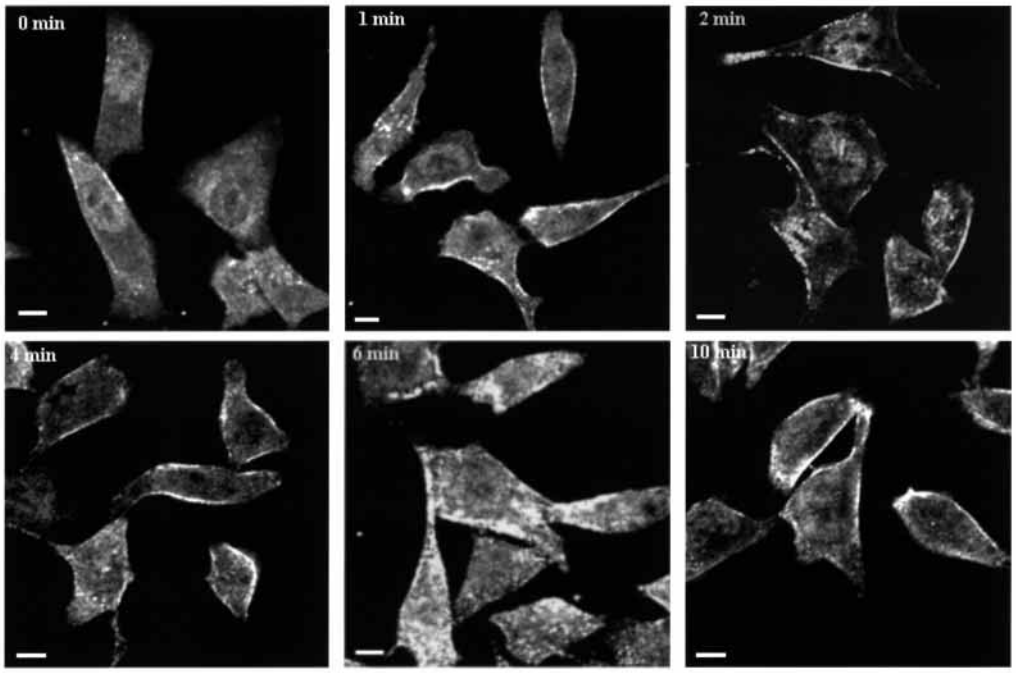

Fig. 4. EGF-dependent MHC-B localization in TSU-pr1 cells. Cells were stimulated with EGF and prepared for indirect immunofluorescence as in Fig. 3. In unstimulated TSU-pr1 cells (0 minutes), MHC-B was localized mainly in the cytoplasm. After EGF stimulation, MHC-B gradually translocated to the cell cortex (1-2 minutes). 4 minutes after EGF stimulation, most of MHC-B localized at the cell cortex. 6 minutes after EGF stimulation, most of MHC-B localized in the cytoplasm. 10 minutes after EGF stimulation, there is another peak of MHC-B localization at the cell cortex. Bar, 10 μm.

stimulation, the amount of MHC-A in the cell cortex approached saturation (Fig. 3).

The kinetics of the EGF-dependent MHC-B translocation to the cell cortex and the patterns of MHC-B staining were very different from those of MHC-A (Figs 3, 4). In unstimulated TSU-pr1 cells, most of the MHC-B was diffusely distributed throughout the cytoplasm (Fig. 4; 0 minutes). Similar results were reported for *Xenopus* cells (Kelley et al., 1996). 1 minute and 2 minutes after EGF stimulation, MHC-B gradually translocated to the cell cortex (Fig. 4). 4 minutes after EGF stimulation, most of MHC-B localized to the cell cortex (Fig. 4). Interestingly, 6 minutes after EGF stimulation, most of the MHC-B returned to the cytoplasm. 10 minutes after EGF stimulation, there is another peak of MHC-B localization to the cell cortex.

These results indicate that the kinetics of the EGF-dependent MHC-A and MHC-B subcellular localization are clearly very differently, possibly suggesting different roles in cell motility and chemotaxis for these myosin II isoforms. Furthermore, the EGF-dependent MHC-B and MHC-A translocation to the cell cortex correlated with the in vivo kinetics of MHC-B phosphorylation but not of MHC-A phosphorylation. The peak of MHC-B localization at the cell cortex (Fig. 4, 4 minutes) took place when MHC-B is almost unphosphorylated (Fig. 2).

By contrast, 6 minutes after EGF stimulation, when MHC-B was at the peak of its phosphorylation (Fig. 2), most of the MHC-B returned to the cytoplasm (Fig. 4). These results might indicate that the EGF-dependent phosphorylation of MHC-B plays a role in the EGF-dependent MHC-B cellular localization. By contrast, there is no correlation between the EGF-dependent phosphorylation and the cellular localization of MHC-A (Figs 2, 3). Once the cells were stimulated with EGF, MHC-A localized to the cell cortex and resided there regardless of the extent of the EGF stimulation (Fig. 3).

Both the different kinetics and extent of EGF-dependent phosphorylation of MHC-A and MHC-B (Fig. 2), and the different kinetics of the EGF-dependent MHC-A and MHC-B subcellular localization (Figs 3, 4) suggest different modes of regulation and different roles for these myosin II isoforms. These results are consistent with recent observation that the filament formation of the 47 kDa C-terminal fragment of MHC-B but not of MHC-A is regulated by phosphorylation (Murakami et al., 2000).

PKC is involved in EGF-dependent phosphorylation

It has been shown that MHC-A and MHC-B are phosphorylated in vivo and in vitro by several kinases, including PKC (Moussavi et al., 1993; Murakami et al., 1998; Murakami et al., 1995). PKC phosphorylates a single serine residue (Ser^{1917}) in MHC-A (Moussavi et al., 1993). Sequence comparison of MHC-A and MHC-B has shown that there is an analogous site in MHC-B, but, in this case, the phosphorylatable residue is a threonine (Thr^{1923}), not a serine (Moussavi et al., 1993; Takahashi et al., 1992). This analysis might suggest that Thr^{1923} is the PKC site on MHC-B (Moussavi et al., 1993). However, Murakami et al., found that a fragment of 47 kDa from the C terminus of MHC-B expressed in *Escherichia coli* can be phosphorylated by a mix of PKC isoforms on serine residues (Murakami et al., 1998; Murakami et al., 1995).

To determine whether the external EGF signal is transmitted to MHC-A and MHC-B via the activation of PKC, we exposed TSU-pr1 cells to the phorbol ester PMA (phorbol myristate) and compared the resulting changes in MHC-A and MHC-B phosphorylation with those caused by EGF stimulation (Fig. 5). Phosphorylation levels of MHC-A and MHC-B were measured 2 minutes and 6 minutes after EGF or PMA stimulation, respectively (see Materials and Methods). Exposure of TSU-pr1 cells to PMA caused increases of MHC-A and MHC-B phosphorylation by 52% and 36% respectively (data not shown). Similar results were obtained for EGF-stimulated TSU-pr1 cells (Figs 2, 5).

To explore the involvement of PKC in the EGF-dependent MHC-A and MHC-B phosphorylation further, we used the PKC-specific inhibitor calphostin C (Kobayashi et al., 1989). TSU-pr1 cells were incubated with ^{32}P in the presence of 100 nM calphostin C and stimulated with EGF. MHC-A and MHC-B were immunoprecipitated 2 minutes and 6 minutes after stimulation, respectively, and analyzed as described in Materials and Methods. Exposure of TSU-pr1 cells to calphostin C caused decreases of MHC-A and MHC-B phosphorylation by 68% and 80%, respectively (Fig. 5). The above results together provide strong evidence that PKC is involved in EGF-dependent MHC-A and MHC-B phosphorylation.

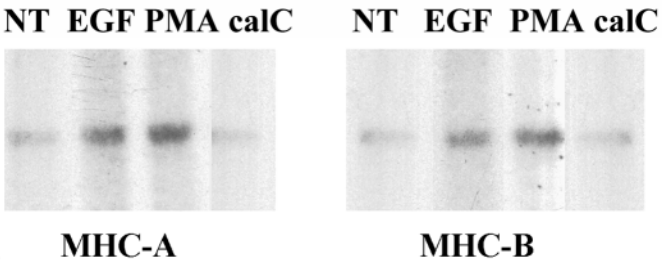

Fig. 5. PMA induced increases in MHC-A and MHC-B phosphorylation of TSU-pr1 cells, whereas calphostin C inhibited this phosphorylation. Images obtained from the PhosphorImager of MHC-A and MHC-B phosphorylation without any treatment (NT) and after the addition of 7 ng ml^{-1} EGF, 200 nM PMA or 7 ng ml^{-1} EGF and 100 nM calphostin C (calC). The experimental conditions and analysis of the phosphorylation levels were as in Fig. 3.

In an attempt to discover whether these PKC(s) had been previously described, we used antibodies against different PKC isoforms (α, βI, βII, γ, ι, ζ, λ, ε, δ, θ) and found that TSU-pr1 cells express PKC isoforms βII, γ, ι, ε and ζ (R.S. and S.R., unpublished). We further found that the addition of ^{32}P-γ-ATP to MHC-A or MHC-B immunoprecipitated from EGF-stimulated TSU-pr1 cells resulted in the phosphorylation of these myosin II isoforms (data not shown). These results indicate that the MHC-A and MHC-B kinase(s) are associated with these myosin II isoforms. To discover whether PKC isoforms βII, γ, ι, ε, ζ are associated with these myosin II isoforms and might therefore be involved in the EGF-dependent phosphorylation, we immunoprecipitated MHC-A and MHC-B and analyzed them on a western blot using antibodies for the above PKC isoforms. The reciprocal experiment was also performed as described in Materials and Methods. None of the PKC isoforms that are produced by TSU-pr1 co-imunoprecipitated with MHC-A or MHC-B (R.S. and S.R., unpublished). Seemingly, these results indicate that MHC-A and MHC-B are not phosphorylated directly by PKC. However, because the antibodies against other known PKCs were not available to us, it is still possible that MHC-A and/or MHC-B are phosphorylated by PKC.

EGF and PMA cause a transient increase in MHC-A and MHC-B kinase activities

The results presented so far suggest that the EGF signal is transmitted to MHC-A and MHC-B by the activation of PKC (Fig. 5). Several studies have also shown that extracellular stimulation of cells increases PKC activity (Mochly-Rosen et al., 1990; Nakamura and Nishizuka, 1994). Therefore, we next tested whether EGF and PMA stimulation of TSU-pr1 cells increased MHC-A and MHC-B kinase activities, and the addition of calphostin C inhibited these activities. For this purpose, we expressed tail domains of MHC-A and MHC-B in *E. coli* that served as substrates for kinase assays. These tail domains contain the MHC-A Ser^{1917} PKC phosphorylation site, the MHC-B Thr^{1923} putative PKC phosphorylation site and the peptides to which the specific antibodies of MHC-A and MHC-B were made. The expressed proteins were purified by a simple technique based on boiling the *E. coli* cell extracts and clearing the denatured proteins (see Materials and Methods). Analysis of the supernatant of the boiled cell extracts indicated that the MHC-A and MHC-B were >90%

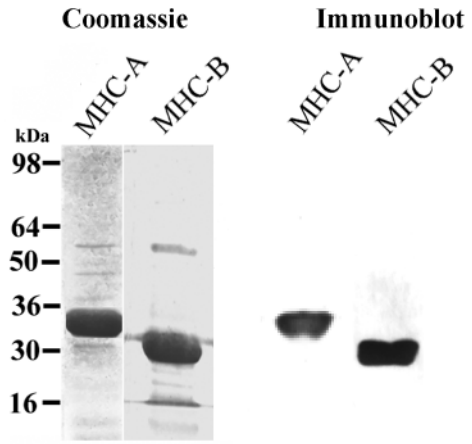

Fig. 6. SDS-PAGE and western blot analysis of the expressed MHC-A and MHC-B tail domains. Purified MHC-A or MHC-B were prepared as described in Materials and Methods. Samples were subjected to SDS-PAGE on 12% gels and blotted on nitrocellulose. The immunoblots were stained with the MHC-A and MHC-B specific antibodies.

pure (Fig. 6). The expressed proteins were also analyzed by western blotting with the MHC-A- and MHC-B-specific antibodies, and it was found that the expressed proteins are the MHC-A and MHC-B tail fragments (Fig. 6).

To study the MHC-A and MHC-B kinase activities, TSU-pr1 cells were stimulated with EGF, PMA or EGF in the presence of calphostin C, and the cells were lysed and subjected to MHCK assay at different times after incubation (see Materials and Methods). The MHC-A or MHC-B tail domains were used as substrates (Fig. 6). Addition of EGF or PMA to TSU-pr1 cells resulted in a transient increase in MHC-A kinase activity with a peak at 1 minute after stimulation (Fig. 7). By contrast, stimulation of TSU-pr1 cells with EGF in the presence of calphostin C abolished the MHC-A kinase activity (Fig. 7). TSU-pr1 cells stimulated with either EGF or PMA and tested for MHC-B kinase activity showed a transient increase in MHC-B kinase with a peak at ~4 minutes (Fig. 7); addition of calphostin C abolished this activity (Fig. 7). These results together with those in Fig. 5 strongly indicate that the EGF signal is transmitted to MHC-A and MHC-B via PKC. The peak of activity of the MHC-A and MHC-B kinase(s) coincided with the localization of MHC-A and MHC-B at the cell cortex, but it preceded the peak of maximum EGF-dependent in vivo phosphorylation of MHC-A and MHC-B. A plausible explanation of these findings is that EGF stimulation of TSU-pr1 cells leads to translocation and activation of MHC-A and MHC-B kinases to the cell cortex, as well as to translocation of MHC-A and MHC-B, leading to phosphorylation of these myosin II isoforms.

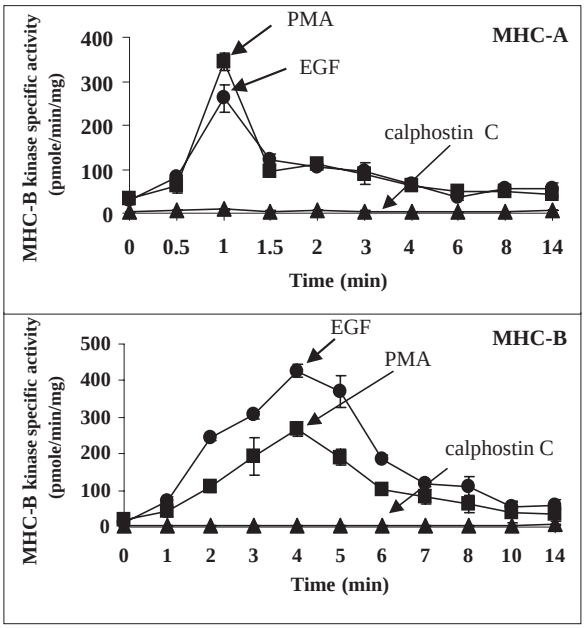

Fig. 7. The effects of EGF, PMA and calphostin C on the activities of MHC-A and MHC-B kinase(s). Specific activity of MHC-A and MHC-B kinases, as determined by lysing TSU-pr1 cells treated with EGF, PMA or EGF and calphostin C, and subjecting them to MHCK assay as described in Materials and Methods. Error bars, ±s.e.m.; *n*=3-6.

Replacement of the MHC-A and MHC-B PKC phosphorylation sites with alanine

To determine whether the EGF-activated MHC-A and MHC-B kinase(s) are members of the PKC family, we produced in *E. coli* MHC-A and MHC-B tail domains in which the previously reported PKC phosphorylation site MHC-A Ser[1917] and the putative PKC phosphorylation site MHC-B Thr[1923] (Moussavi et al., 1993) were replaced with alanine residues (MHC-A S/A and MHC-B T/A, respectively). To test whether MHC-A S/A and MHC-B T/A were effective substrates for the EGF-activated MHC-A and MHC-B kinases, we stimulated TSU-pr1 cells with EGF and subjected them to MHCK assay at different times after incubation using the above mutated MHC tail domains (see Materials and Methods). As shown in Fig. 8, in contrast to MHC-A and MHC-B tail domains, the mutated MHC-A S/A and MHC-B T/A tail domains were

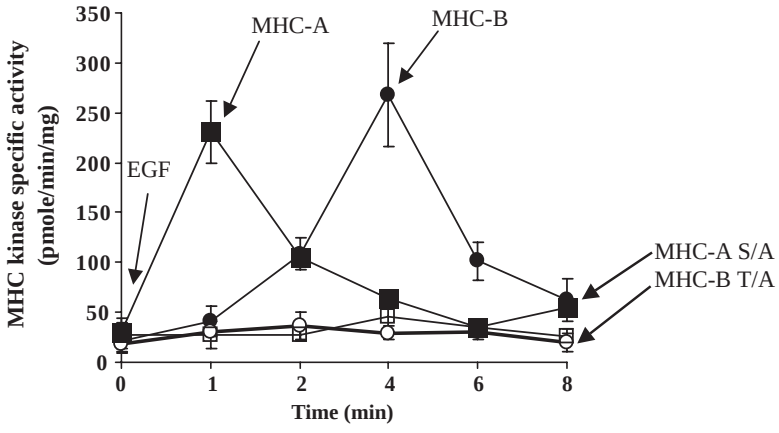

Fig. 8. Replacement of the MHC-A and MHC-B PKC phosphorylation sites with alanine residues prevented their phosphorylation by the EGF-activated MHC-A and MHC-B kinase(s). Specific activity of MHC-A and MHC-B kinases, as determined by lysing TSU-pr1 cells treated with EGF and subjecting them to MHCK assay using MHC-A, MHC-B, MHC-A S/A and MHC-B T/A tail domains as described in Materials and Methods. Error bars, ±s.e.m.; *n*=3.

inefficient substrates for EGF-activated MHCKs. Using the MHC-A and MHC-B tail domains as substrates in the EGF-activated MHCK assay provided similar results to those shown in Fig. 7. However, using MHC-A S/A and MHC-B T/A tail domains as substrates in this assay resulted only in a basal level of activity. These observations indicate that the EGF-activated MHC-A and MHC-B kinases phosphorylate the previously mapped PKC site on MHC-A or the putative PKC site on MHC-B, and further suggest that these kinases are members of the PKC family. In addition, these results indicate that, in response to EGF stimulation of TSU-pr1 cells, there is an activation of PKC(s) that is involved in MHC-A and MHC-B phosphorylation.

As mentioned above, Murakami et al. reported that the C-terminal 47 kDa fragment of brain-type MHC-B can be phosphorylated on serine residues by a mix of PKC isoforms (Murakami et al., 1998; Murakami et al., 2000). These serine residues are within the MHC-B tail domain described here. These observations are inconsistent with the results described above, in which a threonine residue is phosphorylated by cell extract obtained from EGF-stimulated TSU-pr1. A plausible explanation for this discrepancy is that the basal level of phosphorylation of MHC-B T/A (Fig. 7) represents phosphorylation on serine residues reported earlier (Murakami et al., 1998). However, EGF stimulation of TSU-pr1 cells results in the activation of a PKC that phosphorylates Thr^{1923} on MHC-B. Therefore, replacing this threonine with alanine abolished the EGF-dependent increases in MHC-B T/A phosphorylation but, nevertheless, the basal level of phosphorylation of this protein is similar to that of MHC-B (Fig. 7). To explore this possibility, we performed EGF-dependent in vivo phosphorylation of MHC-B but, instead of using ^{32}P to detect the phosphorylated form of MHC-B, we used anti-phosphothreonine antibody. We found that EGF stimulation of TSU-pr1 cells resulted in transient increases in MHC-B phosphorylation on threonine residue(s) (A. Ben-Ya'acov and S.R., unpublished). These results strongly suggest that the EGF-dependent increases in MHC-B phosphorylation occurred on threonine residue(s).

EGF-dependent in vivo phosphorylation is carried out by two different kinases

To characterize further the kinase(s) that phosphorylate MHC-A and MHC-B in response to EGF stimulation, we partially purified these kinases using MHC-A and MHC-B tail fragment affinity columns (columns A and B, respectively) as described in Materials and Methods. To investigate the elution profile of MHC-A and MHC-B kinase(s) from the columns, the fractions obtained from these columns were subjected to MHCK assay as described in Materials and Methods. As shown in Fig. 9, the maximum activity of the kinase purified on column A (MHC-A kinase) and assayed using MHC-A tail fragment as a substrate was eluted with 100 mM NaCl (Fig. 9; 'A-A'). However, the maximum activity of the kinase purified on column B (MHC-B kinase) and assayed using MHC-B tail fragment as a substrate was eluted with 200 mM NaCl (Fig. 9; 'B-B'). The different elution profiles of MHC-A and MHC-B kinases might indicate that they are different kinases. To explore this possibility further, we tested whether the MHC-A kinase is competent to phosphorylate the MHC-B tail fragment (Fig. 9; 'A-B') and whether MHC-B kinase is competent to phosphorylate MHC-A tail fragment (Fig. 9; 'B-A'). As shown in Fig. 9, MHC-A kinase did not phosphorylate the MHC-B tail fragment (Fig. 9; 'A-B') but, by contrast, MHC-B kinase did phosphorylate the MHC-A and MHC-B tail fragments to the same extent (Fig. 9; 'B-A' and 'B-B'). These results further indicate that MHC-A and MHC-B kinases are different. Furthermore, it is plausible that the EGF-dependent MHC-A and MHC-B phosphorylation is carried out by two different kinases, which might explain the different phosphorylation kinetics of these two myosin II isoforms.

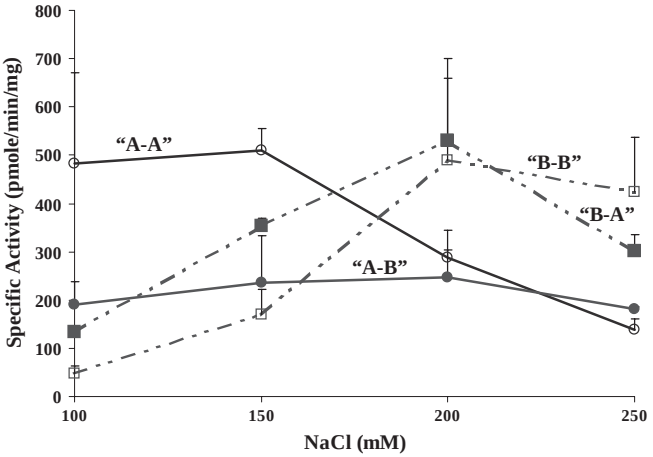

Fig. 9. Specific activity of MHC-A and MHC-B kinases(s) eluted from MHC-A and MHC-B tail domains affinity columns, respectively. Extracts from EGF-stimulated TSU-pr1 cells were loaded on MHC-A or MHC-B tail domain affinity columns. The columns were eluted in a stepwise manner with 100-250 mM NaCl, and the specific activity of MHC-A and MHC-B kinases was determined as described in Materials and Methods. (A-A), MHC-A kinase assayed with MHC-A tail fragment; (A-B), MHC-A kinase assayed with MHC-B tail fragment; (B-B), MHC-B kinase assayed with MHC-B tail fragment; (B-A), MHC-B kinase assayed with MHC-A tail fragment. Error bars, s.e.m.; *n*=6.

DISCUSSION

Cell migration is crucial for embryonic development, the inflammatory immune response, wound repair and tumor formation and metastasis (Lauffenburger and Horwitz, 1996). Cell movement can be modulated by signals from the extracellular environment but the intracellular signal transduction pathways that lead to this biological response are not fully understood.

EGF-induced cell motility requires the presence of a phosphotyrosine motif in the intracellular regulatory region of the EGFR (Chen et al., 1994). This suggests that the immediate downstream effector molecule in the motogenic pathway is activated by SH2-domain interactions. Numerous SH2-domain-containing effector molecules interact with, and are activated by, EGFR (Carpenter, 1992), and at least three of these pathways can be linked to cell motility. Activation of a small GTP-binding protein of the Rho subfamily leads to the formation of filopodia, lamellipodia and focal adhesions, which are required for cell motility and chemotaxis (Burridge,

1999; Horwitz and Parsons, 1999; Van-Aelst and D'Souza-Schorey, 1997). A second pathway involves the kinase phosphatidylinositol 3′ kinase, whose activation is required for chemotaxis mediated by the PDGFβ receptor (Kundra et al., 1994; Wennstrom et al., 1994). A third signaling pathway, which involves PLCγ, might also promote cell motility. PLCγ hydrolysis of PIP_2 releases actin-severing and -sequestering proteins, which lead to the dissolution of stress fibers and focal adhesions, enabling a cell to move (Banno et al., 1992; Goldschmit-Clermont et al., 1991; Stossel, 1993). In addition, activation of PLCγ is required for chemotaxis (Kundra et al., 1994; Wennstrom et al., 1994) or is associated with PDGFβ-receptor-mediated chemotaxis (Bornfeldt et al., 1994). Chen et al. have shown that enhanced inositol phosphate production was observed only in cell lines demonstrating EGFR-mediated cell movement (Chen et al., 1994). This correlation between the biochemical and biological responses suggested that PLCγ was the immediate downstream effector. To identify PLCγ definitively as a necessary intermediary, Chen et al. downregulated this enzyme activity using antisense oligonucleotides (Chen et al., 1994). This treatment partially abrogated both PLCγ activity and EGF-induced motility. These experiments indicate that PLCγ is required for EGFR-mediated movement and place PLCγ directly downstream of the EGFR.

PLCγ activation by EGFR produces diacylglycerol (DAG) and inositol trisphosphate, which activate members of the PKC family (Margolis et al., 1990). This is a large family of at least 12 isoforms differing in their structure, tissue distribution, subcellular localization, mode of activation and substrate specificity (Dekker and Parker, 1994). PKCs phosphorylate a wide variety of substrates including proteins involved in signal transduction (including Ras, GAP and Raf) (Hug and Sarre, 1993), as well as motility-associated cytoskeletal modulators (including Fak, profilin and MARCKS) (Aderem, 1992; Hansson et al., 1988). In addition, a unique PKC phosphorylates MHC from *Dictyostelium* and plays an important role in chemotaxis of this organism (Abu-Elneel et al., 1996; Ravid and Spudich, 1992). All these studies provide a strong evidence for the involvement of PKC isoforms in mediating and regulating chemotaxis and cell motility. Although much is known about the pathway that begins with the EGF receptor and continues through PLCγ and PKC, we know very little of how EGF mediates cell motility. Our results provide the first indication of the mechanism linking the EGF receptor to cell motility. Furthermore, we found that of the two vertebrate myosin II isoforms, MHC-B is the one that is most likely to play a role in chemotaxis; EGF stimulation of TSU-pr1 cells resulted in transient subcellular organization of MHC-B but not of MHC-A. Such a transient response is expected from a cytoskeletal protein that is involved in chemotaxis towards EGF.

Our results are consistent with several recent reports indicating that myosin II plays a role in such cellular processes. Moores et al. have found that green fluorescent protein-myosin II concentration increases in the tips of retracting pseudopodia (Moores et al., 1996). This suggests that myosin II might play an important role in the dynamics of pseudopodia as well as filopodia, lamellipodia and other cellular protrusions. Kelley et al. reported that the lamellipodium in highly polarized, rapidly migrating cells was dramatically enriched for MHC-B (Kelley et al., 1996), suggesting a possible involvement of MHC-B-based contraction in leading extension and/or retraction.

The time course of EGF-mediated MHC-B phosphorylation subcellular localization fits that of EGF-mediated lamellipodium extension. Recently, Segall et al. reported that the addition of EGF to mammary adenocarcinoma cell line MTLn3 cells stopped ruffling and resulted in extension of hyaline lamellipodia containing increased amounts of F-actin at the growing edge. Lamellipodium extension was maximal within 5 minutes, followed by retraction and resumption of ruffling (Segall et al., 1996). Interestingly, the peak of MHC-B associated with the cell cortex occurs at 4 minutes after EGF stimulation (Fig. 4), at that time point MHC-B is almost unphosphorylated (Fig. 2). However, at the peak of MHC-B phosphorylation (Fig. 2, 6 minutes), most of the MHC-B translocated back to the cytoplasm (Fig. 4). It is therefore, plausible that MHC phosphorylation leads to MHC-B filament dissociation such that, when the MHC-B is not phosphorylated, it forms filaments that localized to the cell cortex. By contrast, phosphorylation of MHC-B at the cell cortex possibly by PKC leads to filament dissociation that translocate to the cytoplasm. Phosphorylation of MHC-B might help to destabilize the MHC-B filaments. Such a function is also suggested by the localization of the site of phosphorylation in MHC-B, which is in the α-helical portion of the rod just N terminal to the non-helical tail (Moussavi et al., 1993). This part of MHC-B molecule has been implicated in filament formation (Hodge et al., 1992). Thus, the role of MHC-B phosphorylation is to cause a major cellular rearrangement of this myosin II that is required for MHC-B to function as a motor protein. The MHC-B cellular rearrangement might result in a change in cell shape and, perhaps, in the case of TSU-pr1 cells, in chemotaxis towards EGF. Recently, it has been shown that the single *Drosophila* MHC can be phosphorylated by PKC (Su and Kiehart, 2001). However, similar to mammalian MHC-A this phosphorylation has no effect on filament assembly (Murakami et al., 1998; Su and Kiehart, 2001). These results further indicate that phosphorylation of MHC-B, for which effects on assembly have been observed in vitro, might be more important for different function in the organism that expresses them.

EGF and PMA stimulation of TSU-pr1 cells increased MHC-A and MHC-B kinase activities. These findings, together with the finding that calphostin C inhibited these kinase activities, provide strong evidence that these kinases belong to the PKC family. Further support for this notion was provided by the findings that replacing the previously mapped PKC phosphorylation site of MHC-A and the putative PKC site of MHC-B with alanine residues resulted in tail fragment that cannot be phosphorylated by EGF-stimulated TSU-pr1 cell extract. Furthermore, we found that the increase in the in vivo EGF-dependent MHC-B phosphorylation occurs on threonine residue(s) and not serine residues (A. Ben-Ya'acov and S.R., unpublished), as was previously reported (Murakami et al., 1998). It is therefore, possible that the putative PKC phosphorylation site on MHC-B (Thr^{1923}) is indeed a PKC site.

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