Accelerated Publication

Rac/Cdc42 and p65PAK Regulate the Microtubule-destabilizing Protein Statmin through Phosphorylation at Serine 16*

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We have identified a rapid protein phosphorylation event at residue serine 16 of statmin using two-dimensional gel electrophoresis coupled to matrix-assisted laser desorption/ionization mass spectrometry in combination with post-source decay analysis, which is induced by the epidermal growth factor receptor. Phosphorylation is specifically mediated by the small GTPases Rac and Cdc42 and their common downstream target, the serine/threonine kinase p65PAK. Both GTPases have previously been shown to regulate the dynamics of actin polymerization. Because statmin destabilizes microtubules, and this process is inhibited by phosphorylation at residue 16, Rac and Cdc42 can potentially regulate both F-actin and microtubule dynamics.

Members of the Rho GTPase family, Rho, Rac, and Cdc42, control the assembly of filamentous actin structures in all mammalian cells (1). Their ability to link extracellular signals to the reorganization of the actin cytoskeleton suggests that they are likely to be important regulators of actin-driven cell processes, and Rac, for example, is crucial for growth cone guidance and cell migration both in tissue culture cells and in vivo in Drosophila and Caenorhabditis elegans (2–4). We report here that activation of Rac and to a lesser extent Cdc42 by EGF1 leads to the phosphorylation of statmin at residue 16. Phosphorylation at this site has been shown to inhibit statmin-induced destabilization of microtubules, and our results suggest, therefore, that Rac and Cdc42 can regulate the dynamics of both the actin and the microtubule cytoskeletons (5, 6).

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Plasmids—Human recombinant EGF was from Collaborative Biomedical Products. Toxins B1470 and B10463 were gifts from C. von Eichel-Streiber (Inst. for Microbial Medicine, Mainz, Germany). CNF-1 was a gift from G. Schmidt (University of Freiburg, Germany). Polyclonal antisera directed against statmin and statmin that has been phosphorylated at serines 16, 25, or 38 were described previously (7). Monoclonal phosphospecific anti-ERK1,2 antibody was from Sigma. The PAK1-(83–149) autoinhibitory fragment cloned into pRK5Myc was derived from murine PAK1 (8). All other expression plasmids encoded Myc-tagged proteins and have been described previously (7, 9).

Cell Culture and Transfections—HEp-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. 1 × 106 cells per well were seeded into 12-well dishes and serum-starved 24 h later. For preparative purposes, cells were seeded at 1 × 105 cells per 10-cm dish and were serum-starved 2 days later for another 20 h prior to lysis. For [32P] or [33P]orthophosphate labeling, cells were incubated in phosphate-free medium in the presence of 12.5 μCi/ml [32P], for preparative or 25 μCi/ml [33P], or 100 μCi/ml [33P], for analytical purposes for 3 h prior to lysis. For transfection experiments, HEp-2 cells were seeded at 2 × 105 per well into 6-well dishes 20 h before transfection. Cells were incubated for 4 h in 1.0 ml of serum-free medium containing 6 μl of LipofectAMINE (Life Technologies, Inc.) and 1.5 to 1.7 μg of total DNA per well. The transfection mixture was supplemented with 1 ml of medium containing 20% fetal bovine serum, and cells were lysed 20 h later.

Cell Lysis, Gel Electrophoresis, and Immunoblotting—Cell lysis was performed in 50 mM Tris, pH 7.5, 100 mM dithiothreitol, 0.3% SDS, 5 mM sodium pyrophosphate, 150 units/ml benzonase plus additives (1 mM EDTA, 2 mM MgCl2, 1 mM sodium fluoride, 1 mM orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). For immunoprecipitations, lysis buffer contained 20% fetal bovine serum. prior to lysis. For analytical purposes, the final focusing step was at 8000 V for 4 h.

Protein Purification—Fourteen 10-cm dishes of serum-starved and [32P]- or [33P]-labeled HEp-2 cells were stimulated with 100 ng/ml EGF for 7 min and lysed (350 μl per dish). Lysates were precleared, supplemented with 80 μl of 20% SDS and 120 μl of 87% glycerol plus additives, and the sample containing about 30 mg of total protein was loaded on the Model 491 Prep Cell (Bio-Rad). Preparative gel electrophoresis was performed utilizing a 11% separating gel. Aliquots containing proteins in the 19,000- to 23,000-dalton range were precipitated and analyzed by two-dimensional gel electrophoresis and autoradiography of silver-stained gels (10). The 21,000-dalton phosphoprotein of interest, which was found in two fractions, was dried to one-tenth of its original volume, precipitated, and subjected to preparative two-dimensional gel electrophoresis.

MALDI-MS Analysis—The protein spot of interest was visualized by Coomassie staining and excised, and the gel fragment was washed in water. The gel piece was allowed to shrink in 100 μl of acetonitrile/water (1:1) (both Baker HPLC Analyzed, Mallinckrodt Baker B.V., and 1 The abbreviations used are: EGF, epidermal growth factor; MALDI-TOF-MS, matrix-assisted laser desorption/ionization mass spectrometry; PSD, post-source decay analysis; MAP, mitogen-activated protein; RP-HPLC, reversed phase-high performance liquid chromatography; ERK, extracellular receptor kinase.
Deventer, The Netherlands) for 30 min and dried in a centrifugal vacuum concentrator. 10 μl of 0.1 M ammonium bicarbonate buffer containing 0.05 M g of sequencing grade modified trypsin (Promega) was added followed by ammonium bicarbonate buffer to submerge the gel piece. Digestion proceeded overnight at 37 °C and was stopped by FIG. 2.

**Fig. 2.** Effects of GTPase-modifying toxins on EGF-stimulated stathmin phosphorylation. A, serum-starved HEp-2 cells were incubated with toxin B1470 for 3 h and stimulated with 100 ng/ml EGF for 7 min. Total cell lysates were analyzed by immunoblotting with antisera specific for stathmin phosphorylated at serines 16, 25, or 38 or with an anti-phospho-ERK antibody. B, serum-starved HEp-2 cells were treated with 2 μg/ml CNF-1 for the indicated times, and stathmin phosphorylation was assessed as described above.

Devender, The Netherlands for 30 min and dried in a centrifugal vacuum concentrator. 10 μl of 50 mM ammonium bicarbonate buffer containing 0.05 μg of sequencing grade modified trypsin (Promega) was added followed by ammonium bicarbonate buffer to submerge the gel piece. Digestion proceeded overnight at 37 °C and was stopped by FIG. 2.

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acridination. The supernatant was removed from the gel pieces and a fraction (10%) was purified/concentrated on added Poros® 50 R2 beads (Roche Molecular Biochemicals GmbH, Mannheim, Germany) and used for direct MALDI-MS peptide mass fingerprint analysis (11). The remainder of the digestion mixture was separated on a RP-HPLC column; eluting peptides were automatically collected on Poros® 50 R2 beads and used for MALDI-PSD measurements (12). Measurements were performed on a Bruker Reflex III MALDI-TOF-MS (Bruker Daltonik GmbH, Bremen, Germany) operating in the reflectron mode. RP-HPLC fractions were first scanned in the reflectron mode, and candidate peptides were selected and their PSD spectra recorded (12). The information present in the PSD spectra was used by the SEQUEST algorithm (13) to identify the protein in a public nonredundant protein database.

RESULTS AND DISCUSSION

Rac interacts with numerous cellular targets including at least three families of Ser/Thr kinases, p65PAK, MLK, and p70S6kinase. To identify phosphorylation events controlled by Rac, metabolically labeled HEp-2 cells were pretreated with either control vector or Myc-tagged N17Rac1 or N17Cdc42 (1.0 μg/well each). After 24 h, cells were stimulated with 100 ng/ml EGF for 7 min and lysed. Myc-tagged proteins were immunoprecipitated from cell extracts and immunoblotted with either anti-[phospho-Ser-16]stathmin (upper) or anti-Myc (lower) antibodies. B, HEp-2 cells were transfected with Myc-tagged stathmin (0.5 μg/well) plus either control vector or Myc-tagged L63RhoA, L61Rac1, or L61Cdc42 (0.5 μg/well each). After 24 h, cells were lysed, and anti-Myc immunoprecipitates were analyzed as described above.

As shown in Fig. 1B, EGF treatment resulted in the appearance of two new forms of stathmin and a significant increase in the intensity of at least one other form. Stathmin has been shown to be phosphorylated on four distinct serines: 16, 25, 38, and 63, resulting in a variety of migration patterns on two-dimensional gels (Fig. 1C and Refs. 7 and 16). To identify which residues are phosphorylated in response to EGF, antibodies specific for three phosphorylated forms (serines 16, 25, and 38) were used on Western blots (7). As seen in Fig. 1D, EGF induces a dramatic increase in phosphorylation at Ser-16 and Ser-25. Ser-38 is phosphorylated in control cells and does not increase significantly upon EGF addition. The lack of a significant change in the P3 isoform at 19 kDa (Refs. 25, 38, 63 and 16) coupled with the strong increase in the P3 isoform at 23 kDa (16, 25, 38) suggests that very little phosphorylation is induced at Ser-63 by EGF.

To identify which phosphorylation event is mediated by Rho GTPases, phosphospecific antibodies were used to analyze Western blots of one-dimensional gels of lysates from cells treated with the two clostridial toxins. As can be seen in Fig. 2A, 160 ng/ml toxin B1470 results in a complete inhibition of EGF-induced phosphorylation of Ser-16 although at even 10-fold higher concentrations, the toxin does not inhibit EGF-induced phosphorylation at Ser-25. Ser-25 has been reported to be phosphorylated by ERK MAP kinases and the EGF-stimu-
lated activation of ERK1 and ERK2 was not changed by toxin treatment (Fig. 2A, bottom left and Ref. 17). Toxin B10463 gave similar results (data not shown). The basal levels of phosphorylated Ser-38 were unaffected by both toxins. Cytotoxic necrotizing factor 1 (CNF1) from *Escherichia coli* activates all members of the Rho family and when added to HEp-2 cells results in increased phosphorylation of stathmin only on Ser-16 (Fig. 2B and Ref. 18). We conclude that EGF-induced phosphorylation at Ser-16 of stathmin is mediated by Rac and/or Cdc42.

To confirm that Rac and Cdc42 can induce phosphorylation at Ser-16, HEp-2 cells were transfected with a Myc-tagged stathmin expression vector either alone or with dominant-negative Rac (N17Rac1) or Cdc42 (N17Cdc42) and treated 24 h later with EGF. Inhibition of Rac almost completely prevented phosphorylation on Ser-16, whereas inhibition of Cdc42 inhibited phosphorylation by around 50% (Fig. 3A). Next, stathmin was cotransfected with constitutively activated GTPases, L61Rac and L61Cdc42, but not L63Rho, induced phosphorylation specifically at Ser-16 (Fig. 3B).

Three isoforms (1, 2, and 3) of the Ser/Thr kinase p65PAK interact directly with Rac and Cdc42 leading to a variety of cellular effects (8, 19). To determine whether p65PAK mediates Ser-16 phosphorylation, HEp-2 cells were transfected with stathmin along with an autoinhibitory fragment (residues 83–149) derived from p65PAK (8). As seen in Fig. 4, A and B, inhibition of p65PAK completely prevented EGF-, L61Rac-, and L61Cdc42-dependent phosphorylation on Ser-16.

In conclusion, we have show that the addition of EGF to HEp-2 cells leads to a Rac/Cdc42 and p65PAK-dependent phosphorylation of stathmin at Ser-16. Whether p65PAK directly phosphorylates stathmin or whether it activates another downstream kinase is not currently known. Two kinases have been reported to phosphorylate stathmin at Ser-16, the cAMP-dependent kinase A and the Ca<sup>2+</sup>/calmodulin-dependent kinase isozymes, CaMK IV/Gr and CaMKII (5, 16, 20). The cAMP-dependent kinase A is unlikely to be involved because it preferentially phosphorylates Ser-63, and pretreatment of HEp-2 cells with EGTA to block Ca<sup>2+</sup> influx had no effect, suggesting that CaM kinases are not involved (data not shown).

Stathmin plays an important role in controlling microtubule dynamic either by sequestering α/β tubulin heterodimers or by increasing catastrophe frequency at the plus ends of microtubules or both (21–23). As a result, stathmin causes destabilization of growing microtubules and phosphorylation at Ser-16 appears to block this activity (5, 6). Rac and Cdc42 regulate actin polymerization and form membrane protrusions at the leading edge of migrating cells and neuronal growth cones. The results described here suggest, therefore, that Rac and Cdc42 might control both F-actin and microtubule dynamics in localized regions associated with cell protrusions (24, 25).

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