The Interaction of Epsin and Eps15 with the Clathrin Adaptor AP-2 Is Inhibited by Mitotic Phosphorylation and Enhanced by Stimulation-dependent Dephosphorylation in Nerve Terminals

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Clathrin-mediated endocytosis was shown to be arrested in mitosis due to a block in the invagination of clathrin-coated pits. A Xenopus mitotic phosphoprotein, MP90, is very similar to an abundant mammalian nerve terminal protein, epsin, which binds the Eps15 homology (EH) domain of Eps15 and the α-adaptin subunit of the clathrin adaptor AP-2. We show here that both rat epsin and Eps15 are mitotic phosphoproteins and that their mitotic phosphorylation inhibits binding to the appendage domain of α-adaptin. Both epsin and Eps15, like other cytosolic components of the synaptic vesicle endocytic machinery, undergo constitutive phosphorylation and depolarization-dependent dephosphorylation in nerve terminals. Furthermore, their binding to AP-2 in brain extracts is enhanced by dephosphorylation. Epsin together with Eps15 was proposed to assist the clathrin coat in its dynamic rearrangements during the invagination/fission reactions. Their mitotic phosphorylation may be one of the mechanisms by which the invagination of clathrin-coated pits is blocked in mitosis and their stimulation-dependent dephosphorylation at synapses may contribute to the compensatory burst of endocytosis after a secretory stimulus.

Recent studies have implicated several cytosolic proteins besides clathrin and the clathrin adaptor AP-2 in clathrin-mediated endocytosis, including the endocytosis of synaptic vesicles in nerve terminals (1–5). Two such proteins are Eps15 and epsin (6, 7). Eps15 was first identified as an endogenous substrate for EGFR (E) receptor kinase (8) and was subsequently found to be an interacting partner for the "appendage domain" of the AP-2 subunit α-adaptin. Binding of Eps15 to AP-2 is mediated by its COOH-terminal region (9, 10), whereas the NH2-terminal region of Eps15 includes three Eps15 homology (EH) domains (11). Via these modules, Eps15 binds proteins with the consensus amino sequence NPF (12). Epsin, which contains three NPF motifs in its COOH-terminal region (NPF domain), is a major binding partner for Eps15 (7). Its NH2-terminal portion comprises an evolutionary conserved domain of unknown function, the ENTH domain (epsin NH2-terminal homology domain), whereas its central region, which contains eight DPW repeats (DPW domain), binds the appendage domain of α-adaptin at a site that overlaps with the Eps15-binding site (7). Perturbation of the interactions of both Eps15 and epsin with AP-2, as well as disruption of the function of both proteins by antibody injection, block clathrin-mediated endocytosis (7, 13–16). It was proposed that Eps15 and epsin play an important role in clathrin-mediated endocytosis, possibly by participating in dynamic rearrangements of the clathrin coat during bud invagination and fission (7, 17, 18).

Clathrin-mediated endocytosis is blocked during mitosis. In mitotic cells clathrin coats assemble, but their invagination is impaired (19, 20). The identification of substrates of mitotic kinases responsible for this effect may therefore shed new light on the still elusive mechanisms underlying the invagination reaction. Epsin is highly homologous to the Xenopus mitotic phosphoprotein MP90, which was identified in a screen for substrates of mitotic kinases (21), and contains a single putative consensus site for Cdc2 kinase, which is conserved in mammalian epsin. These considerations prompted us to investigate whether epsin undergoes mitotic phosphorylation. We report here that both epsin and Eps15 are phosphorylated in mitosis and that their phosphorylation inhibits binding to the clathrin adaptor AP-2. We also report that both epsin and Eps15, like other accessory proteins of clathrin-mediated endocytosis, undergo stimulation-dependent dephosphorylation in nerve terminals (22–25), with a resulting increase in their binding to each other and to AP-2. Their dephosphorylation may facilitate endocytosis of synaptic vesicle membranes following an exocytic burst.

MATERIALS AND METHODS

Cells and Reagents—B82 mouse fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 20% calf serum and 5 mM methotrexate. Antibodies directed against epsin, Eps15, amphiphysin 1, and GST fusion proteins of α-adaptin and the DPW domain of epsin were previously described (7). Antibody against α-adaptin was purchased from Sigma.

Analysis of Interphase and Mitotic Cells—B82 mouse fibroblasts were grown to 80–80% confluency and synchronized with 50 ng/ml nocodazole for 4 h. Mitotic cells were collected by detaching rounded up cells. Interphase (G1 phase) cells were obtained by washing away the nocodazole from the mitotic cells, replating cells in fresh medium, and allowing cells to grow for 4 h before harvesting. Particulate and soluble fractions from these cells were obtained as described (7). Cells extracts...
for affinity purification were prepared by lysing cells in 50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl₂, 5 mM EGTA, 10% glycerol, protease inhibitors (100 µg/ml aprotinin, 100 µg/ml leupeptin, 100 µg/ml pepstatin, 100 µg/ml antipain, and 1 mM PMSF), and phosphatase inhibitors (1 mM sodium orthovanadate, 2 µM cyclosporin, and 100 µM okadaic acid) for 15 min on ice followed by centrifugation.

**Generation of a Mutant DPW Domain**—The mutant DPW domain was obtained by polymerase chain reaction-based site-directed mutagenesis. A pair of primers harboring the serine 328 to aspartate mutation were generated: 5'-GACCCCTGGGGAGGTGATCCT-3' and 5'-AGGATCACCTTCCCAAGGCTC-3'. Using 5'-GACCCCTGGGGAGGTGATCCT-3' together with 5'-AAACCGAATTCGGATCCGCTTGAGGTGATCCT-3' and 5'-AAACCGAATTCGGATCCGCTTGAGGTGATCCT-3' together with 5'-AGGATCACCTTCCCAAGGCTC-3', two cDNA fragments were obtained and purified by a QIAGEN kit (QIAGEN). These two DNA fragments were used as both primers and templates in a standard polymerase chain reaction to generate a full-length mutant DPW domain, which was subsequently cloned into the PGE831 vector (Amersham Pharmacia Biotech). The sequence of the mutant DPW domain was confirmed by standard double-strand sequencing.

**p34<sup>cdc2</sup>-Cyclin B Kinase Reaction**—10 µg of recombinant wild type or mutant DPW domain were obtained from a GST-DPW fusion protein by thrombin (Novagen) cleavage and incubated at 30 °C for 30 min with 3 mM ATP, 5 mM MgCl₂, 1 mM CaCl₂, 100 nM okadaic acid, and 50 nM p34<sup>cdc2</sup>-cyclin B kinase (26) (a kind gift of M. Solomon, Yale University) in the presence of 0.25 µCi/µl [γ<sup>32</sup>P]ATP, 0.4 mM ATP, 15 mM MgCl₂, 20 mM EGTA, 10 mM dithiothreitol, 80 mM potassium β-glycerophosphate (pH 7.3), and 1 mg/ml ovalbumin. The reaction was stopped by addition of a 20-fold excess of 10 mM Hepes (pH 7.4), 150 mM NaCl, and 5 mM EDTA.

**Phosphorylation of Brain Cytosol**—Brain cytosol was prepared by homogenizing rat brains in 2 volumes of 10 mM Hepes (pH 7.4), 1 mM EDTA, and a protease inhibitor mixture (3 µg/ml each of aprotinin, antipain, leupeptin, and pepstatin). The lysate was centrifuged at 100,000 × g for 2 h, and the resulting supernatant (cytosol) was desalted on Sephacryl G-25 (Amersham Pharmacia Biotech) into 20 mM Hepes (pH 7.4), 120 mM KCl, and 1 mM MgCl₂ at room temperature. The desalted cytosol was incubated at 30 °C for 30 min with a general protein kinase inhibitor (K252a) (dephospho-cytosol) or with 5 mM ATP, 100 units of calf intestinal phosphatase (Boheringer Mannheim) for 1 h at 37 °C in a buffer containing 10 mM Tris-HCl (pH 8), 10 mM MgCl₂, 50 mM NaCl, 0.1% Nonidet P-40, 100 µg/ml aprotinin, 100 µg/ml leupeptin, and 1 mM PMSF. Treatment with phosphorylation inhibitors was performed with 10 mM sodium orthovanadate, 50 mM sodium fluoride, and 20 mM sodium pyrophosphate.

**Miscellaneous Procedures**—Affinity purification of brain extracts and experiments on intact synaptosomes were performed as described previously (27).

**RESULTS**

We investigated whether mammalian epsin undergoes phosphorylation in mitotic cells as predicted by the property of *Xenopus* MP90 to act as a substrate for mitotic kinases (21). The mitotic phosphorylation of MP90 was shown to lower its mobility in SDS-PAGE (21). Cell extracts from mitotic and interphase (G₁) phase B82 cells (a mouse fibroblastic cell line) were separated by SDS-PAGE, and the mobility of epsin was analyzed by Western blotting. As shown by Fig. 1A, epsin from mitotic B82 cells had a slower mobility than epsin from interphase cells. A similar shift was observed for Eps15. A putative Cdc2 phosphorylation site (28) is present in the COOH-terminal region of Eps15 (threonine 779 of mouse Eps15 (8)), which is the α-adaptin-binding region.

To confirm that the electrophoretic shifts were due to phosphorylation, epsin and Eps15 were immunoprecipitated from interphase and mitotic cell extracts. The immunoprecipitates obtained from mitotic extracts were then incubated with or without alkaline phosphatase or with both alkaline phosphatase and protein phosphatase inhibitors (F, I.) as indicated.

**FIG. 1. Mitotic phosphorylation of epsin and Eps15 in fibroblastic B82 cells.** A, epsin and Eps15 Western blots of total extracts of interphase (G₁) and mitotic (M) cells demonstrating the upper mobility shift of the two proteins. B, epsin and Eps15 immunoprecipitates generated from Triton X-100 extracts from interphase (G₁) and mitotic (M) B82 cells were processed by Western blotting after incubation in the absence or in the presence of alkaline phosphatase (Alk. P.) or alkaline phosphatase and protein phosphatase inhibitors (F, I.) as indicated.

**FIG. 2. The mitotic phosphorylation of epsin and Eps15 inhibits their interaction with α-adaptin and decreases their recovery in particulate fractions.** A, Western blots of the proteins indicated, whole (S) and particulate (P) fractions from interphase (G₁) and mitotic (M) B82 cells. B, Triton X-100 extract of interphase and mitotic B82 cells was affinity-purified on the immobilized appendage domain (AD) of α-adaptin, and the bound material was reacted by Western blotting.
Regulated Phosphorylation of Epsin and Eps15

Fig. 3. Epsin is an in vitro substrate for the Cdc2 kinase, and its phosphorylation inhibits binding to α-adaptin. A, wild type (wt) and S326D DPW domains of epsin were incubated with purified Xenopus p34\(^{cdk2}\)-cyclin B complex and separated by SDS-PAGE. The figure shows autoradiography and immunolabeling by an alkaline phosphatase method of the two proteins. B, the wild type DPW of epsin was phosphorylated in vitro by the p34\(^{cdk2}\)-cyclin B complex and subsequently affinity-purified on the bead-immobilized appendage domain of α-adaptin. The figure shows \(^{32}P\) autoradiograph and anti-epsin Western blotting of the phosphorylated starting material (SM) and of the material bound (B) and not bound (NB) by the beads. C, a Triton X-100 rat brain extract (SM, starting material) was affinity-purified on three different amounts (3, 6, and 9 μg) of immobilized GST fusion proteins of wild type and S326D DPW domains of rat epsin, and bound α-adaptin was revealed by Western blotting.

Epsin and MP90 contain a single putative phosphorylation site for Cdc2 kinase in the DPW domain (serine 328 of rat epsin) (7, 21). To determine whether this site acts as a substrate for the Cdc2 kinase and mediates the inhibition of AP-2 binding in mitosis, a mutant DPW domain of rat epsin was generated harboring a S328D mutation. Purified wild type and mutant DPW domains were incubated with \(\gamma^{-32}P\)ATP in the presence of the purified Xenopus p34\(^{cdk2}\)-cyclin B kinase complex (26). As shown in Fig. 3A, a very strong difference was observed in the \(^{32}P\) incorporation of the two proteins, indicating that serine 328 is a key substrate site for the kinase in vitro. Furthermore, affinity purification on the immobilized α-adaptin appendage domain of the \(^{32}P\)-labeled wild type DPW domain revealed no binding of the \(^{32}P\)-labeled protein, proving that its phosphorylation by Cdc2 kinase blocks the interaction (Fig. 3B). Finally, as shown by Fig. 3C, affinity purification of a brain cytosolic extract on wild type and mutant DPW domains demonstrated a significant decrease in the binding of α-adaptin to the mutant domain, confirming that the introduction of an acidic charge at position 328 affects the epsin-AP-2 interaction.

Fig. 4. Epsin and Eps15 undergo stimulation-dependent dephosphorylation in brain, and their phosphorylation in brain extracts decreases the interaction with AP-2. A, Western blots of total proteins of rat synaptosomes incubated in vitro for 1 min in the control condition or in the presence of 55 mM K\(^{+}\). B, epsin immunoprecipitates generated from rat brain dephtospho- (D) and phospho- (P) cytosol (see “Materials and Methods”) were processed by anti-epsin Western blotting after incubation in the absence or in the presence of alkaline phosphatase (Alk. P.) or alkaline phosphatase and protein phosphatase inhibitors (P.I.) as indicated. C, rat brain dephospho- (black bars) and phospho-cytosol (shaded bars) was affinity-purified on three different amounts of immobilized α-adaptin appendage domain, and the amount of bound epsin and Eps15 was determined by Western blotting after incubation in the absence or in the presence of alkaline phosphatase (Alk. P.) or alkaline phosphatase and protein phosphatase inhibitors (P.I.) as indicated. D, starting material used for the immunoprecipitations. We investigated whether epsin and Eps15 as well undergo dephosphorylation. Fig. 4A shows immunoblots of rat synaptosomes incubated for 1 min in either control buffer or high K\(^{+}\) buffer. A downward shift of epsin in depolarized synaptosomes can be seen. This shift correlates with the concomitant downwards shift of amphiphysin 1 previously shown to reflect its Ca\(^{2+}\)-dependent dephosphorylation (23). In control synaptosomes, Eps15 migrated as a doublet. Depolarization resulted in a decreased immunoreactivity on the upper bands with a corresponding increase of the immunoreactivity in the lower band, suggesting that Eps15 as well undergoes depolarization-dependent dephosphorylation.

The phosphorylation of amphiphysin, dynamin 1, and synaptotagmin 1 in brain extracts inhibits their property to interact with partners proteins implicated in endocytosis (25). The effect of epsin and Eps15 phosphorylation in brain extracts on their interaction with the AP-2 subunit α-adaptin was therefore investigated. Brain cytosolic extracts were incubated in either phosphorylating or dephosphorylating conditions as described (25). Western blots of anti-epsin immunoprecipitates generated at the end of these incubations demonstrated an upper mobility shift of epsin in the phosphorylation conditions that could be reversed by treatment of the immunoprecipitates.
with alkaline phosphatase (Fig. 4B) consistent with the interpretation that the mobility shift of epsin and Eps15 observed in Fig. 4A is due to dephosphorylation. Aliquots of the phospho- and dephospho-extracts were then incubated with the appendage domain of α-adaptin, and the bound material was analyzed by Western blotting. Quantitation of the blots demonstrated that binding to α-adaptin is inhibited by phosphorylation (Fig. 4C).

We next performed immunoprecipitation experiments from these extracts to test the effect of phosphorylation on the endogenous interactions within the cytosolic extract of epsin and Eps15 with AP-2. In both anti-Eps15 and anti-epsin immunoprecipitates coprecipitation of AP-2 was clearly decreased by the previous phosphorylation of the extracts (Fig. 4D). This result is consistent with an inhibitory effect of the phosphorylation of epsin and Eps15 on their interaction with AP-2, although they do not rule out a contribution of phosphorylation of AP-2 itself to their reduced interaction.

DISCUSSION

The results of this study demonstrate that the interaction of Eps15 and epsin with the α-adaptin subunit of AP-2 is modulated by phosphorylation.

Both Eps15 and epsin undergo phosphorylation in mitosis, a stage of the cell cycle where clathrin-mediated endocytosis is blocked (19, 20). In mitosis, clathrin-coated pits are still present, but early stages of clathrin-coated pits (shallow domes and wide necks) predominate over late stages (narrow neck). This observation led to the speculation that invagination of clathrin-coated pits (shallow domes and wide necks) predominate over late stages (narrow neck). This may result from dephosphorylation (22, 23, 25, 30, 31). Based on the recent observation led to the speculation that invagination of clathrin-coated pits (shallow domes and wide necks) predominate over late stages (narrow neck). This may result from dephosphorylation. Aliquots of the phospho- and dephospho-extracts were then incubated with the appendage domain of α-adaptin, and the bound material was analyzed by Western blotting. Quantitation of the blots demonstrated that binding to α-adaptin is inhibited by phosphorylation (Fig. 4C).

Finally, dephosphorylation by a Ca²⁺-dependent phosphatase calcineurin (32) produced by depletion of internal Ca²⁺ (34). Eps15 is a substrate for EGF receptor kinase (8). We have found that epsin as well undergoes phosphorylation in response to EGF stimulation of fibroblastic cells, although this phosphorylation does not occur on tyrosine residues, indicating an indirect effect of EGF receptor kinase.² Based on our preliminary experiments, however, the EGF-dependent phosphorylation of Eps15 and epsin does not appear to decrease their interaction with AP-2, suggesting that these phosphorylation reactions play a role in cell physiology different from those described in this study.

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