Amphiphysin1, which can simultaneously bind to dynamin1 and the clathrin adaptor AP-2, is essential for dynamin1 recruitment during receptor-mediated endocytosis, but little is known about its regulatory mechanism. Here, we purified a 120-kDa mitogen-activated protein kinase (MAPK) substrate protein from porcine brains and identified the protein as amphiphysin1. Serine phosphorylation of amphiphysin1 was rapidly induced by nerve growth factor (NGF) in PC12 cells, and the induction was blocked by a MAPK inhibitor. Furthermore, when phosphorylated by MAPK in vitro or by NGF treatment in vivo, amphiphysin1 failed to bind to AP-2, but its association with dynamin1 was unaffected. Consistent with this, mutation of consensus MAPK phosphorylation sites increased amphiphysin1 binding to AP-2 and their intracellular colocalization. Thus, we propose that MAPK phosphorylation of amphiphysin1 controls NGF receptor/TrkA-mediated endocytosis by terminating the amphiphysin1-AP-2 interaction. This perhaps helps to regulate the availability of amphiphysin1-dynamin1 complexes for binding to the endocytic vesicle.

Clathrin-mediated endocytosis, which involves the orchestration of several molecular components, is crucial for various intracellular communications including the control of the levels of transmembrane receptors and their ligands, the recycling of synaptic vesicles in nerve terminals, and signal transduction (1). Among endocytic proteins, amphiphysin1 has recently been postulated to perform an essential function in endocytosis (2). Amphiphysin1 interacts with dynamin1 through its SH3 (Src-homology 3) domain (3) and thereby recruits dynamin1 to the site of clathrin-dependent endocytosis (4) by binding to the clathrin adaptor AP-2 (α-adaptin subunit), which is associated with plasma membrane receptors. Phosphorylation and dephosphorylation events play an important role in the regulation of the assembly of endocytic protein complexes; phosphorylation of amphiphysin1 negatively regulates its association with AP-2, and phosphorylation of dynamin1 results in its dissociation from amphiphysin1 (5). These modifications may contribute to the availability of amphiphysin1 and dynamin1 for binding to the coated vesicle. Recently, cylin-dependent kinase Cdk5 was identified as a protein kinase responsible for this phosphorylation reaction (6–8). Cdk5 phosphorylated amphiphysin1 and thereby promoted the dissociation of amphiphysin1 from AP-2. Likewise, Cdk5-phosphorylation of dynamin1 inhibited its binding to amphiphysin1. The results indicate that Cdk5 plays an important physiological role in endocytosis at nerve terminals by altering protein-protein interaction. However, the exact molecular mechanism underlying the regulation of endocytosis through phosphorylation reaction has not fully been elucidated.

The 42/45-kDa MAPK1 is activated by extracellular signals. Activation of MAPK requires its dual phosphorylation on threonine and tyrosine residues catalyzed by MAPK kinase (MAPKK), and MAPKK is activated by phosphorylation by Raf-1 (9). These kinases constitute a so-called MAPK cascade pathway, which controls cell proliferation, cell differentiation, and early embryonic development. In the current search for protein substrates for MAPK, we found that amphiphysin1 but not dynamin1 was phosphorylated by MAPK in response to NGF stimulation of PC12 cells. MAPK-dependent phosphorylation of amphiphysin1 negatively regulated the association of amphiphysin1 with AP-2 adaptor both in vitro and in vivo. Thus, we speculate that MAPK controls NGF receptor-mediated endocytosis by terminating the interaction between amphiphysin1 and AP-2.

EXPERIMENTAL PROCEDURES

Cell Fractionation and Phosphorylation Reaction—One rat brain was homogenized in 5 ml of homogenization buffer (0.25 M sucrose, 3 mM imidazole, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). The particulate fraction was isolated by a low and high speed centrifugation (10) and extracted in 5 ml of buffer A (25 mM Tris·HCl, 20 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na3VO4, 1 mM PMSF, 1 mM dithiothreitol (DTT), 1 μg/ml leupeptin, pH 7.5) for 30 min. The extract was spun at 30,000 g for 20 min at 4 °C, and the supernatant was used for the assay. Glutathione S-transferase (GST)-Xenopus MAPK (1 μg) was preincubated with GST-Xenopus MAPKK (1 μg) in 10 μl of the kinase buffer (25 mM Tris·HCl, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, 10 mM MgCl2, 5 mM MnCl2, pH 7.5) containing 50 μM ATP for 30 min at 30 °C, and the particulate extract and 0.44 TBq of (γ-32P)ATP (110 TBq/mmol, Amersham Biosciences) were added to the reaction mixture for 30 min.
at 30°C. The reaction products were analyzed by 10% SDS-PAGE followed by autoradiography.

In another approach (Fig. 2), 5 μg of GST-human amphiphysin1 fusion proteins were reacted with GST-MAPK, which had been activated by GST-MAPKK as described above. For in vivo phosphorylation study, PC12 cells (5 × 10^6) or primary rat embryo brain neuronal cells (E18) (2 × 10^7) were labeled with [32P]orthophosphate and stimulated with NGF (50 ng/ml) for 10 min in the presence or absence of PD98059 (100 μM). Phosphorylation products and immunocomplexes prepared with anti-GFP (Sigma) or anti-amphiphysin1 (N8-2) antibodies were analyzed by SDS-PAGE followed by autoradiography. Note the upward shift of the amphiphysin1 bands in response to NGF-induced phosphorylation. B, time course of amphiphysin1 phosphorylation and MAPK activation. Cells were stimulated with NGF (50 ng/ml) for the indicated periods, and lysates were immunoprecipitated with anti-phospho-MAPK (Sigma) and N8-2 antibodies. C, phosphoamino acid analysis. GST-amphiphysin1 and GFP-wt-amphiphysin1 were phosphorylated as described in A, and phosphoamino acid analysis was performed as described under "Experimental Procedures." The phosphorylated amino acids were identified by autoradiography. The phosphoamino acid standards (P-Thr, P-Ser, and P-Tyr) were visualized by ninhydrin.

In some experiments, cells were pretreated with 100 μM PD98059 (Calbiochem) for 1 h prior to the addition of NGF. Extracts were immunoprecipitated with anti-GFP antibodies (Sigma) or mouse monoclonal anti-amphiphysin1 antibodies (N8-2) (12). In vitro phosphorylated samples and immunocomplexes were subjected to SDS-PAGE and autoradiography.

Protein Purification—Four porcine brains were homogenized in homogenization buffer by a Dounce homogenizer, the particulate fractions were extracted in 200 ml of buffer A, and the extract was clarified by centrifugation as described previously (11). 50% (w/v) ammonium sulfate precipitates of the supernatant were applied to a DEAE-Sepharose column (Amersham Biosciences, 100 ml) equilibrated with buffer B (25 mM Tris-HCl, 20 mM NaCl, 1 mM DTT, 1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin, 1 μM E64).

**Fig. 1. Identification and purification of MAPK substrate proteins.** A, phosphorylation of proteins in rat brain extracts by MAPK. Rat brain particulate extracts were prepared and processed for the kinase assay in the presence of GST-MAPK and/or GST-MAPKK as described under “Experimental Procedures.” The reaction products were analyzed by SDS-PAGE and autoradiography. An asterisk and closed circles indicate p120 and other proteins phosphorylated by MAPK, respectively. B, elution of porcine p120 from an FPLC MonoQ column. Each fraction (20 μl) was subjected to the kinase reaction in the presence (+) or absence (−) of GST-MAPKK/MAPK. The inset shows Coomassie Brilliant Blue staining of the pooled peak fractions. C, phosphorylation pattern of p120 at the peak fractions. D, amino acid sequences of p120 peptides. Peptides were sequenced by Edman degradation. Amino acid sequences were compared with those of human amphiphysin1 (15).

**Fig. 2. Analysis of amphiphysin1 phosphorylated by MAPK.** A, MAPK phosphorylation of amphiphysin1. GST-wt-amphiphysin1 was phosphorylated by activated GST-MAPK in the presence of [γ-32P]ATP as described in the legend for Fig. 1. PC12 cells (5 × 10^6) or primary rat embryo brain neuronal cells (E18) (2 × 10^7) were labeled with [32P]orthophosphate and stimulated with NGF (50 ng/ml) for 10 min in the presence or absence of PD98059 (100 μM). Phosphorylation products and immunocomplexes prepared with anti-GFP (Sigma) or anti-amphiphysin1 (N8-2) antibodies were analyzed by SDS-PAGE followed by autoradiography. Note the upward shift of the amphiphysin1 bands in response to NGF-induced phosphorylation. B, time course of amphiphysin1 phosphorylation and MAPK activation. Cells were stimulated with NGF (50 ng/ml) for the indicated periods, and lysates were immunoprecipitated with anti-phospho-MAPK (Sigma) and N8-2 antibodies. C, phosphoamino acid analysis. GST-amphiphysin1 and GFP-wt-amphiphysin1 were phosphorylated as described in A, and phosphoamino acid analysis was performed as described under “Experimental Procedures.” The phosphorylated amino acids were identified by autoradiography. The phosphoamino acid standards (P-Thr, P-Ser, and P-Tyr) were visualized by ninhydrin.
FIG. 3. Effects of MAPK phosphorylation of amphiphysin1 on its interaction with AP-2 and dynamin1. A, PC12 cells were stimulated with or without NGF (50 ng/ml) for 10 min in the presence or absence of PD98059 (10 μM), and lysate proteins were immunoblotted or immunoprecipitated with anti-amphiphysin1 antibodies as in Fig. 2. Immunoprecipitates were analyzed by immunoblotting with mouse monoclonal anti-adaptin antibodies (Sigma) and mouse monoclonal anti-dynamin1 antibodies (Sigma). B, GST-amphiphysin1-immobilized resins were phosphorylated by GST-MAPK and incubated with PC12 cell lysates. Bound proteins were analyzed by AP-2 and dynamin1 immunoblotting. C and D, PC12 cells were transfected with amphiphysin1 constructs, labeled with [32P]-orthophosphate and stimulated with NGF for 10 min as in Fig. 2. Cell lysate proteins were immunoprecipitated with anti-GFP antibodies, and immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography (C) or subjected to immunoblotting with anti-AP-2 antibodies (D). The ratio (arbitrary unit) of phosphorylated exogenous amphiphysin1 and co-precipitated AP-2 per unit of exogenous amphiphysin1 is shown. E, GST-wt-amph1 and GST-amph1 mutants at Ser-285 and Ser-293 (1 μg each) were phosphorylated by activated GST-MAPK in the presence of [γ-32P]ATP (0.44 TBq) as described under “Experimental Procedures,” and the reaction products were analyzed by SDS-PAGE followed by autoradiography. The amounts of protein were normalized by Coomassie Brilliant Blue (CBB) staining.

pH 7.5), and proteins (8 ml/fraction) were eluted with a linear NaCl gradient (0.02–0.5 M). 20 μl of the fraction was subjected to the kinase assay at each step. The peak fraction containing p120 eluted at 0.25 M NaCl was applied to a heparin-Sepharose column (Amersham Biosciences, 20 ml) equilibrated with buffer C (20 mM HEPES-NaOH, 0.2 mM EDTA, 1 mM DTT, 5 mM MgCl2, pH 8.0) (13). Elution (0.5 ml/fraction) was performed with a linear KCl gradient (0.05–0.6 M), and a major peak was eluted at 0.35 M NaCl, and purified p120 proteins were processed for microsequencing (11).

Phosphoamino Acid Analysis—Amphiphysin1 proteins phosphorylated in vitro and in vivo were eluted from gel pieces and hydrolyzed in 6 N HCl at 105 °C for 2 h followed by high voltage electrophoresis as described previously (14). The phosphorylated amino acids were detected by autoradiography.

Immunoprecipitation and Immunoblotting—PC12 cells were lysed in either a lysis buffer (20 mM Tris-HCl, 1% Triton X-100, 120 mM KCl, 1 mM PMSF, pH 7.5) or radiimmune precipitation buffer, and the lysates were immunoblotted or immunoprecipitated with various antibodies as indicated.

GST Pull-down Assay—GST-wt-amphiphysin1 or GST (5 μg) bound to glutathione beads (Amersham Biosciences) were phosphorylated by activated GST-MAPK in the presence of 50 μM ATP as described in the legend for Fig. 1. The resins were incubated with PC12 cell lysates or purified p120 proteins (40 μg) for 90 min. AP-2 and dynamin1 bound to the resins were analyzed by immunoblotting with antibodies against these proteins.

Mutational Analysis—To create the amphiphysin1 mutants amph-285 and amph-293, Ser-285 and Ser-293, respectively, were converted to alanine by PCR mutagenesis, and the resulting DNAs were cloned into pEGFPC1. All constructions were verified by DNA sequencing. To create the amphiphysin1 mutants amph-285 and amph-293, Ser-285 and Ser-293, respectively, were converted to alanine by PCR mutagenesis, and the resulting DNAs were cloned into pEGFPC1. All constructions were verified by DNA sequencing. 48 h after transfection of the wild type and mutant amphiphysin1 constructs (5 μg) into PC12 cells (1×10^6 cells/dish) with LipofectAMINE 2000 (Invitrogen), cells were labeled with [32P]-orthophosphate in phosphate-free medium and stimulated with NGF prior to phosphorylation analysis as described above. Alternatively, transfected cells were stimulated with or without NGF and subjected to immunostaining.

Immunocytochemistry—Cells on coverslips were fixed in methanol at −20 °C for 5 min and permeabilized with 0.1% Triton X-100 for 10 min. Immunostaining was performed with the indicated antibodies as described previously (11).

RESULTS

Identification and Purification of p120 Phosphorylated by MAPK—To identify the potential cellular target proteins for MAPK in neuronal cells, we first examined which proteins in rat brain particulate fractions were phosphorylated by MAPK in vitro. Extracts prepared from particulate fractions were subjected to phosphorylation reaction in the presence or absence of GST-MAPK that had been preactivated by GST-MAPK. The data indicated that several proteins were preferentially phosphorylated in response to MAPK activation (Fig. 1A). We identified one of these with a molecular mass of 120 kDa (p120) as amphiphysin1 by three-step column chromatography. Fig. 1 (B and C) shows the elution profile from an FPLC MonoQ at the final step, and peak fractions contained partially purified p120 proteins. The doublet structure may be due to the protein degradation or posttranslational modification. Microsequencing revealed that amino acid sequences of two peptides prepared from p120 were identical to residues 142–158 and 671–681 of human amphiphysin1 (Fig. 1D), a component of endocytic machinery (15). Moreover, anti-amphiphysin1 antibodies recognized purified p120 in immunoblots (data not shown).

Amphiphysin1 Is Phosphorylated by NGF-regulated MAPK—To confirm whether amphiphysin1 is a substrate for MAPK, GST-amphiphysin1 fusion proteins were created and incubated with GST-MAPK and [γ-32P]ATP. As predicted, amphiphysin1...
was phosphorylated by MAPK following its activation by MAPKK (Fig. 2A). Immunoprecipitation studies with 32P-orthophosphate-labeled PC12 cells showed that NGF treatment rapidly induced amphiphysin1 phosphorylation, as manifested in a slower mobility. A MAPKK inhibitor, PD98059, blocked this NGF action, indicating that MAPK mediates NGF-induced amphiphysin1 phosphorylation. Furthermore, time course experiments showed sustained phosphorylation of amphiphysin1 by NGF treatment (Fig. 2B), which correlated well with the NGF-induced duration of MAPK activation (16). Induction of amphiphysin1 phosphorylation was similarly detected in NGF-stimulated primary neuronal cells isolated from rat embryos (E18) (Fig. 2A). Phosphoamino acid analysis demonstrated that phosphorylation of amphiphysin1 occurred primarily on serine residues both in vitro and in vivo (Fig. 2C). Two-dimensional tryptic peptide mapping of amphiphysin1 showed similar phosphopeptide distribution between MAPK-dependent phosphorylation in vitro and NGF-dependent phosphorylation in vivo (data not shown). Collectively, these results indicate that amphiphysin1 is a physiological substrate for NGF-regulated MAPK.

MAPK Phosphorylation of Amphiphysin1 Blocks Its Association with AP-2—We next tested whether MAPK phosphorylation of amphiphysin1 influences its interaction with AP-2 and dynamin1. PC12 cells were stimulated with NGF for 10 min, and cell lysates were subjected to immunoprecipitation followed by immunoblotting. Fig. 3A shows that amphiphysin1 was phosphorylated upon NGF stimulation, exhibiting a slower migration in gel electrophoresis; this phosphorylation was blocked by PD98059 treatment. In parallel, co-precipitation of amphiphysin1 and AP-2 was markedly inhibited following NGF treatment, whereas amphiphysin1 tightly bound to AP-2 in untreated cells. PD98059 treatment prevented NGF-induced dissociation of amphiphysin1 from AP-2. In contrast, neither NGF treatment nor a MAPKK inhibitor had significant effects on amphiphysin1 binding to dynamin1 (Fig. 3A). Furthermore, GST-amphiphysin1-immobilized resins were prepared, phosphorylated by activated MAPK, and incubated with lysates from PC12 cells. The amount of AP-2 retained to the phosphorylated amphiphysin1 resins was markedly reduced compared with that retained to unphosphorylated ones (Fig. 3B). Again, MAPK phosphorylation of amphiphysin1 had no inhibitory effect on dynamin1 association (Fig. 3B).

Since amphiphysin1 contained Ser-285 and Ser-293 as potential sites (PXSP) of MAPK phosphorylation (17), Ser-285 and Ser-293 were replaced with alanine by site-directed mutagenesis to examine the effect of phosphorylation on amphiphysin1 binding to AP-2. Although GFP-wt-amphiphysin1 was phosphorylated in response to NGF, the mutations lowered the level of phosphorylation (Fig. 3C). On the contrary, the ability of the amphiphysin1 mutants to bind AP-2 was increased as compared with that in the wild type proteins (Fig. 3D), which is in agreement with the observation that inhibition of the MAPK activity enhances the amphiphysin1-AP-2 complex formation (Fig. 3A). MAPK phosphorylation of amphiphysin1 was not completely inhibited by the mutations, which may reflect the presence of other phosphorylation sites for MAPK or a contribution from a kinase other than MAPK. We performed phosphorylation of wt amphiphysin1 and its mutated counterparts by activated MAPK in vitro. The data indicate that the phosphorylation level of Ser-285 and Ser-293 mutants decreased by 60–70% compared with that of wt amphiphysin1, but the phosphorylation of the mutants was not completely abolished (Fig. 3E). Thus, the incomplete inhibition of NGF-induced amphiphysin1 phosphorylation by the mutations could be attributed to the existence of an additional phosphorylation site(s). However, since we cannot rule out the possibility that this additional phosphorylation is due to the nonphysiological, nonspecific phosphorylation of amphiphysin1 by MAPK that occurs in vitro, it is still possible that a kinase(s) other than MAPK also contributes to the NGF-dependent phosphorylation

Fig. 4. Effects of PD98059 and amphiphysin1 mutation on co-localization of amphiphysin1 and AP-2. PC12 cells were treated with or without NGF for 10 min in the presence or absence of PD98059 (PD (A)). PC12 cells were transfected with GFP-wt-amph1 (B, a–c), GFP-amph-285 (B, d–f), or GFP-amph-293 (B, g–i) for 36 h, as described in legend for Fig. 2, and stimulated with NGF for 10 min. Then cells were fixed, permeabilized, processed for AP-2 (A, b, e, h, and k; B, b, c, and h) and amphiphysin1 (A, a, d, g, and j; B, a, d, and g) immunostaining, and observed using a confocal microscope. AP-2 was visualized with rabbit anti-a-adaptin (Santa Cruz Biotechnology) and Cy3-anti-rabbit IgG antibodies (Sigma) and amphiphysin1 with N8-2 and fluorescein isothiocyanate-anti-mouse IgG antibodies (Sigma).
of amphiphysin1 in vivo. Further analysis of the exact phosphorylation sites of amphiphysin1 will be necessary to reach a conclusion about this issue.

MAPK Phosphorylation of Amphiphysin1 Affects Colocalization of Amphiphysin1 and AP-2 in Vivo—To further assess and complement these results, we next examined the subcellular localization of amphiphysin1 and AP-2 in PC12 cells by immunostaining. A large fraction of both proteins colocalized in the cytoplasmic region in unstimulated cells (Fig. 4A, a–c). However, NGF treatment of cells resulted in an increased segregation of amphiphysin1 from AP-2 (Fig. 4A, d–f). When cells were pretreated with PD98059, this NGF action was blocked, and a colocalization of amphiphysin1 with AP-2 was restored (Fig. 4A, g–i). Two proteins remained colocalized in PD98059-treated cells (Fig. 4A, g–i). Consistent with these results, the increased colocalization of the GFP-amphiphysin1 mutants at Ser-285 or Ser-293 with AP-2 was observed in the mutant-transfected PC12 cells even in the presence of NGF (Fig. 4B, d–i). Conversely, expression of GFP-wt-amphiphysin1 resulted in its dissociation from AP-2 in NGF-stimulated cells (Fig. 4B, a–c). These results are consistent with the biochemical study of the physical interaction between two proteins (Fig. 3) as well as the previous observation that dephosphorylation of rat brain extracts promotes the assembly of amphiphysin1 with AP-2, whereas phosphorylation of amphiphysin1 inhibits its binding to AP-2 (5).

Effects of MAPK Phosphorylation of Amphiphysin1 on Endocytosis—We next examined whether phosphorylation of amphiphysin1 by MAPK affects NGF receptor-mediated endocytosis. PC12 cells were stimulated with NGF for 20 min in the presence or absence of PD98059 and subjected to immunostaining. Although NGF treatment of cells triggered internalization of TrkA and caused a moderate increase in colocalization of TrkA into EEA1-positive endosomes (Fig. 5, d–f), the addition of PD98059 significantly enhanced this colocalization (Fig. 5, g–i). Increased TrkA internalization was also detected in cells transfected with GFP-amphiphysin1 mutants at Ser-285 and Ser-293 as compared with cells transfected with wt amphiphysin1 (data not shown). The finding suggests that inhibition of MAPK phosphorylation of amphiphysin1 results in accelerated internalization of TrkA into endosomes, perhaps

![Figure 5](image-url)
because of the increased formation of amphiphysin1-AP-2 complexes. In another word, MAPK phosphorylation of amphiphysin1 seems to have a suppressive effect on endocytosis of the NGF receptor.

DISCUSSION

In this study, we provide evidence for a functional link between amphiphysin1 and the MAPK signaling pathway. We have demonstrated phosphorylation of amphiphysin1 by MAPK in vitro and NGF-stimulated MAPK in vivo. We have also shown that although MAPK phosphorylation of amphiphysin1 disassembled amphiphysin1-AP-2 complexes during NGF stimulation of PC12 cells, a MAPK inhibition rescued the association of AP-2 with amphiphysin1. Thus, our findings point to an important possibility that MAPK negatively regulates the interaction between amphiphysin1 and AP-2 during progression of endocytosis. To our knowledge, this is the first report that one of the cellular targets for MAPK is integrated into NGF receptor-mediated endocytosis.

Our results indicate that phosphorylation of amphiphysin1 at Ser-285 and/or Ser-293 affects the function of amphiphysin1. Since these serine residues are contained in the proline-rich domain (PRD) of amphiphysin1 and the PRD of unphosphorylated amphiphysin1 binds to AP-2 (5), MAPK phosphorylation of Ser-285 and Ser-293 could modulate the interaction between PRD and AP-2, resulting in the dissociation of amphiphysin1 from AP-2. Recent studies show that phosphorylation of amphiphysin1 PRD by Cdk5 inhibited the association of amphiphysin1 with AP-2 in synaptic vesicle endocytosis (7, 8) similar to that by MAPK (present report). Cdk5 appears to phosphorylate amphiphysin1 at serines 261, 272, 276, and 285 and threonine 310, located in the PRD (6, 8). Thus, it is possible that MAPK and Cdk5 target the overlapping phosphorylation sites (Ser-285) leading to a similar regulatory effect on amphiphysin1. Furthermore, the mutation of Ser-285 and Ser-293 to alanine did not completely abolish the phosphorylation of amphiphysin1 in vivo, which implies the involvement of another kinase or the existence of additional phosphorylation sites for MAPK (Fig. 3C). Although the incomplete inhibition in MAPK phosphorylation of the amphiphysin1 mutants in vitro suggests the latter possibility, it is still possible that another kinase also participates in NGF-dependent amphiphysin1 phosphorylation. Therefore, we do not rule out the possibility that Cdk5, as well as MAPK, is involved in phosphorylation of amphiphysin1 in NGF receptor-mediated endocytosis. Another feature of MAPK-dependent phosphorylation is that whereas Cdk5, in addition to phosphorylation of amphiphysin1, phosphorylated dynamin1 and thereby blocked its binding to amphiphysin1 (8), MAPK was unable to phosphorylate dynamin1 and had no inhibitory effect on the formation of amphiphysin1-dynamin1 complexes (Fig. 3A). This suggests that the two kinases exert distinct regulatory roles in some aspects of endocytosis.

Endocytosis through clathrin-coated pits has been regarded as a mechanism to switch off and renew plasma membrane receptor signaling (18). In this scheme, the supply and availability of dynamin1 and accessory proteins of clathrin-coated vesicles would be a critical limiting factor in clathrin-mediated endocytosis. We therefore propose the following model for the role of MAPK phosphorylation of amphiphysin1 (Fig. 6). In NGF-stimulated PC12 cells, the ligand stimulation of the NGF receptor tyrosine kinase TrkA transmits activation signals through the Ras-Raf-MAPKK-MAPK pathway to the nucleus, leading to neurite outgrowth (19). Thus, the activated MAPK also phosphorylates amphiphysin1 following internalization of the NGF receptor, and this phosphorylation event contributes to disassembly of endocytic coat proteins by terminating the interaction of amphiphysin1 with AP-2, which would lead to recycling of amphiphysin1-dynamin1 complexes. Possibly, MAPK-dependent phosphorylation of amphiphysin1 also affects internalization of the NGF receptor into endosomes by controlling amphiphysin1-AP-2 complexes, because MAPK inhibition caused the increased trafficking of TrkA into EEA1-positive endosomes (Fig. 5). Our discovery that MAPK is involved in the regulation of clathrin-mediated endocytosis is consistent with accumulating evidence that functional links exist between the machinery of receptor-mediated signaling and endocytosis via clathrin-coated pits (18).

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REFERENCES

1. Marsh, M., and McMahon, H. T. (1990) Science 285, 215–220H. T.
2. Wigge, P., and McMahon, H. T. (1998) Trends Neurosci. 21, 339–344
3. Wigge, P., Vallis, Y., and McMahon, H. T. (1997) Curr. Biol. 7, 554–560
4. Shupliakov, O., Low, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., Camilli, P. D., and Bredin, L. (1997) Science 276, 259–263
5. Slepnev, V. I., Ochoa, G. C., Butler, M. H., Grabs, D., and Camilli, P. D. (1998) Science 281, 821–824
6. Floyd, S. R., Porro, E. B., Slepnev, V. I., Ochoa, G. C., Tsai, L. H., and Camilli, P. D. (2001) J. Biol. Chem. 276, 8104–8110
7. Tan, T. C., Valova, V. A., Malladi, C. S., Graham, M. E., Berven, L. A., Jupp, O. J., Hanara, G., McClure, S. J., Suraceva, B., Boudle, R. A., Larsen, M. R., Cousin, M. A., and Robinson, P. J. (2003) Nat. Cell Biol. 5, 701–710
8. Tomizawa, K., Sunada, S., Lu, Y. F., Oda, Y., Kinuta, M., Ohshima, T., Saito, T., Wei, F. Y., Matushita, M., Li, S. T., Tsutsui, K., Hisanaga, S., Miko-
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9. Nishida, E., and Gotoh, Y. (1993) Trends Biochem. Sci. 18, 128–131
10. Suzukiwa, K., Miura, K., Mitsushita, J., Resau, J., Hirose, K., Crystal, R., and Kamata, T. (2000) J. Biol. Chem. 275, 13175–13178
11. Miura, K., Miyazawa, S., Furuta, S., Mitsushita, J., Kamijo, K., Ishida, H., Miki, T., Suzukiwa, K., Resau, J., Copeland, T. D., and Kamata, T. (2001) J. Biol. Chem. 276, 46276–46283
12. Jin, Y., Kim, K. Y., Soong, N. K., Shin, E. Y., Kim, E. G., and Kim, S. R. (2001) Exp. Mol. Med. 33, 69–75
13. Otsuka, A., Hirose, K., Kilimann, M. W., and Kamata, T. (2003) Biochem. Biophys. Res. Commun. 301, 769–775
14. Adachi, Y., Copeland, T. D., Takahashi, C., Nosaka, T., Ahmed, A., Oroslan, S., and Hatanaka, M. (1992) J. Biol. Chem. 267, 21977–21981
15. Lichte, B., Veh, R. W., Meyer, H. E., and Kilimann, M. W. (1992) EMBO J. 11, 2521–2530
16. Marshall, C. J. (1995) Cell 80, 179–185
17. Gonzalez, F. A., Raden, D. L., and Davis, R. J. (1991). J. Biol. Chem. 266, 22159–22163
18. Di Fiore, P. P., and De Camilli, P. (2001) Cell 106, 1–4
Regulation of Amphiphysin1 by Mitogen-activated Protein Kinase: ITS SIGNIFICANCE IN NERVE GROWTH FACTOR RECEPTOR-MEDIATED ENDOCYTOSIS
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