CMLS, Cell. Mol. Life Sci. 62 (2005) 461–470
1420-682X/05/040461-10
DOI 10.1007/s00018-004-4456-6

CMLS Cellular and Molecular Life Sciences

Research Article

Cofilin activity during insulin-like growth factor I-stimulated neuroblastoma cell motility

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Received 18 October 2004; received after revision 3 December 2004; accepted 16 December 2004

Abstract. Insulin-like growth factor I (IGF-I) is a potent stimulator of neuroblastoma cell motility. Cell motility requires lamellipodium extension at the leading edge of the cell through organized actin polymerization, and IGF-I stimulates lamellipodial elaboration in human neuroblastoma cells. Rac is a Rho GTPase that stimulates lamellipodial formation via the regulation of actin polymerization. In this study, we show that IGF-I-stimulated phosphatidylinositol 3-kinase (PI-3K) activity promotes rac activation and subsequent activation of the downstream effectors LIM kinase and cofilin. Overexpression of wild-type LIM kinase and wild-type *Xenopus* ADF/cofilin (XAC) suppresses IGF-I-stimulated motility in SH-SY5Y cells, while expression of dominant negative LIM kinase and constitutively active XAC increases SH-SY5Y motility in the absence of IGF-I stimulation. These results suggest that regulation by cofilin of actin depolymerization is important in the process of neuroblastoma cell motility, and IGF-I regulates cofilin activity in part through PI-3K, rac, and LIM kinase.

Key words. LIMK; rac; cytoskeleton; tumor; PAK.

Motility is the process by which cells translocate through tissue spaces and is dependent upon organized actin polymerization at the leading edge of the moving cell. This actin polymerization forms the architecture for a membrane process called the lamellipodium, which pushes the cell margin forward in the direction of migration [1]. Actin-based lamellipodial extension is a key component of motility in numerous cell types, including neurons and neuronal processes that migrate during development, pathogen-tracking leukocytes, and metastatic cancer cells. Extracellular cues that activate cell surface receptors, including peptide growth factors [2] are capable of modulating the motility of many cells, in part by affecting actin polymerization and lamellipodial extension.

Growth factor-mediated regulation of cellular function plays an important part in the development of the tumorigenic and metastatic properties of neuroblastoma (NBL) cancer cells. Insulin-like growth factor I (IGF-I) is highly expressed in bone [3, 4], the primary site of NBL metastasis [5], and is a mitogenic and anti-apoptotic factor for human SH-SY5Y and SHEP NBL cells [6–13]. IGF-I also stimulates actin-rich lamellipodial formation, dynamic changes in morphology, and motility in SH-SY5Y and SHEP cells [14–16]. IGF-I serves as a motility stimulant for other cancers, including rhabdomyosarcoma [17], melanoma [18], and breast cancer [19, 20]. Characterizing the signaling mechanisms activated by IGF-I that lead to changes in actin architecture and cell motility is important for an overall understanding of the process that leads to metastasis in NBL and other forms of cancer.

A key regulator of the actin meshwork, composed of filamentous F-actin, in lamellipodia is the small GTPase rac. Rac, when GTP bound, stimulates the formation of lamellipodia via actin polymerization [21, 22]. Hydrolysis of bound GTP leads to rac inactivation. Rac activity is

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implicated in the signaling processes of several growth factors, including platelet-derived growth factor [23, 24], nerve growth factor [25], and IGF-I [26, 27]. Phosphatidylinositol 3-kinase (PI-3K) activation by growth factors serves as a link to rac activation, as rac may directly bind the phospholipid product of PI-3K (phosphatidylinositol 3,4,5-phosphate, PIP_3) [28]. Proteins called guanine nucleotide exchange factors (GEFs) stimulate rac to bind GTP and may be activated by PI-3K signaling. For example, one rac-GEF known as vav is activated by PIP_3 [29]. As PI-3K signaling is required for IGF-I-mediated motility in NBL cells [16], rac activation may be controlled by IGF-I and lead to actin reorganization and lamellipodium extension.

One route by which rac modifies the actin cytoskeleton in fibroblasts is through regulation of the LIM kinase-actin depolymerizing factor (ADF)/cofilin system. GTP-bound rac promotes LIM kinase (LIMK) activation by p21-activated kinase-mediated phosphorylation of its threonine 508 [30, 31]. Exogenous expression of LIMK in fibroblasts leads to accumulation of actin filaments in aggregates [32], likely regulated by ADF/cofilin. LIMK phosphorylates and inactivates ADF/cofilin [33], two proteins with similar but somewhat distinct activities [34]. ADF/cofilin, when not phosphorylated on serine 3, binds to ADP-actin in actin filaments and increases the rate of actin depolymerization from the pointed ends of the filaments. ADF/cofilin activity is crucial for the in vivo rate of actin treadmilling observed within the lamellipodial actin meshwork. This treadmilling occurs by releasing monomers from the pointed end that are recharged with ATP and added to the actively growing barbed ends at the leading edge of the meshwork [35]. IGF-I activation of rac downstream from PI-3K could regulate LIMK and cofilin activity, thus altering actin polymerization in motile NBL cells.

As IGF-I is a potent stimulator of lamellipodial elaboration and NBL motility [16], IGF-I likely activates signaling proteins that modify actin architecture in the lamellipodium. Our hypothesis is that IGF-I stimulates NBL cell motility through regulation of the PI-3K-rac-LIMK-cofilin pathway. IGF-I, via PI-3K, could stimulate rac activity, which in turn could activate LIMK and cause cofilin phosphorylation, affecting the dynamics of actin treadmilling. This study examines the ability of IGF-I to activate rac and LIMK downstream of PI-3K, leading ultimately to cofilin phosphorylation. The effects of LIMK and cofilin on IGF-I mediated motility in NBL are also tested. Regulation of LIMK and cofilin activity by IGF-I is a novel component of the mechanism leading to increased NBL motility and metastasis.

Materials and methods

Cell culture and transfections

Human SH-SY5Y and SHEP NBL cells were cultured in DMEM with 10% calf serum and maintained in a humidified incubator with 10% CO_2 at 37 °C. SH-SY5Y cells were transiently transfected with pMHneo containing V12 Rac or N17 Rac (gift of Dr. L. Petruzzelli, University of Michigan) using lipofectamine 2000 reagent (Invitrogen, Carlsbad, Calif.). SH-SY5Y cells were also stably transfected with pcDNA3.1, or pcDNA3.1 containing wild-type or dominant negative (D460N) LIMK (gift of Dr. G. Gill, University of California San Diego). Stable transfectants were made using lipofectamine 2000 reagent, selected in 500 µg/ml G418, and maintained in 250 µg/ml G418. Adenovirus containing GFP, GFP-linked *Xenopus* ADF/cofilin (XAC), GFP-A3 XAC, or GFP E3 XAC (gifts of Dr. J. Bamburg, Colorado State University) was used to infect cells. GFP-A3 XAC has the serine at position 3 replaced by alanine, and cannot be phosphorylated by LIMK. It is, thus, continuously active. GFP-E3 XAC contains a glutamate at the phosphorylation site, which resembles a phosphorylated serine and is thus inactive. Cells were exposed to 1000 MOI of virus in a minimal volume of serum-free DMEM for 1 h, then returned to serum-containing medium and used in experiments the following day. Expression of all constructs introduced, either through transfection or infection, was confirmed by either fluorescent observation of GFP or by Western immunoblotting.

Measurement of actin polymerization

SH-SY5Y cells were cultured in six-well dishes until 80% confluent, serum starved for 6 h, and then treated with 1 nM IGF-I for 0–30 min. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with 2 units/ml Texas Red-conjugated phalloidin (Molecular Probes, Eugene, Ore.). Cells were rinsed three times with PBS to remove unbound Texas Red phalloidin. Texas Red phalloidin fluorescence in each well was measured using an Ascent Fluoroskan fluorimeter (Thermo LabSystems, Franklin, Mass.). Time points were run in triplicate, and each experiment repeated three times.

Rac activation assays

Rac activity was measured using a kit from Cytoskeleton (Denver, Col.) according to the manufacturer's protocol. SH-SY5Y cells were serum starved for 6 h, then treated with or without 1 nM IGF-I for 0–30 min. In other experiments, cells were pre-treated with 10 µM LY294002 (LY; Sigma, St. Louis, Mo.) or 10 µM PD98059 (PD; Sigma) for 1 h, then stimulated with or without 1 nM IGF-I for 20 min. As both LY and PD are dissolved in DMSO, a DMSO treatment control was included. Lysates were im-

mediately collected in lysis buffer and incubated at 4 °C for 1 h with a GST-conjugated peptide corresponding to the rac-binding domain of PAK (PBD). Activated (GTP-bound) rac in the lysates binds the GST-PBD fusion protein. Lysates were incubated for an additional hour with glutathione-conjugated agarose beads. The beads were pelleted and washed, boiled in SDS sample buffer, and bound proteins separated using SDS-PAGE. The amount of rac pulled out of the lysates was detected by immunoblotting with anti-rac antibody (Cytoskeleton). Experiments were repeated in triplicate.

Measurement of cofilin phosphorylation

SH-SY5Y cells were serum starved and treated with or without 10 µM LY294002 or 10 µM PD98059 for 1 h. In other experiments, SH-SY5Y cells were transfected with rac mutants or LIMK mutants and serum starved. Cells were then treated with or without 10 nM IGF-I for 0–20 min, and lysates were collected in salt-free RIPA buffer (20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% Triton-X 100) with inhibitors as described above. Lysate samples containing equal amounts of protein were mixed with 2× isoelectric focusing sample buffer, pH 3–7 (Invitrogen), and separated on a pre-cast pH 3–7 isoelectric focusing gel (Invitrogen). Proteins were transferred to nitrocellulose and immunoblotted with anti-cofilin antibodies (Cytoskeleton and Cell Signaling Technologies). Isoelectric focusing was chosen to separate phosphorylated cofilin from unphosphorylated cofilin, as the two have different pIs. Unlike SDS-PAGE, more negatively charged (phosphorylated) proteins appear slightly lower on the gel than the unphosphorylated, positively charged proteins. Pixel density was measured on films exposed to the immunoblotted nitrocellulose using Scion Image (Scion Corporation, Frederick, Md.). Quantitative results are expressed, after subtracting out the background pixel density, as a ratio of phosphorylated cofilin to total cofilin expression.

Immunofluorescence

Immunocytochemistry was performed as described previously [14].

Morphology of cells expressing LIMK or XAC constructs

SH-SY5Y and SHEP cells expressing LIMK constructs or XAC constructs were plated on glass coverslips, allowed to adhere for 1 day, then serum starved and treated with 10 nM IGF-I for 15 min. The cells were then fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X 100, and stained with 2 units/ml Texas Red-conjugated phalloidin (Molecular Probes). Cells were imaged for GFP staining (in the case of XAC constructs) and Texas Red phalloidin staining using a Zeiss Axiophot inverted microscope.

Phagokinetic track assays

Cells were plated on gold particle-coated coverslips [prepared as described in ref. 36] at a density of 25,000 cells per coverslip. The cells were incubated for 2 h to allow adhesion to the coverslip. Wells were treated with or without 1 nM IGF-I, previously shown to maximally stimulate NBL cell motility [16]. Incubation continued for 6 h, followed by fixation with 3.5% glutaraldehyde. Coverslips were mounted on glass slides and viewed on a Lietz Orthoplan inverted microscope attached to a Sony videoprocessor. Digital images of the tracks etched into the gold by the cells from three separately treated coverslips per condition were collected at ×200 magnification using Adobe Photoshop 4.0 software. For each condition, the areas of 120 tracks made by individual cells were measured using NIH Image 1.61 software.

Results

IGF-I increases actin polymerization and rac activation in SH-SY5Y cells

IGF-I stimulates actin-rich lamellipodium formation in SH-SY5Y cells [15, 16]. To quantify actin polymerization in response to IGF-I, SH-SY5Y cells were serum starved and stimulated with 1 nM IGF-I, and fixed at time points up to 30 min. The cells were stained with Texas Red-conjugated phalloidin, which binds to polymerized, filamentous F-actin. The amount of fluorescence incorporated at each time point was measured in a fluorimeter. IGF-I stimulated increased Texas Red-phalloidin incorporation, which rapidly increased at 5 min, steadily increased to a peak at 20 min, then dropped to control levels at 30 min (fig. 1). Our previous results show that IGF-I stimulates lamellipodial elaboration in serum-starved SH-SY5Y cells over this same time course, and these lamellipodium contain an intense band of phalloidin-stained F-actin at the leading edge [16, 26]. Together, these results suggest that IGF-I induces a localized increase in actin polymerization that leads to lamellipodial elaboration in SH-SY5Y cells.

Rac is a small GTPase of the rho family of GTPases, and is known to organize lamellipodia via actin polymerization when in the GTP-bound state [21, 22]. To determine if rac is activated by IGF-I, SH-SY5Y cells were serum starved and stimulated with 1 nM IGF-I for 0–30 min. Rac activity was measured in these cells using a GST pull-down assay as described in Materials and methods. IGF-I increased rac activation to a peak of 2.9-fold higher than the control at 10 min, which remained 2.3-fold higher than the control at 15 min, followed by a return to baseline by 30 min (fig. 2A). This occurred at a time just prior to maximal IGF-I-stimulated increases in lamellipodial elaboration [16] and actin polymerization

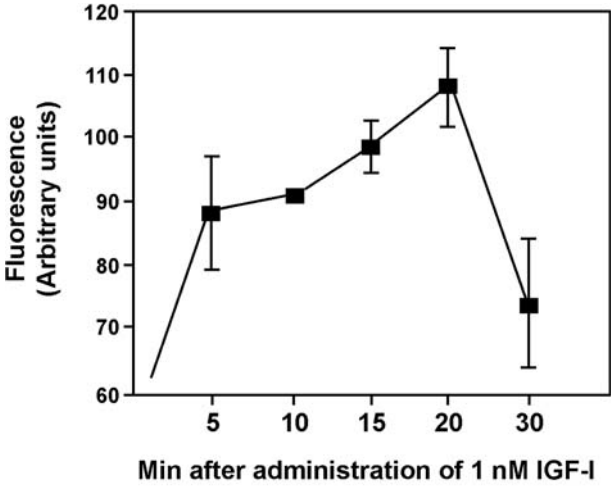

Figure 1. IGF-I causes increased actin polymerization in SH-SY5Y cells. Serum-starved SH-SY5Y cells were treated with 1 nM IGF-I at the indicated time points, fixed with 4% paraformaldehyde, and stained with Texas Red-phalloidin. F-actin content of the cells was measured by detecting phalloidin fluorescence with an Ascent Fluorskan fluorimeter. Graph shows the compiled data from three separate experiments. Error bars are SE.

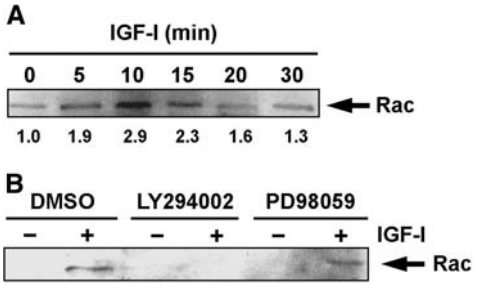

Figure 2. IGF-I activates rac downstream of PI-3K. (*A*) Serum-starved SH-SY5Y cells were treated with 1 nM IGF-I for various time points. Lysates were collected and incubated with the GST-PBD, which binds activated rac. GST-PBD was bound to agarose beads, pelleted, and co-precipitating proteins were separated by SDS-PAGE. Rac was detected with anti-rac antibody. Pixel density was measured using Scion Image, background subtracted, and value set relative to control. Each pixel density is listed underneath the corresponding band. (*B*) Serum-starved SH-SY5Y cells were treated with 10 µM LY294002, an inhibitor of PI-3K signaling, or 10 µM PD98059, an inhibitor of MAP signaling for 1 h, then incubated with or without 1 nM IGF-I for 15 min. Activated rac was detected as described above. LY blocks IGF-I-mediated rac activation. Representative immunoblots from three separate experiments are shown.

(fig. 1), suggesting that rac-mediated signaling could be responsible for these events.

We have previously shown that IGF-I-mediated lamellipodial elaboration and motility are largely dependent upon PI-3K activation [15, 16]. To determine if rac activation is also dependent upon PI-3K in SH-SY5Y cells, serum-starved cells were treated for 1 h with 10 µM LY294002, an inhibitor of PI-3K, or 10 µM PD98059, an inhibitor of the MAPK signaling pathway, followed by IGF-I stimulation for 15 min. Rac activation by IGF-I was abolished only when the PI-3K pathway was blocked (fig. 2B). Thus, IGF-I-mediated rac activation is dependent upon PI-3K signaling, not MAPK, in SH-SY5Y cells and is a potential mechanism for organizing IGF-I-mediated lamellipodial actin.

Rac and the p85 regulatory subunit of PI-3K localized to regions of intense F-actin staining at the leading edge of lamellipodia following stimulation with IGF-I (fig. 3). Rac staining was most intense along the leading edge of the lamellipodium up to 15 min after treatment with IGF-I (fig. 3). F-actin, rac, and p85 remained localized to the leading edge at 30 min (fig. 3).

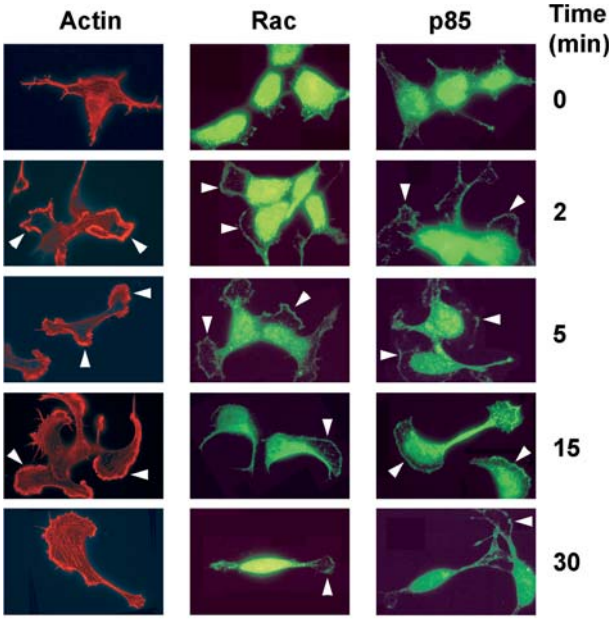

Figure 3. PI-3K and rac localize to the membrane-ruffling region. Cells were serum starved, then treated with IGF-I for 0, 2, 5, 15, and 30 min. The cells were then fixed in 4% paraformaldehyde, and stained using Texas Red-phalloidin to visualize F-actin, or immunostained for the p85 subunit of PI-3K or Rac. Both the p85 subunit of PI-3K and rac showed strong staining at the tip of the lamellipodia where membrane ruffling is most active. The arrowheads indicate the ruffling region.

IGF-I causes cofilin phosphorylation via PI-3K and rac

LIMK affects actin organization by phosphorylating and inactivating cofilin on serine 3 [33]. Cofilin, when active, severs actin filaments and facilitates actin depolymerization from the pointed ends of the filaments [35, 37–39]. Isoelectric focusing can separate the phosphorylated (inactive) form of cofilin from the unphosphorylated (active) form, and was used to determine if IGF-I leads to an increases in cofilin phosphorylation in SH-SY5Y cells. IGF-I increased cofilin phosphorylation twofold over a 20-min period (fig. 4A). The lower, phosphorylated cofilin band was lost when the samples were treated with alkaline phosphatase prior to isoelectric focusing separa-

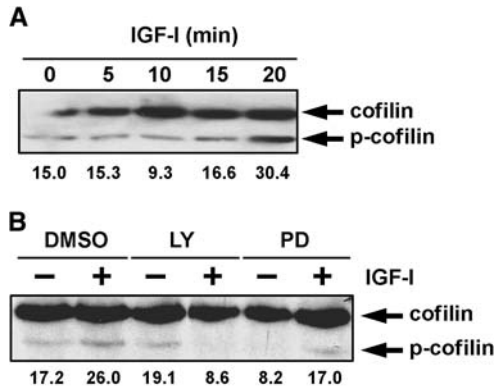

Figure 4. IGF-I causes cofilin phosphorylation downstream of PI-3K. (*A*) Serum-starved SH-SY5Y cells were treated with 1 nM IGF-I for the indicated time periods. Lysates were collected and separated by isoelectric focusing, to separate cofilin from phosphorylated cofilin. Cofilin was detected using anti-cofilin antibody. (*B*) Serum-starved SH-SY5Y cells were treated with DMSO, 10 µM LY294002, or 10 µM PD98059 for 1 h, then stimulated with or without IGF-I. Lysates were collected and phospho-cofilin detected as described above. LY prevents IGF-I-mediated cofilin phosphorylation. Compared to controls, LY-treated cells show a decrease in cofilin phosphorylation when stimulated with IGF-I, suggesting an IGF-I mechanism for cofilin dephosphorylation. Representative immunoblots from three separate experiments are shown.

tion (data not shown). Inhibition of PI-3K with LY294002 blocked IGF-I-mediated phosphorylation of cofilin; cofilin phosphorylation in the LY294002 + IGF-I-treated samples was 55% lower than in IGF-I-untreated controls (fig. 4B). PD98059 treatment lowered both unstimulated baseline and IGF-I-stimulated cofilin phosphorylation by 52% and 35%, respectively. Therefore, the MAPK pathway likely regulates baseline cofilin phosphorylation, while the PI-3K pathway regulates IGF-I-mediated phosphorylation.

Expression of constitutively active V12 rac (fig. 5) increased cofilin phosphorylation. IGF-I-stimulated cofilin phosphorylation was blocked by expression of dominant negative N17 rac (fig. 5). These results show that IGF-I-stimulates cofilin phosphorylation by activating PI-3K and rac, and suggest that one part of the mechanism of

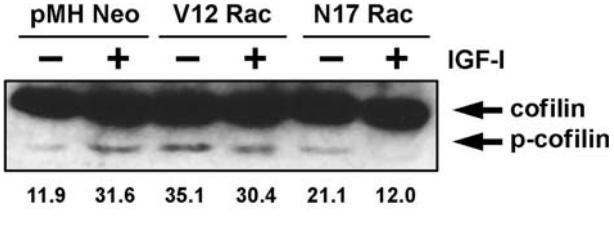

Figure 5. Rac activity is required for IGF-I-stimulated cofilin phosphorylation. SH-SY5Y cells were transiently transfected with vector, active rac (V12), or dominant negative rac (N17), serum starved, and treated with 10 nM IGF-I for 0 or 20 min. Lysates were collected and proteins separated by isoelectric focusing. N17 rac prevents IGF-I-stimulated cofilin phosphorylation.

IGF-I-stimulated lamellipodial elaboration may involve controlling the actin-depolymerizing activity of cofilin.

Motility of cells expressing LIMK and cofilin constructs

Lamellipodial elaboration is required for cell motility. As LIMK and cofilin activity regulate actin polymerization, and actin polymerization underlies lamellipodial elaboration, normal LIMK and cofilin activity may be required for the process of cell motility. The motility of SH-SY5Y and SHEP NBL cells is increased by IGF-I [16], and IGF-I caused cofilin phosphorylation (figs 4, 5). In NIH 3T3 cells, expression of wild-type and constitutively active LIMK causes actin to aggregate. This effect is due to inactivation of ADF/cofilin, which facilitates depolymerization of actin from the pointed end of filaments [32, 35, 37]. We therefore evaluated the morphology of SH-SY5Y cells expressing wild-type and dominant negative LIMK. Our results (fig. 6A) show there is little effect of these mutants on lamellipodial extension following IGF-I administration.

To examine the role of LIMK in NBL cell motility, SH-SY5Y NBL cells expressing LIMK constructs were plated on coverslips coated with a fine layer of gold particles (fig. 6B). The cells adhere to the coverslips in serum-free medium for 2 h, and are then stimulated with 1 nM IGF-I and incubated for 6 h, according to our previously published results [16]. The phagocytic tracks made by moving cells during the incubation are photographed and measured. SH-SY5Y cells overexpressing wild-type LIMK showed markedly inhibited IGF-I-stimulated motility (fig. 6B). In contrast, dominant negative D460N LIMK dramatically increased the motility of unstimulated cells, to levels comparable to treatment with IGF-I (fig. 6B).

Actin morphology was also observed in NBL cells using wild-type XAC, which has a similar function to cofilin in mammalian cells [40], A3-XAC, which cannot be phosphorylated and thus resembles a continuously active cofilin, and E3-XAC, which mimics phosphorylation and acts as an inactive cofilin. Serum-starved cells expressing GFP alone show numerous stress fibers, and some lamellipodia. Addition of 10 nM IGF-I for 30 min increased the elaboration of actin-based lamellipodia and localization of XAC to the lamellipodium (fig. 7A). Addition of 10 nM IGF-I for 30 min increased lamellipodial formation, and led to more actin accumulation in the lamellipodia. IGF-I treatment also caused XAC localization to the lamellipodia. E3-XAC caused accumulation of actin in serum-starved cells (fig. 7B), and this accumulation did not extend into the lamellipodium, but was rather just internal to the lamellipodium. Interestingly, E3 cofilin was also localized to the lamellipodium by exposure to IGF-I, despite its decreased affinity for actin filaments. Therefore, modulation of normal cofilin activity disrupts IGF-I-mediated lamellipodial extension.

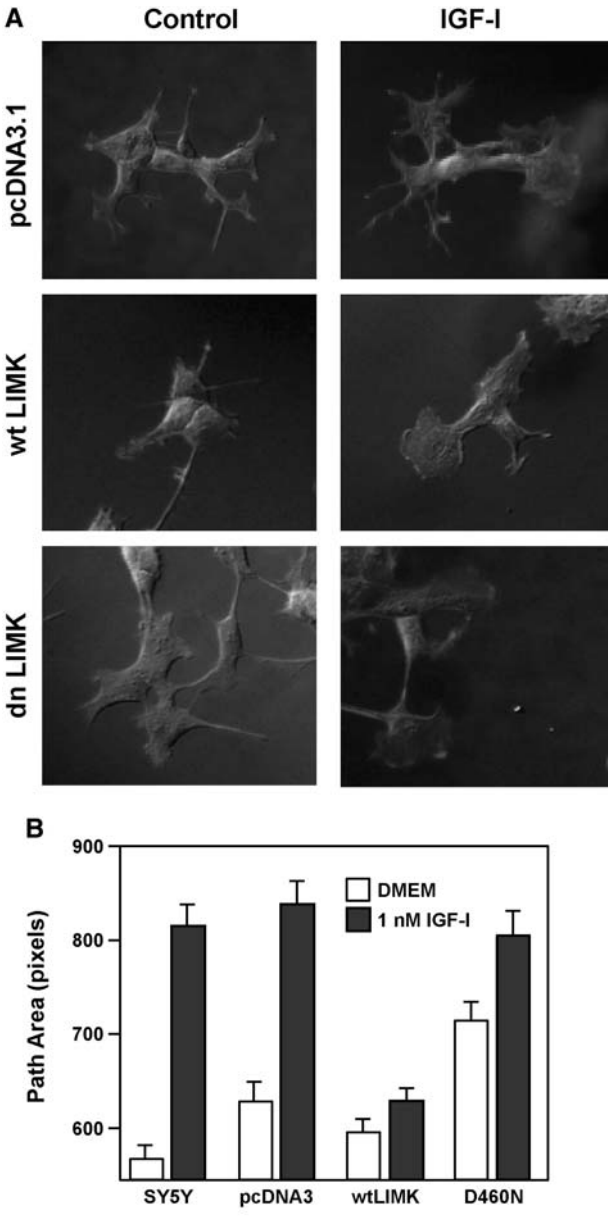

Figure 6. LIMK inhibits IGF-I-mediated motility. *(A)* SH-SY5Y cells stably transfected with pcDNA3.1, wild-type (wt) LIMK, or dominant negative (dn) LIMK were grown on glass coverslips, serum starved, and treated with 1 nM IGF-I for 0 or 15 min before being fixed with 4% paraformaldehyde and stained with Texas red-phalloidin to label F-actin. DIC and fluorescent images were taken and overlayed. IGF-I stimulated the formation of large lamellipodia regardless of LIMK expression. (*B*) Stably transfected SH-SY5Y cells were plated on coverslips coated with gold particles. Motility was measured by photographing and measuring the areas of tracks left in the gold 6 h after stimulation with 1 nM IGF-I. Dominant negative LIMK (D460N) increases cell motility in the absence of stimulation. Wild-type LIMK (wtLIMK) blocks IGF-I-stimulated motility. Bars represent the averaged areas from 150 cells from three experiments with SE.

SH-SY5Y cells expressing wild-type XAC also showed markedly decreased motility in both serum-starved and IGF-I-stimulated conditions (fig. 7C). Expression of A3-XAC, which cannot be phosphorylated and inactivated and is therefore constitutively active, increased motility in unstimulated cells, similar to the effect of inactive LIMK (fig. 7C). In contrast, inactive E3-XAC had a moderately inhibitory effect on motility in both unstimulated and IGF-I-stimulated cells (fig. 7C). Therefore, cofilin activation is necessary for maximal IGF-mediated motility.

Collectively, these results show that cells maintain the ability to elaborate lamellipodia when LIMK mutants and wild-type XAC are expressed, suggesting that perturbing the normal balance of cofilin phosphorylation by expression of mutant constructs is insufficient to completely alter the ability of the cell to elaborate lamellipodia, or that the normal functioning of LIMK and cofilin is not completely required for lamellipodial elaboration. In contrast, increasing the potential for cofilin inactivation through increased phosphorylation, either by LIMK overexpression or cofilin overexpression, is inhibitory to motility. Alternatively, decreasing cofilin phosphorylation by either inactivating LIMK or expressing a non-phosphorylatable XAC increases cell motility in the absence of exogenous stimulators (IGF-I). Therefore, both LIMK and cofilin play important regulatory roles in IGF-I-mediated motility in NBL.

Discussion

Organized actin polymerization is the driving event behind lamellipodial formation at the leading edge of motile cells [41]. IGF-I causes lamellipodial formation [15] and increased motility [16] in SH-SY5Y and SHEP NBL cells, potentially contributing to their ability to metastasize and invade tissues. We have previously reported increases in dynamic membrane extension caused by IGF-I in NBL cells within 20 min [16]. Also, in the current report, actin polymerization peaks after 20 min of IGF-I exposure (fig. 1). Therefore, IGF-I must quickly activate signaling mechanisms that lead to actin-driven lamellipodial extension, potentially affecting long-term changes in cell motility that increase the metastatic properties of NBL cells.

A well-characterized regulator of actin-driven lamellipodial extension is the rho GTPase rac. Rac, in its active GTP-bound state, causes actin polymerization and lamellipodial elaboration in fibroblasts [21, 22]. Rac is a component of the intracellular signaling pathways of a number of extracellular cues that stimulate lamellipodial formation, including nerve growth factor [25], platelet-derived growth factor [23, 24], semaphorin 3D [42, 43], acetylcholine [44], and laminin [45]. Given the

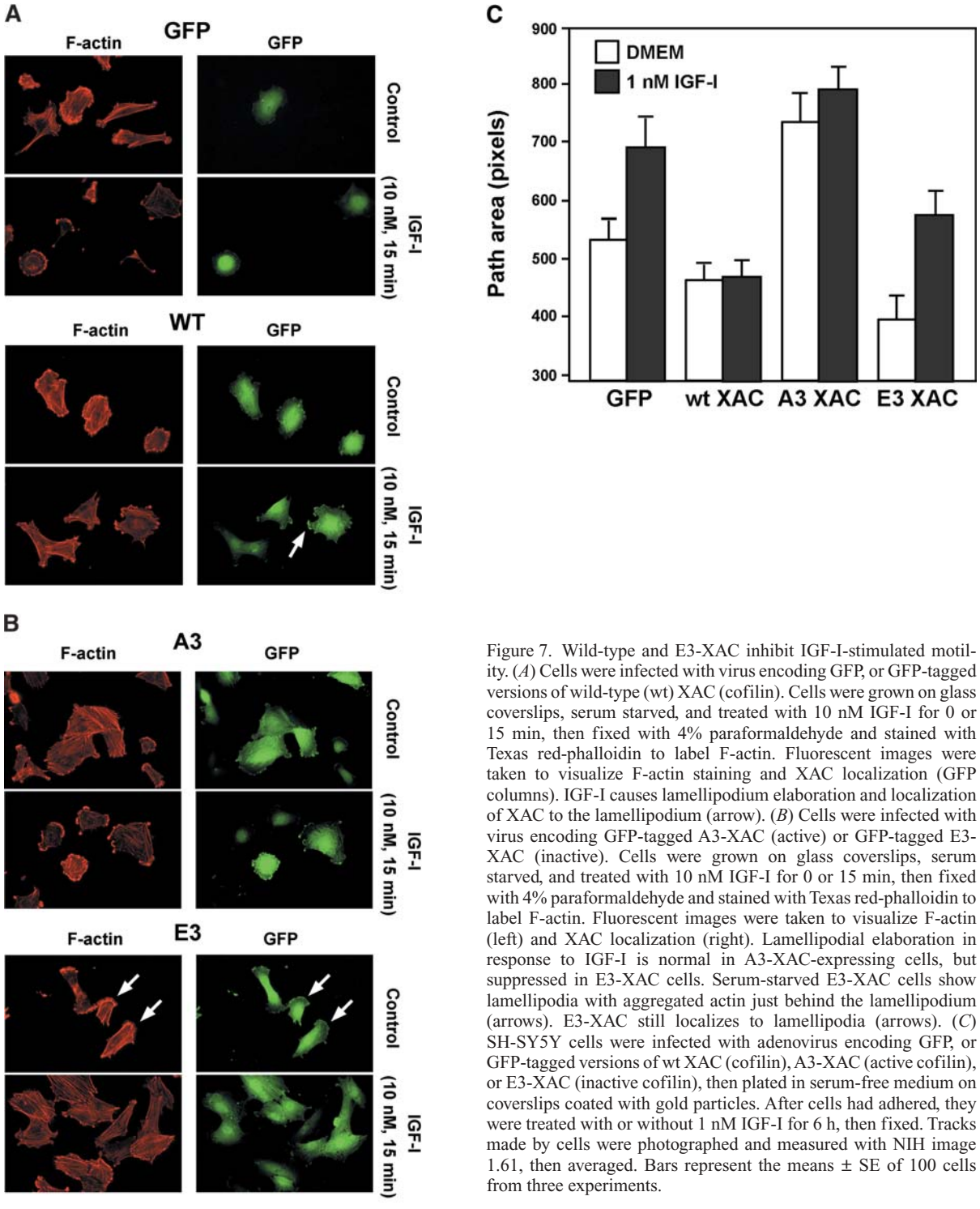

Figure 7. Wild-type and E3-XAC inhibit IGF-I-stimulated motility. (*A*) Cells were infected with virus encoding GFP, or GFP-tagged versions of wild-type (wt) XAC (cofilin). Cells were grown on glass coverslips, serum starved, and treated with 10 nM IGF-I for 0 or 15 min, then fixed with 4% paraformaldehyde and stained with Texas red-phalloidin to label F-actin. Fluorescent images were taken to visualize F-actin staining and XAC localization (GFP columns). IGF-I causes lamellipodium elaboration and localization of XAC to the lamellipodium (arrow). (*B*) Cells were infected with virus encoding GFP-tagged A3-XAC (active) or GFP-tagged E3-XAC (inactive). Cells were grown on glass coverslips, serum starved, and treated with 10 nM IGF-I for 0 or 15 min, then fixed with 4% paraformaldehyde and stained with Texas red-phalloidin to label F-actin. Fluorescent images were taken to visualize F-actin (left) and XAC localization (right). Lamellipodial elaboration in response to IGF-I is normal in A3-XAC-expressing cells, but suppressed in E3-XAC cells. Serum-starved E3-XAC cells show lamellipodia with aggregated actin just behind the lamellipodium (arrows). E3-XAC still localizes to lamellipodia (arrows). (*C*) SH-SY5Y cells were infected with adenovirus encoding GFP, or GFP-tagged versions of wt XAC (cofilin), A3-XAC (active cofilin), or E3-XAC (inactive cofilin), then plated in serum-free medium on coverslips coated with gold particles. After cells had adhered, they were treated with or without 1 nM IGF-I for 6 h, then fixed. Tracks made by cells were photographed and measured with NIH image 1.61, then averaged. Bars represent the means ± SE of 100 cells from three experiments.

effect of IGF-I on actin polymerization and lamellipodial elaboration in SY-SY5Y cells, rac is a plausible activator of these events downstream of IGF-I. IGF-I activates rac in SH-SY5Y cells in a time course consistent with the observed changes in actin polymerization and cell morphology (fig. 2A). Ideally, expression of dominant-negative (N17) rac could be used to block the IGF-I-stimulated changes in actin polymerization and cell morphology to further test the involvement of rac in these events. However, expression of N17 rac in unstimulated SH-SY5Y cells causes such severe changes in the actin cytoskeleton (the cells become rounded with no extensions; data not shown) that one could not say how IGF-I-mediated changes in the cytoskeleton or motility are affected when

signaling is blocked at the level of rac. Nonetheless, since IGF-I is a potent activator of rac, and expression of active rac in SH-SY5Y (data not shown and Cheng et al. [26]) and other cell types causes increased actin polymerization and lamellipodial elaboration, rac activation by IGF-I is plausibly at least partly involved in IGF-I-stimulated lamellipodial extension.

PI-3K is an effector of IGF-I signaling that is important in SH-SY5Y lamellipodial extension and motility [15, 16]. The lipid second messenger product of PI-3K, PIP_3, binds rac and causes rac activation [28]. We showed that rac activation by IGF-I is dependent upon PI-3K signaling (fig. 2B). The p85 subunit of PI-3K and rac also localize to the leading edge of IGF-I-induced lamellipodia, where the most intense F-actin staining is found (fig. 3). Rac activation is also facilitated by GEFs, many of which are under the control of extracellular signals [41]. Potentially, GEF activity may also be affected by PI-3K signaling, although little is known about the expression and activity of GEFs in NBL cells.

Rac activates several groups of proteins with distinct functions that directly modify actin polymerization dynamics and architecture. One such series of effectors includes Scar/WAVE and the Arp2/3 complex, which nucleate the formation of new actin branches that polymerize toward the leading edge and push the leading edge membrane forward [41]. Another activity important for lamellipodial actin dynamics downstream of rac is the regulation of pointed-end depolymerization by LIMK and cofilin. Cofilin binds to ADP-actin monomers in F-actin and causes filaments to depolymerize from the pointed end. Cofilin also severs actin filaments. Both of these activities may be critical for the rapid rate of actin treadmilling observed in vivo. In one model, cofilin-mediated increases in the rate of monomer release from the pointed end leads to reincorporation of these monomers at the actively growing barbed end at the leading edge of the actin meshwork [35, 37]. Another model proposes that severed actin filaments from aging parts of the meshwork are caught by Arp2/3 at the leading edge and thus promote forward extension of the meshwork [39]. LIMK regulates cofilin activity by an inactivating phosphorylation at serine 3. LIMK is activated by phosphorylation downstream of rac and its target kinase, PAK [31]. Increased LIMK expression in fibroblasts leads to accumulation of actin filaments in aggregates, as cofilin is no longer actively depolymerizing filaments [32]. Furthermore, LIMK1 expression increases, and subsequently modulates cofilin phosphorylation, within the growth cones of primary hippocampal neurons extending neurites, a process which requires reorganization of actin at the leading edge [46]. The depolymerizing activity of cofilin is thus regulated downstream of rac via activation of LIMK and may play a role in IGF-I-mediated actin polymerization and lamellipodial extension. We show in this report that cofilin phosphorylation is increased by IGF-I, dependent upon PI-3K and rac activity (figs 4, 5). As mentioned, cofilin activity is important for maintaining the rapid rate of actin treadmilling of the lamellipodium. Why, then, is the increased inactivation of cofilin by IGF-I part of a mechanism for lamellipodial extension? One possibility is that IGF-I is involved in actin turnover through regulation of cofilin phosphorylation. Figures 4 and 5 suggest this scenario may occur. To address this question further, we began investigating the effects of cofilin and LIMK activation on IGF-I-mediated actin morphology and motility in SH-SY5Y NBL cells. Increased LIMK expression in other cell types causes actin aggregation [32], presumably because cofilin becomes more inactive and does not facilitate actin turnover as well. We find that LIMK expression does not cause any detectable changes in short-term lamellipodial elaboration in response to IGF-I. We also did not observe any changes in the dynamics of membrane ruffling in time-lapse video microscopy (data not shown). IGF-I does cause GFP-linked XAC to localize to lamellipodia. Interestingly, cells expressing E3-XAC show thickened bands of actin around the cell periphery that lie just inside many of the extended lamellipodia. XAC localization was most intense within the lamellipodia, and not in the region of thickened actin. These results suggest a role for cofilin in depolymerizing the older, more internal parts of the lamellipodial actin meshwork. When inactive cofilin is increased (hypothetically in the experiments by increasing the expression of E3, which mimics phospho-cofilin), actin builds up in the more internal parts of the lamellipodium. Lamellipodial extension obviously continued in the time course we examined (30 min), suggesting that if actin monomer recycling is perturbed by the experimental conditions, it is not sufficient to completely block lamellipodial extension. This idea is supported by a recent publication reporting that the requirements for G-actin in non-motile lamellipodium are quite low, whereas more G-actin is required for lamellipodium in motile cells [47]. At the time points observed in our experiments, the cells have not begun to translocate [16]. This same group also reported that LIMK and cofilin do not affect G-actin enough to inhibit the lamellipodium in non-motile cells, but can affect lamellipodia in moving cells. Thus, expression of LIMK and cofilin mutants may not affect cell morphology in the short term, but could still affect long-term changes in cell motility.

While the short-term experiments examining actin morphology did not show a strong effect on lamellipodial elaboration, LIMK and cofilin activity do appear to contribute to the overall process of cell motility. Increasing cofilin activity by expressing dominant negative LIMK or non-phosphorylatable XAC causes increased cell motility in serum-starved SH-SY5Y cells, comparable to vector-transfected cells treated with IGF-I (figs 6, 7). In-

creased expression of wild-type LIMK, which increases cofilin phosphorylation, or increased expression of wild-type XAC and E3-XAC decrease cell motility and markedly diminish the ability of IGF-I to induce more cell motility. The inhibition of motility by wild-type LIMK or E3-XAC overexpression is probably due to increased deactivation of cofilin. Zebda et al. [48] have found that overexpression of LIMK inhibits the epidermal growth factor-stimulated extension of lamellipodia in MTLn3 cells. Why overexpression of wild-type XAC is inhibitory to motility is less clear, but it may be the result of compensatory increases in cofilin phosphorylation by LIMK. The monomer recycling activity of cofilin does appear to be involved in the motility process [35], potentially by increasing the availability of actin monomers for incorporation into actively growing branches at the leading edge. In addition, a recent study, performed in prostate carcinoma cells containing a chemically engineered form of cofilin that mimics phosphorylated cofilin when photoactivated, suggests that cofilin activity also polymerizes actin and induces lamellipodial protrusion and directed motility, although the exact mechanism is unknown [49].

Figure 8 summarizes the model for IGF-I regulation of cofilin activity in NBL cells. IGF-I activates PI-3K, which either directly stimulates rac to become GTP bound, or works through the control of GEFs. Rac in turn stimulates PAK-mediated LIMK activation, which increases cofilin phosphorylation. PI-3K also signals to an uncharacterized cofilin phosphatase mechanism. Thus, IGF-I may increase the turnover of cofilin phosphorylation, and this contributes to lamellipodial actin dynamics by controlling the location and rate of pointed-end depolymerization and filament severing.

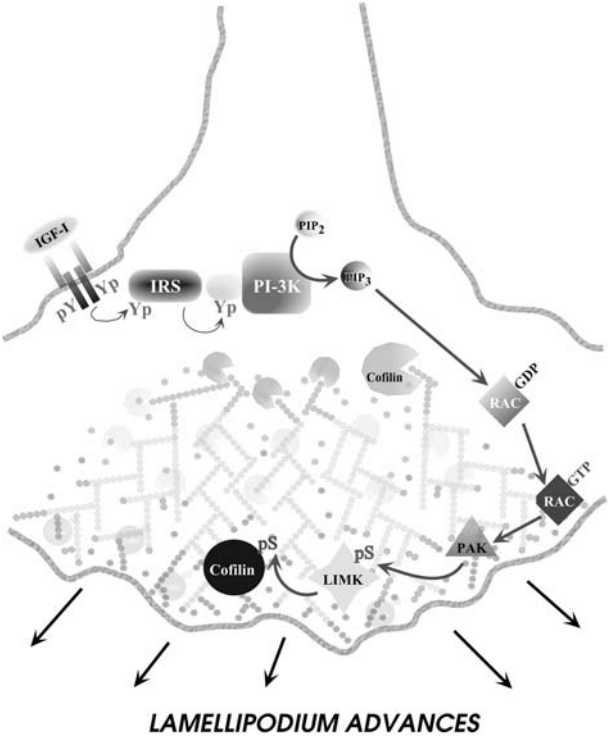

Figure 8. Model for IGF-I regulation of cofilin activity. IGF-I activates the IGF-I receptor and causes subsequent activation of PI-3K. PI-3K activates rac, either directly or through the activity of GEFs. Rac causes PAK to autophosphorylate, which then activates LIMK. Active LIMK phosphorylates and inactivates cofilin, preventing actin depolymerization and severing.

Acknowledgements. The authors thank Ms. J. Boldt for excellent secretarial assistance. This work was supported by the National Institutes of Health (NIH NS38849 and NS36778), and the Program for Understanding Neurological Diseases (PFUND).

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