Src-dependent Tyrosine Phosphorylation Regulates Dynamin Self-assembly and Ligand-induced Endocytosis of the Epidermal Growth Factor Receptor

Endocytosis of ligand-activated receptors requires dynamin-mediated GTP hydrolysis, which is regulated by dynamin self-assembly. Here, we demonstrate that phosphorylation of dynamin I by c-Src induces its self-assembly and increases its GTPase activity. Electron microscopic analyses reveal that tyrosine-phosphorylated dynamin I spontaneously self-assembles into large stacks of rings. Tyrosine 597 was identified as being phosphorylated both in vitro and in cultured cells following epidermal growth factor receptor stimulation. The replacement of tyrosine 597 with phenylalanine impairs Src kinase-induced dynamin I self-assembly and GTPase activity in vitro. Expression of Y597F dynamin I in cells attenuates agonist-driven epidermal growth factor receptor internalization. Thus, c-Src-mediated tyrosine phosphorylation is required for the function of dynamin in ligand-induced signaling receptor internalization.

Vital cellular responses including cell metabolism, proliferation, and differentiation are regulated by specific interactions between extracellular ligands and their plasma membrane-anchored receptors. Accessibility of ligand to receptor, is therefore, an important facet of biological regulation. Receptor expression on the cell surface is dictated, at least in part, by vesicle trafficking via regulation of receptor internalization. Receptor internalization (also termed endocytosis) is implicated in receptor desensitization, down-regulation, and more recently signal transduction (1, 2). Endocytosis of G protein-coupled receptors and receptor tyrosine kinases is dependent on the invagination, constriction, and fission of vesicles (clathrin-coated and caveolae) from the plasma membrane into the cytosol. Dynamin, a large GTPase, plays a crucial role in these steps of receptor-mediated endocytosis (3, 4).

The GTPase activity of dynamin is stimulated 5–10-fold over basal levels by self-assembly (5). In the native state, dynamin exists as a homotetramer (6). At low ionic strength conditions (7), or in the presence of GDP and γ-phosphate analogues at physiological salt conditions (8), concentrated dynamin tetramers spontaneously self-assemble into spiral stacks of rings, similar to the structure which appears as an electron dense “collar” around the necks of endocytic pits observed at synaptic nerve terminals (9, 10). Purified dynamin also assembles onto phospholipid liposomes to generate dynamin-coated helical tubes that constrict and vesiculate upon GTP addition (11, 12). On the other hand, dynamin-decorated phospholipid nanotubes undergo nucleotide-dependent conformational changes causing extension in pitch along the dynamin helix without constriction or fragmentation (13, 14).

Recently, the GTPase effector domain of dynamin was shown to play a role in self-assembly and subsequent increase in GTPase activity (6, 15). The GTPase effector domain has an intrinsic GTPase activating protein activity and interacts with the N-terminal GTPase domain to stimulate GTP hydrolysis. Although the molecular details governing the action of dynamin in the fission of vesicles from the plasma membrane have been controversial, it is, nonetheless, clear that GTP binding and hydrolysis play critical roles (3, 4).

Tyrosine phosphorylation plays an important, if poorly understood, role in the internalization of cell surface receptors. Exposure of cells to tyrosine kinase inhibitors profoundly attenuates B cell receptor (16) and asialoglycoprotein receptor (17) endocytosis. Furthermore, inhibition of the non-receptor tyrosine kinase c-Src attenuates stem cell factor-induced c-Kit internalization in hematopoietic cells (18), antibody-induced internalization of neural cell adhesion molecule L1 in neuroblastoma cells (19), and EGFR endocytosis (20). Conversely, overexpression of c-Src causes an increase in EGFR internalization rate following EGF treatment (21).

Some components of the cellular endocytic machinery have been shown to undergo regulated tyrosine phosphorylation. In the case of EGFR internalization, one such target is clathrin. Stimulation with EGF increases c-Src-mediated tyrosine phosphorylation of clathrin, which regulates clathrin translocation to the plasma membrane (20). Another target is dynamin, which can directly interact with c-Src (22) and becomes tyrosine-phosphorylated in response to insulin (23) and lysophosphatidic acid (24) stimulation. The c-Src-mediated tyrosine phosphorylation of dynamin is required for agonist-induced endocytosis of β₂-adrenergic (25) and M1 muscarinic acetylcholine (26) receptors. However, unlike clathrin, the mechanisms whereby phosphorylation of dynamin regulates receptor-mediated endocytosis are unclear. Here, we show that tyrosine

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1 The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; β₂AR, β₂-adrenergic receptor; SH, Src homology; PH, pleckstrin homology; Pipes, 1,4-piperazineethanesulfonic acid; ATPγS, adenosine 5′-O-(thiotriphosphate); App(NH)p, 5′-adenyl-β,γ-imidodiphosphate.
phosphorylation of dynamin induces its self-assembly and subsequent increase in GTPase activity, and is required for agonist-induced EGFR internalization.

EXPERIMENTAL PROCEDURES

Expression Plasmide—Expression vectors for wild type and K44A dynamin have been described before (25). To generate single tyrosine mutants (Y231F, Y354F, and Y597F), each tyrosine residue of the wild type or K44A dynamin I was mutated to phenylalanine by overlapping polymerase chain reactions (UAC or UAU (Y) → UUC or UUU (F)). The double tyrosine mutant (Y231F/Y354F and Y231F/Y597F) dynamin was constructed by recombination of the two single tyrosine mutant constructs. All dynamin I cDNAs were subcloned in pCDNA3 expression vector. Baculovirus transfer vector containing dynamin I cDNA (wild type or Y231F/Y597F) was constructed in EcoRI/NotI sites of pVL1393 plasmid. The plasmid encoding the FLAG epitope-tagged EGFR was generated by polymerase chain reactions. The FLAG epitope coding sequences were introduced at the 5′ end of EGFR cDNA in pBR expression vector.

Cell Culture and Transfection—LipofectAMINE and tissue culture reagents were from Invitrogen. COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 μg ml−1 gentamicin at 37 °C in a humidified 5% CO2 atmosphere. 70–80% confluent COS-7 cells in 100-mm plates were transiently transfected with 20 μg of plasmid DNA using LipofectAMINE (Invitrogen). Once transfected, cells were cultured for 24 h before stimulation.

Proteins were visualized using Supersignal chemiluminescence reagent (Pierce) and quantified to provide samples for dynamin expression determined by immunoblotting with a 1:250 dilution of an anti-dynamin II monoclonal antibody (Sigma) as described (26). The Src kinase inhibitor PP2 and the EGFR kinase inhibitor tyrphostin AG1478 were from Calbiochem. Sodium orthovanadate (Na3VO4) on ice, and clarified by centrifugation. Cell lysates were mixed with 100 mM phenylmethylsulfonyl fluoride, 100 μg ml−1 trypsin inhibitor, and mixed for 15 min and incubated with 100 μM of a colorimetric alkaline phosphatase substrate (Bio-Rad) for 105 min. Background signal was concurrently determined using empty vector-transfected cells and subtracted from each of the values for the receptor-transfected cells. Receptor sequestration was defined as the fraction of total cell surface receptors that were removed from the plasma membrane following agonist treatment and thus were not accessible to antibody/antigen interaction. Background signal was greater than 90% as judged by Coomassie Blue staining.

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In Vivo Dynamin Phosphorylation—Purified c-Src (obtained from Dr. J. F. Work and Dr. S. Rohan), lacking the first 85 amino acid residues) was added to 750 μg of calf thymus histones and 150 μg of calf thymus DNA, in the presence of 0.4 M NaOH, and absorbance was determined using a microplate reader (Bio-Rad) at 405 nm. Background signal was concurrently determined using empty vector-transfected cells and subtracted from each of the values for the receptor-transfected cells. Receptor sequestration was defined as the fraction of total cell surface receptors that were removed from the plasma membrane following agonist treatment and thus were not accessible to antibody/antigen interaction. Background signal was greater than 90% as judged by Coomassie Blue staining.

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In Vitro Dynamin GPase Assay—Dynamin GPase activity was determined by measuring the release of 32P from [γ-32P]ATP in in vitro assays. Dynamin GPase activity was measured as described (30). Purified recombinant dynamin I and c-Src were mixed in 50 μl of kinase reaction buffer (10 mM Pipes, pH 7.0, 10 mM MnCl2, 50 mM NaCl, 100 μM ATP, 10 μM of [γ-32P]ATP) on ice. Reactions were performed at either 37 °C or room temperature for the indicated times and terminated by addition of Laemmli sample buffer followed by 5 min boiling. Phosphorylated dynamin was resolved by SDS-PAGE, visualized by autoradiography, and quantified using a Storm PhosphoImager (Amersham Biosciences) to calculate phosphorylation stoichiometry.

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FIG. 1. EGFR-regulated tyrosine phosphorylation of transiently expressed dynamin I. A, EGFR promotes phosphorylation of wild-type K44A dynamin. COS-7 cells transiently expressing wild-type K44A dynamin were serum-starved overnight and pretreated with 100 μM Na3VO4 for 1 h followed by stimulation with EGFR (10 ng ml\(^{-1}\)) for an additional 1 h. Dynamin immunoprecipitates (IP) were resolved by SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted (IB) with anti-phosphotyrosine (pTyr) antibody (PY20H). Values shown are expressed as percent of the EGF-induced tyrosine phosphorylation signal of K44A dynamin. B, time-dependent tyrosine phosphorylation of K44A dynamin by EGFR. COS-7 cells expressing K44A dynamin were serum-starved, exposed to Na3VO4 for a total of 2 h, and treated with EGFR at 37°C for the indicated times. Tyrosine phosphorylation content of K44A dynamin was determined as above. Values are presented as percent of maximal signal obtained after 2 h stimulation. C, EGFR-regulated tyrosine phosphorylation of dynamin involves Src kinase. Serum-starved K44A dynamin-expressing cells were treated with Na3VO4 for 30 min before adding either PP2 (5 μM) or AG1478 (250 nM). After an additional 30-min incubation, cells were stimulated with EGF for 1 h and analyzed for dynamin tyrosine phosphorylation. Data shown are expressed as percent of EGF-induced tyrosine phosphorylation of K44A dynamin from control cells, treated with the vehicle dimethyl sulfoxide only. A representative immunoblot is shown on the right of each panel. In each box, the lower panel shows total dynamin expression in lysates from the corresponding sample. Each data point represents the mean ± S.E. from at least three independent experiments.

RESULTS

EGFR Stimulation Induces Tyrosine Phosphorylation of Dynamin—Src-mediated tyrosine phosphorylation of clathrin (20) and dynamin (25) are required for EGFR and β2-AR internalization, respectively. However, the involvement of tyrosine phosphorylation of dynamin in the process of EGFR endocytosis has not been reported. To test this possibility, we examined EGF-induced tyrosine phosphorylation of dynamin I transiently expressed in COS-7 cells after pretreatment with the tyrosine phosphatase inhibitor sodium orthovanadate. Stimulation with EGF caused a 5–10-fold increase in the tyrosine phosphorylation of dynamin (Fig. 1A). Interestingly, we found that the tyrosine phosphate content of K44A dynamin was 2–3-fold higher than that of wild type dynamin (Fig. 1A). After targeting to the plasma membrane, K44A dynamin remains there because of its impaired ability to bind and hydrolyze GTP (32, 33). This may suggest that tyrosine phosphorylation of dynamin was stabilized at the plasma membrane.

Using K44A dynamin I transiently expressed in COS-7 cells, we determined the time course of its EGF-induced tyrosine phosphorylation. In the presence of sodium orthovanadate, tyrosine phosphorylation of K44A dynamin was significantly increased within 5 min and reached a plateau 30 min after EGF stimulation (Fig. 1B). The signal of K44A dynamin tyrosine phosphorylation was attenuated by the EGFR kinase inhibitor AG1478 as well as the Src kinase inhibitor PP2 (Fig. 1C), indicating that EGF-induced tyrosine phosphorylation of dynamin I was resuspended in phosphorylation reaction buffer as described. The concentrations of salt and dynamin in the reaction mixture were 50 μM and 10 μg ml\(^{-1}\), respectively. The mixture was assayed to carbon-coated EM grids that had been glow-discharged using a Hummer X glow-discharge unit (Technics West Inc.) just prior to use as described (31), and negatively stained with 2% aqueous uranyl acetate before air-drying. Images were obtained using a Philips 301 electron microscope at 80 kV with 50-μm objective aperture.
Tyrosine Phosphorylation-regulated Dynamin Function

Dynamin was EGFR- and Src kinase activity-dependent.

Because the data shown in Fig. 1 were obtained using transient expression of exogenous dynamin I, we confirmed these results by examining the EGF-induced tyrosine phosphorylation of endogenous dynamin. Endogenous dynamin II was isolated using GST-Grb2 affinity binding (24, 25) from COS-7 cells after stimulation with EGF for the indicated times (Fig. 2A), following pretreatment with sodium orthovanadate. Tyrosine phosphorylation of endogenous dynamin II was determined as described above. Data shown are expressed as EGF-induced fold increases in tyrosine phosphorylation over unstimulated samples. A representative immunoblot is shown on the right of each panel. In each box, the lower panel shows the amount of dynamin II isolated on the GST-Grb2 beads in the corresponding samples. Each data point represents the mean ± S.E. from five independent experiments. pTyr, phosphotyrosine.

**Fig. 2.** EGF-induced tyrosine phosphorylation of endogenous dynamin II. A, time-dependent tyrosine phosphorylation of endogenous dynamin II by EGF. COS-7 cells were serum-starved overnight, exposed to Na3VO4 for a total of 2 h, and then stimulated with EGF at 37 °C for the indicated times. Endogenous dynamin II was isolated using GST-Grb2 affinity beads and the tyrosine phosphorylation content of isolated dynamin II was determined by immunoblotting (IB) as outlined under Fig. 1. Values are presented as EGF-induced fold increases in tyrosine phosphorylation over unstimulated samples. B, EGF-induced tyrosine phosphorylation of endogenous dynamin II requires Src activity. Serum-starved cells were treated with Na3VO4 for a total of 2 h. PP2 (5 μM) or AG1478 (250 nM) were added 30 min prior to stimulation with EGF for 10 min. The tyrosine phosphorylation of endogenous dynamin II was determined as described above. Data shown are expressed as EGF-induced fold increases in tyrosine phosphorylation over unstimulated samples. A representative immunoblot is shown on the right of each panel. In each box, the lower panel shows the amount of dynamin II isolated on the GST-Grb2 beads in the corresponding samples. Each data point represents the mean ± S.E. from five independent experiments. pTyr, phosphotyrosine.

Sepharose chromatography. Purified dynamin was competent in GTP hydrolysis and was not tyrosine phosphorylated (data not shown). As shown in Fig. 3A, dynamin could serve as a direct substrate of c-Src. Maximum stoichiometry of phosphorylation achieved was calculated to be 0.39 mol of P/ mol of dynamin. Because the basic unit of the dynamin oligomer is a homotetramer (6), it is reasonable to assume that only a fraction of dynamin molecules are phosphorylated by c-Src in vitro.

Previously, two dynamin tyrosine residues (Tyr231 and Tyr597) were identified as being phosphorylated following β2-AR stimulation in HEK293 cells (25). To determine whether the tyrosine-phosphorylated residues by c-Src in vitro are the same as those identified in cultured cells, we compared the in vitro phosphorylation pattern of recombinant wild type and Y231F/Y597F dynamin proteins. The mutant protein was impaired by ~40–50% in its tyrosine phosphorylation compared with wild type protein (Fig. 3B). Mass spectrometric analysis of tryptic-digested fragments of in vitro c-Src-phosphorylated dynamin and immunoblotting of the phosphorylated dynamin with an antibody generated specifically against phospho-Tyr597 revealed phosphorylation of two residues, Tyr354 and Tyr597. Tyr231 was previously identified to be phosphorylated in response to β2-AR activation in cultured cells, whereas Tyr354 was not detected in the prior in vivo analysis (25) (Fig. 3C). Tyr231, another residue that becomes phosphorylated following β2-AR stimulation in cultured cells (25), was not found to be phosphorylated by c-Src in vitro, suggesting that Tyr231 could be phosphorylated by another kinase rather than c-Src. Together, these data suggest the relevance of c-Src-mediated phosphorylation of Tyr597 in the in vivo function of dynamin.
EGF-induced Tyrosine Phosphorylation of Dynamin Is Required for EGFR Internalization—To identify the physiological significance of the tyrosine phosphorylation sites in dynamin following receptor activation, we examined EGF-induced tyrosine phosphorylation content of the single tyrosine mutant dynamins, Y354F and Y597F, as well as the double tyrosine mutant dynamin Y231F/Y597F, transiently expressed in COS-7 cells. In addition, we examined the tyrosine phosphorylation of the K44A dynamin version of each tyrosine mutant, because K44A dynamin showed a higher tyrosine phosphorylation signal compared with wild type dynamin following EGF stimulation (Fig. 1). EGF stimulation induced tyrosine phosphorylation of both wild type and K44A dynamin (Fig. 4A), consistent with the data shown in Fig. 1A. Replacement of Tyr354 with Phe showed no substantial effect on the tyrosine phosphorylation level of either wild type or K44A dynamin in response to EGF stimulation (Fig. 4A and B). In contrast, the Y597F mutation resulted in 50–70% reduction in the EGF-induced dynamin tyrosine phosphorylation signal. Furthermore, replacement of both Tyr231 and Tyr597 with Phe caused a more dramatic attenuation (75–90%) in the tyrosine phosphorylation of dynamin than the Y597F single mutation following EGF stimulation. These results suggest that EGF stimulation induces phosphorylation of both Tyr597 and Tyr231, but not Tyr354, in COS-7 cells.

To establish the physiologic relevance of dynamin tyrosine phosphorylation in signaling receptor endocytosis, we tested the effect of the tyrosine mutant proteins on agonist-induced EGFR internalization in COS-7 cells. Expression of Y597F dynamin caused 60% reduction in EGF-induced EGFR internalization, whereas expression of the Y354F dynamin had no effect (Fig. 4C), as would be predicted from the lack of Tyr354 phosphorylation following EGF stimulation. Furthermore, expression of the Y231F/Y597F dynamin exhibited additional attenuation (80–90%) of EGF-induced EGFR internalization, which was equivalent to the degree of inhibition observed with GTPase-deficient K44A dynamin. Taken together, these results demonstrate that phosphorylation of both Tyr597 and Tyr231 was required for EGFR internalization following EGF stimulation, whereas phosphorylation of Tyr354 was not involved in EGFR endocytosis.

Tyrosine Phosphorylation of Dynamin Potentiates Its GTPase Activity in Vitro—Tyrosine phosphorylation of dynamin (Fig. 4C and Refs. 25 and 26) and GTP hydrolysis (3, 4) are both
required for ligand-induced endocytosis of signaling receptors. To test whether c-Src-mediated tyrosine phosphorylation regulates dynamin GTPase activity, we compared the rate of GTP hydrolysis by in vitro tyrosine-phosphorylated and unphosphorylated recombinant dynamin proteins. Fig. 5A shows that preincubation of dynamin with c-Src and ATP induced a 4–5-fold increase in steady-state GTP hydrolysis rate compared with the unphosphorylated control protein. Assays performed using dynamin alone, dynamin mixed with ATP or c-Src, or dynamin mixed with non-hydrolyzable ATP analogues (ATPγS) and c-Src, all failed to stimulate GTPase activity of dynamin (Fig. 5A), demonstrating that tyrosine phosphorylation of dynamin is required for the enhanced GTP hydrolysis.

Our results demonstrate that the Tyr597 residue of dynamin is phosphorylated by c-Src in vitro (Fig. 3), and that this phosphorylation regulates ligand-induced EGFR endocytosis (Fig. 4C), albeit by undefined mechanisms. To determine the role of Tyr597 phosphorylation on the function of dynamin, we compared the GTPase activities of in vitro tyrosine-phosphorylated wild type and Y231F/Y597F recombinant dynamin proteins. Because Tyr597 was not phosphorylated by c-Src in vitro, mutation of this residue (Y231F) would not be expected to influence the tyrosine phosphorylation-regulated GTPase activity of dynamin. After 2 min incubation with ATP at 30 °C, Y231F/Y597F dynamin showed −40% reduction in its tyrosine phosphorylation-induced initial rate of GTP hydrolysis compared with the wild type protein (Fig. 5B). Unphosphorylated wild type and Y231F/Y597F dynamin had equally low basal GTPase activities. These results demonstrate that in vitro tyrosine phosphorylation of dynamin stimulates its GTPase activity, and that phosphorylation of Tyr597 significantly controls this process.

Tyrosine-phosphorylated Dynamin Spontaneously Self-assembles to Generate Spiral Stacks of Interconnected Rings—At low ionic strength, purified dynamin self-assembles into structures composed of rings and spiral stacks, which are sedimentable by high-speed centrifugation (>100,000 × g) (5, 7). Because self-assembly of dynamin is known to regulate its GTP hydrolysis rate (5, 6, 15), we hypothesized that the increased GTPase activity of tyrosine-phosphorylated dynamin involves its enhanced self-assembly. Purified recombinant dynamin protein was phosphorylated, diluted in GTPase reaction buffer, and incubated at 30 °C, exactly as described for the GTPase assay. Self-assembled dynamin was collected by sedimentation. Initial results, utilizing high-speed centrifugation (150,000 × g for 10 min), showed no difference in self-assembly of in vitro tyrosine-phosphorylated or unphosphorylated dynamin proteins (Fig. 6A, small gel). However, when centrifugal force was reduced to 3,000 × g, a striking difference was noted; dynamin incubated with c-Src and ATP was sedimentable at 3,000 × g, whereas unphosphorylated proteins precipitated in trace amounts only (Fig. 6A).

To further establish a relationship among dynamin GTPase activity, self-assembly, and tyrosine phosphorylation, we examined the effect of tyrosine phosphorylation of dynamin on its self-assembly using wild type and Y231F/Y597F recombinant dynamin proteins that were phosphorylated by c-Src. Fig. 6B shows that the Y231F/Y597F dynamin has −40% diminution in its tyrosine phosphorylation-promoted sedimentation compared with wild type protein, exactly paralleling the effect on GTPase activity (Fig. 5B). Unphosphorylated Y231F/Y597F dynamin showed the same extent of precipitation as wild type control. Taken together, these data demonstrate that tyrosine-phosphorylated dynamin polymerizes into large molecular mass struc-
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Fig. 5. Tyrosine phosphorylation of dynamin potentiates the rate of GTP hydrolysis. A, c-Src-catalyzed phosphorylation of dynamin increases its GTPase activity. Purified recombinant rat dynamin I was co-incubated with c-Src (molar ratio of c-Src/dynamin = 1/5) and ATP for 6 h at room temperature to generate tyrosine-phosphorylated dynamin protein. Controls included incubation of dynamin alone (CN), or together with ATP, c-Src, or c-Src and the non-hydrolyzable ATP analogues App(NH)P and ATPγS. At the end of reaction, dynamin (0.2 μM) from each sample was incubated in GTPase assay buffer at 30 °C for 15 min, and GTP hydrolysis rates were then determined as described under “Experimental Procedures.” B, GTP hydrolysis by tyrosine-phosphorylated wild type and Y231F/Y597F dynamin proteins. Wild type and Y231F/Y597F-phosphorylated dynamin proteins were generated as outlined in A. Control samples (–c-Src) were prepared by incubating each dynamin protein alone in kinase reaction buffer. The rate of GTP hydrolysis was determined after incubation in GTPase buffer at 30 °C for 2 min. The asterisk indicates p < 0.05 versus tyrosine-phosphorylated wild type dynamin. Data represent mean ± S.E. from five independent experiments done in triplicate (A) or duplicate (B).

To confirm that tyrosine phosphorylation induces formation of real self-assembled structures of dynamin large enough to be precipitated at relatively low (3,000 × g) centrifugal force, we employed negative-staining electron microscopy. As shown in Fig. 7A, clustered stacks (50–300 nm length) of tightly interconnected dynamin rings were observed in solutions of tyrosine-phosphorylated samples obtained by incubation with c-Src and ATP. High magnification of a large stack showed a well ordered spiral array of dynamin rings (Fig. 7B). In contrast, unphosphorylated control dynamin was composed of short curved structures, some of which developed partial rings and globules (Fig. 7C).

Helical stacks of dynamin have been observed upon dilution into low ionic strength buffer (<50 mM salt) (7) or in the presence of GDP and γ-phosphate analogues at physiological salt conditions (8). However, those structures were prevalent only at high dynamin protein concentrations (>1 mg ml−1), whereas the tyrosine phosphorylation-induced assembled structures shown here were generated at much lower protein concentrations (0.16 mg ml−1). Furthermore, the helical stacks appeared tightly ordered (Fig. 7, A and B), similar to spirals obtained using a C terminus truncated 90-kDa fragment of dynamin, which was compactly arranged compared with intact dynamin protein in the presence of GDP and γ-phosphate analogues at physiological salt conditions (8). Dimensions of the stacks (~50 nm) were comparable not only to the structures obtained at high dynamin protein concentrations in vitro, but also to the collars observed at the neck of invaginated coated pits that accumulate at synaptic nerve terminals (9, 10). It has also been shown that helically coated phospholipid tubes generated from purified dynamin and liposomes in vitro have the same diameter (11). Taken together, these results demonstrate that tyrosine phosphorylation of dynamin induces its spontaneous self-assembly into large helical structures composed of interconnected rings.

DISCUSSION

Several models exist to describe the role of dynamin in the fission of vesicles from the plasma membrane to the cytosol, including dynamin being a pinchase (7, 11, 12), a poppase (13, 14), or an adapter (15) to recruit downstream effectors, which mediate the fission step. Regardless of which model is correct, it is an indisputable fact that control of dynamin GTPase activity is the key for endocytic vesicle formation (3, 4). Similarly, it is well established that dynamin self-assembles to form spiral structures on the neck of invaginated pits during endocytosis (9, 10, 33), which has been demonstrated to stimulate its GTPase activity (5, 6, 15). Although a certain conformation of GTP-bound dynamin was proposed to favor self-assembly (8), regulatory mechanisms inducing and stabilizing the assembled dynamin structures remain unclear. The present data show that c-Src-mediated tyrosine phosphorylation promotes significant increases in dynamin self-assembly into helically interconnected rings and also stimulates the rate of GTP hydrolysis, both of which are hallmarks for dynamin execution of vesicle budding from the plasma membrane.

Agonist stimulation triggers recruitment of c-Src and dynamin into the vicinity of activated receptor on the plasma membrane. Some receptors, such as EGFR (34) and β2-AR (35), bind c-Src directly, whereas others, such as β1-AR (36), form complexes with c-Src via bridging adapter proteins. Dynamin translocates to the plasma membrane by direct interaction with phospholipids (37) and adapter proteins, such as amphiphysin (3, 38). Once in close proximity to each other on the plasma membrane, c-Src phosphorylates dynamin. In support of this hypothesis is our finding that K44A dynamin, which is competent to translocate to invaginated pits on the plasma membrane following receptor activation, but which does not recycle to the cytosol because of its impaired ability to bind and hydrolyze GTP (32, 33), displays a significant increase in tyrosine phosphorylation compared with wild type dynamin (Fig. 1A). Furthermore, disruption of c-Src recruitment to activated β2-AR inhibits tyrosine phosphorylation of dynamin (39). These data suggest that dephosphorylation of dynamin occurs in the cytosol following GTP hydrolysis-induced disassembly.

Phosphoamino acid analysis of in vitro c-Src-phosphorylated
Tyrosine phosphorylation-regulated Dynamin Function

Tyrosine phosphorylation of dynamin on Tyr597, but not Tyr354, appears to be required for EGF-induced EGFR internalization (Fig. 4). Tyr597 in dynamin also needs to be phosphorylated to mediate β2-AR internalization (25) and expression of Y231F/Y597F dynamin inhibits m1 muscarinic acetylcholine receptor internalization (26). Replacement of Tyr597 with Phe reduces the tyrosine phosphorylation-induced GTPase activity (Fig. 5B) and self-assembly of dynamin (Fig. 6B), suggesting that phosphorylation of Tyr597 regulates dynamin function in endocytosis of ligand-activated receptors by controlling self-assembly and subsequent GTP hydrolysis. Does tyrosine phosphorylation of dynamin play a role in clathrin-mediated endocytosis of constitutively recycling nutrient receptors, similar to ligand-induced internalization of signaling receptors? Vallis et al. (40) showed that expression of bovine Y596F mutant dynamin I has no effect on transferrin uptake, suggesting that phosphorylation of Tyr596, which corresponds to the Tyr597 residue of rat dynamin I, is not required for constitutive endocytosis of nutrient receptors. It is possible that the function of dynamin is regulated differently for constitutive endocytosis of nutrient receptors, in the absence of tyrosine phosphorylation. In support of this hypothesis, the existence of two functionally and biochemically distinct subpopulations of clathrin-coated pits that mediate agonist-regulated internalization of the β2-AR and constitutive endocytosis of the transferrin receptor has been demonstrated (41). Alternatively, the tyrosine phosphorylation-induced assembled structure of dynamin may have an unidentified specific role for endocytosis of activated signaling receptors, which is not required for constitutive endocytosis of recycling receptors. In the case of EGFR endocytosis, one possibility is that tyrosine phosphorylation-induced dynamin self-assembly leads to recruitment of the ligand-activated receptor to the endocytic machinery. It has been suggested that biochemical differences between EGF and transferrin uptake mostly reflect specific requirements for the recruitment of the EGFR to coated pits (42). We also cannot exclude the possibility that the effect of Y597F dynamin on EGFR endocytosis may be indirect, such as by interfering with receptor activation, because expression of K44A dynamin was shown to alter high affinity binding of EGF to the receptor (43). Currently, however, what determines this specificity of tyrosine phosphorylation-regulated function of dynamin in signaling receptor internalization is not known.

How does tyrosine phosphorylation induce dynamin self-assembly? It is well established that phosphorytrosine residues primarily serve as docking sites for proteins containing Src covalent attachments and as recognition elements for signaling pathways involving the Src family of oncogenes and their non-receptor family members. Further experiments are needed to elucidate the role of Src in dynamin assembly and its interaction with other SH2 and SH3 domain proteins involved in clathrin-mediated endocytosis (44). A representative gel is shown for each panel. Each data point represents mean ± S.E. from five independent experiments.

**Fig. 6. Tyrosine phosphorylation promotes dynamin self-assembly.** A. c-Src-phosphorylated dynamin precipitates at low centrifugal force. Tyrosine-phosphorylated dynamin and appropriate controls were prepared as outlined under Fig. 5A, and diluted in GTPase buffer to exactly mimic conditions used in the GTPase assay. After incubating at 30 °C for 2 min, each dynamin sample was centrifuged at 3,000 × g for 10 min. Pelleted dynamin was visualized by Coomassie Blue staining and quantified. Values shown are expressed as fold-increase over control (CN) in which dynamin was incubated alone during the in vitro phosphorylation reaction. Additional controls included the high speed centrifugation (150,000 × g for 10 min) of dynamin proteins prepared in the absence or presence of c-Src with ATP. B. tyrosine phosphorylation-promoted self-assembly of wild type and Y231F/Y597F dynamin proteins. Wild-type and Y231F/Y597F dynamin proteins were subjected to in vitro phosphorylation by c-Src kinase, diluted in GTPase buffer, and incubated at 30 °C for 2 min, exactly as described under Fig. 5B. In each sample, precipitated dynamin was visualized by Coomassie Blue staining and quantified after centrifugation at 3,000 × g for 10 min. Values are expressed as fold increase over the wild type control (−c-Src). The asterisk indicates p < 0.05 versus tyrosine-phosphorylated wild type dynamin. A representative gel is shown for each panel. Each data point represents mean ± S.E. from five independent experiments.
homology (SH) 2 or phosphotyrosine-binding domains (44, 45). Indeed, some dynamin-binding proteins stimulate the GTPase activity of dynamin (3, 38). However, our *in vitro* results using purified dynamin show that tyrosine phosphorylation *per se* increases the GTPase activity of dynamin. Additionally, dynamin itself does not contain SH2 or phosphotyrosine-binding domains, indicating a lack of potential intramolecular SH2 or phosphotyrosine-binding domain-mediated interactions in tyrosine-phosphorylated dynamin. Several lines of evidence have suggested that tyrosine phosphorylation of proteins may stimulate their enzymatic activity by inducing a local conformational change rather than by creating binding sites for other partners. For example, most protein-tyrosine kinases, including receptor tyrosine kinases and non-receptor tyrosine kinases such as Src, are activated by phosphorylation of tyrosine residues in the activation loop (45). Furthermore, it has been reported that c-Src phosphorylates the Gαs, Gαo, Gα13, and Gα11 GTPases (47, 48). In *in vitro* reconstitution assays, tyrosine-phosphorylated Gαs shows an increased rate of binding to GTPγS as well as an increased rate of receptor-stimulated GTP hydrolysis (47). Together, the present results suggest the possibility that tyrosine phosphorylation may induce a conformational change of dynamin, which favors self-assembly.

TyR<sup>354</sup> is well conserved among dynamin isoforms and resides in the PH domain. This domain is required for endocytosis (49, 50), presumably to recruit dynamin to the plasma membrane via binding to phospholipids (37). The contribution of the PH domain to dynamin self-assembly is not clear. *In vitro*, the PH domain was shown either not to participate (51, 52) or to even be a negative regulator (6, 53) of dynamin-dynamin interaction. However, it is not clear whether the dynamin proteins used in these studies were tyrosine phosphorylated. Interaction of the PH domain phosphorylated on Tyr<sup>397</sup> with other domains of dynamin remains to be determined. On the other hand, whereas phosphorylation of Tyr<sup>354</sup> in dynamin appears to be physiologically irrelevant, at least with regard to EGFR endocytosis, the *in vitro* results demonstrate that phosphorylation of this residue exerts a positive effect on dynamin self-assembly and GTPase activity. Thus, it will be of interest to test whether phosphorylation of Tyr<sup>354</sup> alters other functions of dynamin, such as rapid endocytosis of synaptic vesicles in nerve terminals (3, 38), because the Tyr<sup>354</sup> residue is exclusively found in neuronal dynamin I.

In addition to being phosphorylated on tyrosine residues, neuronal dynamin I is also phosphorylated on a serine residue by protein kinase C in intact synaptosomes (54). *In vitro*, protein kinase C-mediated phosphorylation stimulates dynamin I GTPase activity (55), and increases binding to calcium (56), but blocks binding to phospholipids (57). Upon synaptic membrane depolarization, serine-phosphorylated dynamin I was dephosphorylated by the calcium-dependent phosphatase calcineurin (58), which was required for endocytosis in nerve terminals (59). Calcineurin-mediated dephosphorylation restores the ability of dynamin to bind phospholipids (57) and inhibits its GTPase activity (58) *in vitro*. Thus, it will be of interest to examine the relationship between tyrosine and serine phosphorylation of dynamin I in nerve terminals and their impact on endocytosis.

The effect of GTP binding on dynamin self-assembly is not entirely clear. *In vitro*, addition of GDP with γ-phosphate analogues induces dynamin self-assembly to generate spiral stacks (8), and treatment of isolated nerve terminals with GTPγS promotes tubular membrane invaginations decorated with electron-dense dynamin rings (10). However, the binding of guanine nucleotides GTPγS, GTP, or GDP destabilizes assembled dynamin structures (5), and GTP binding-impaired K44A dynamin self-assembles, like wild type protein under low ionic strength conditions (5). Our data show that tyrosine phosphorylation controls dynamin self-assembly (Fig. 6) even in the absence of guanine nucleotides (Fig. 7). Furthermore, stimulation with EGF promotes tyrosine phosphorylation of GTP binding-deficient K44A dynamin (Fig. 1), suggesting that nucleotide binding was not required for tyrosine phosphorylation. It is not clear at this juncture whether c-Src-regulated tyrosine phosphorylation exerts any effect on the affinity of dynamin to bind these nucleotides. Dynamin can also generate helically assembled structures around phospholipid liposomes (11, 12) or nanotubes (13, 14) *in vitro*, which were shown to undergo conformational changes in a nucleotide-dependent manner (11–14). Furthermore, the recently resolved crystal structure of the dynamin GTPase domain (60) and three-dimensional reconstruction of dynamin in the constricted state (12) provides additional evidence for the requirement of dynamin self-assembly and conformational change in endocytic vesicle formation. Thus, it remains to be determined how tyrosine phosphorylation collaborates with the guanine nucleotide in the context of dynamin self-assembly and conformational change.

Together, our results establish the novel paradigm that post-translational tyrosine phosphorylation of dynamin serves as a regulator of dynamin function in endocytosis of signaling receptors via control of self-assembly. Furthermore, the agonist-dependent tyrosine phosphorylation of dynamin provides a
mechanism by which tyrosine kinase receptors regulate their own accessibility to external stimulation and, as a result, cellular response.

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