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Regulatory Mechanisms of Dynamin-Dependent Endocytosis

Kohji Takei*, Yumi Yoshida and Hiroshi Yamada

Department of Neuroscience, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikatacho, Okayamashi, Okayama 700-8558

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Extensive studies on endocytosis in the last decade have resulted in identification of several key molecules that function in clathrin- and dynamin-dependent endocytosis. Most endocytic molecules contain multiple binding motifs that mediate protein–protein or protein–lipid interactions, which must be modulated spatially and temporally during endocytosis. Regulation of these interactions is the molecular basis of regulatory mechanisms involved in endocytosis. This review first describes current models of the mechanism of dynamin-dependent fission, then introduces several mechanisms that modulate dynamin GTPase activity and dynamin-dependent vesicle formation. Such mechanisms include regulation by inositol phospholipids, especially phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], and their metabolism. It concludes by describing the regulation of dynamin 1 by its binding partner, amphiphysin 1, and regulation by cyclin-dependent kinase 5 (Cdk5)–dependent phosphorylation of dynamin 1 and amphiphysin 1. These mechanisms help endocytic molecules to function properly, and cooperatively regulate dynamin-dependent endocytosis.

Key words: amphiphysin, clathrin, cyclin-dependent kinase 5, endocytosis, dynamin.

Clathrin-mediated endocytosis is initiated by the recruitment of clathrin coat components to the plasma membrane, followed by the invagination of the membrane to form a clathrin-coated pit. It is completed by the fission reaction, in which dynamin GTPase plays a key role (*1*). Extensive studies on clathrin/dynamin-mediated endocytosis in the last decade have identified two categories of novel proteins that function in endocytosis: clathrin coat components such as clathrin, and accessory proteins (*2*). Endocytic molecules generally contain multiple binding motifs that mediate protein–protein or protein–lipid interactions. Spacio-temporal regulation of these interactions during endocytosis constitutes the molecular basis for the process of endocytosis.

Dynamin was first isolated from brain as a microtubule-binding protein (*3*). The protein was then identified as a GTPase and as a mammalian homologue of the *shibire* gene product in *Drosophila*. As mutations in the *shibire* gene in *Drosophila* resulted in blockage of endocytosis, dynamin was proposed to function in endocytosis (*4*, *5*). Implication of dynamin in the fission of clathrin-coated pits was demonstrated by electron microscopic observation that dynamin polymerizes to form rings and spirals around the neck of the pits (*6*). The function of dynamin in the fission process was further demonstrated in a simple *in vitro* experiment, in which large unilamellar liposomes were incubated with purified dynamin. Dynamin bound to and tubulated the liposomes. Addition of GTP to the reaction mixture resulted in fragmentation of the tubules, clearly demonstrating the GTP-dependent fission activity of dynamin (*7*, *8*).

As shown in Fig. 1, the GTPase module of dynamin is located at its N-terminus and is followed by several functional domains: the pleckstrin homology (PH) domain, the GTPase effector domain (GED), and the C-terminal proline/arginine-rich domain (PRD). The PH domain binds to PtdIns(4,5)P_2, and the interaction strongly stimulates the dynamin GTPase activity (*9*). The region following the PH domain interacts with the GTPase module of adjacent dynamin molecules within dynamin polymers, and this interaction also stimulates dynamin GTPase activity. Because it functions as a GTPase-activating protein, this domain is referred to as the GTPase effector domain or GED (*10*). The C-terminal proline/arginine-rich domain (PRD) binds a variety of proteins containing Src-homology 3 (SH3) domain (*11*). The domain structures and related interactions are schematized in Fig. 1. This review first describes current models of dynamin-dependent fission, then discusses several important factors that affect the dynamin GTPase activity.

Mechanism of dynamin-dependent vesicle formation

Fission of clathrin-coated pits is mediated by dynamin, which polymerizes into rings around the neck of the pits. However, its precise mechanism of action in the fission remains controversial. *In vitro* studies have shown that dynamin binds to lipids, deforms the lipid bilayers into narrow tubules, and fragments them in a GTP-hydrolysis–dependent manner (*7*, *8*, *12*). Thus, one model proposes that dynamin acts as a mechanoenzyme: GTP hydrolysis causes a conformational change of dynamin, which generates the driving force of the fission reaction. The conformational change is thought to cause constriction of the dynamin ring, by which the coated pit is pinched off (pinchase model). The recent observation that dynamin rings are smaller in diameter in presence of GTP strongly supports the pinchase model (*13*). An alternative mechanoenzyme model assumes extension of the dynamin spiral, by which the coated pit is popped off (popase model) (*14*). In either case, fission is thought to

*To whom correspondence should be addressed. Phone: +81-86-235-7120, Fax: +81-86-235-7126, E-mail: kohji@md.okayama-u.ac.jp

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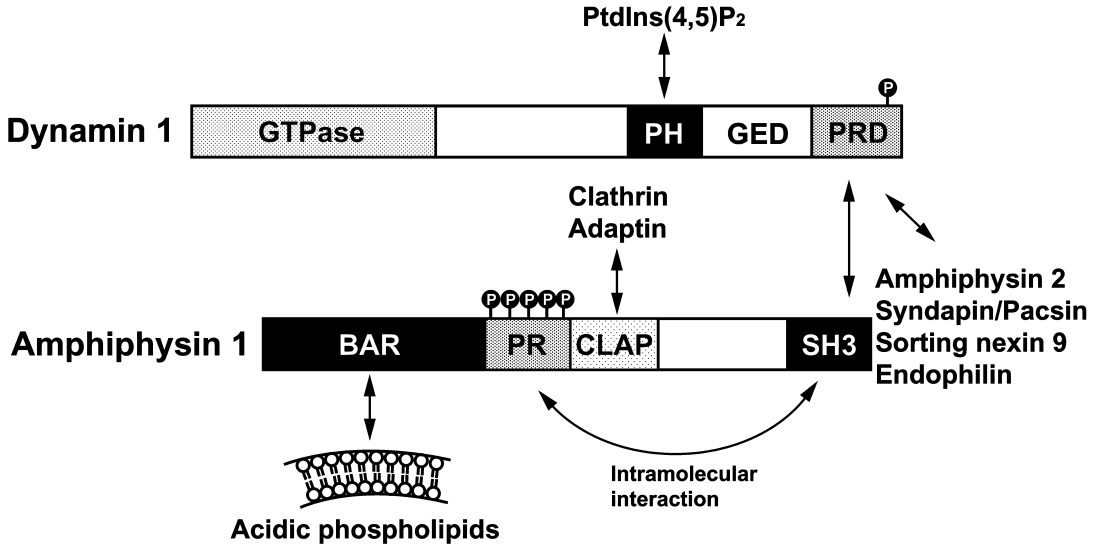

Fig. 1. **Domain organization of dynamin 1 and amphiphysin 1 and their interaction with other molecules.** PH, pleckstrin homology domain; GED, GTPase effector domain; PRD, proline/arginine-rich domain; BAR, BIN/amphiphysin/Rvs domain; PR, proline-rich stretch; CLAP, clathrin AP-2–binding domain; SH3, Src-homology 3 domain; P, phosphorylation sites.

occur upon GTP hydrolysis, and therefore the fission activity correlates with the dynamin GTPase activity.

Another model proposes that dynamin acts as a molecular switch in the same way as small GTPases, *i.e.*, dynamin is active only in its GTP-bound state. This model assumes downstream effectors, which are activated by the "active" dynamin and perform the fission reaction (*15*, *16*). However, such effector molecules currently remain unidentified.

PtdIns(4,5)P_2 metabolism regulates dynamin-dependent endocytosis

Interactions of dynamin with acidic phospholipids stimulate the dynamin GTPase activity (*17*). Among acidic phospholipids, PtdIns(4,5)P_2, is known to bind specifically to the PH domain of dynamin (*9*) and stimulate its GTPase activity more potently than other acidic phospholipids. Interaction between dynamin and acidic phospholipids might be implicated in recruitment of dynamin on the membrane, raising the local concentration of dynamin. Dynamin tends to oligomerize at high concentrations, and self-assembly of dynamin synergistically stimulates its GTPase activity (*18*). The presence of PtdIns(4,5)P_2 stimulates dynamin-dependent vesicle formation *in vitro* (*19*) and *in vivo* (*20*).

A number of other endocytic proteins interact with inositol phospholipids. Clathrin adaptor protein 2 (AP-2), a tetramer composed of α, β2, μ2, and σ2 subunits, binds to polyphosphoinositides (*21*). The interaction is mediated by positively a charged region containing multiple lysine residues in the α and μ2 subunits, as revealed by crystallographic study of AP-2 bound to inositol polyphosphate (*22*). Neuron-specific adaptor protein AP180 and its homologue expressed in multiple tissues, CALM (clathrin assembly lymphoid myeloid leukemia protein), also bind to PtdIns(4,5)P_2 at their N-terminal ANTH (AP180 N-terminal homology) domain, which contains an K(X)$_9$KX(K/R)(H/Y) motif (*23*, *24*). Epsin isoforms share a structurally similar domain, epsin N-terminal homology (ENTH) domain, which also binds to PtdIns(4,5)P_2 (*25*). Proteins containing the ANTH/ENTH domain may function in recruitment of cargo molecules to clathrin-coated pits independently or in concert with AP-2 (*26*).

Some endocytic proteins catalyze reactions in inositol phospholipid metabolism. Synaptojanin 1, an inositol 5-phosphatase, and phosphatidylinositol 4-phosphate 5-kinase Iγ [PI(4)P5KIγ], which is the major isoform in brain, catalyze degradation and synthesis of PtdIns(4,5)P_2, respectively. These proteins are both enriched in the synapse, and their implication in synaptic vesicle endocytosis has been reported (*27*, *28*). Other factors that indirectly regulate PtdIns(4,5)P_2 metabolism could be involved in endocytosis. For example, ADP-ribosylation factor 6 (ARF6) directly binds to PI(4)P5KIγ and indirectly activates its activity. A GTP-bound active form of ARF6 stimulates phospholipase D, which catalyzes phosphatidic acid synthesis. Phosphatidic acid in turn stimulates PI(4)P5KIγ-mediated PtdIns(4,5)P_2 synthesis, and this cascade finally results in increased recruitment of clathrin coat proteins (*29*).

Amphiphysin regulates dynamin GTPase activity

Amphiphysin, a major binding partner of dynamin, has recently been found to regulate dynamin GTPase activity as well as dynamin-dependent vesicle formation (*30*). Amphiphysin, which is present in brain primarily as a homo- or hetero-dimer of two similar isoforms, amphiphysin 1 and 2, is comprised of several functional domains (Fig. 1). The C-terminal SH3 domain of amphiphysin is a binding motif for PRD of dynamin, and the SH3 domain–mediated interaction is probably important physiologically in nerve terminals, because disruption of the interaction by microinjection of the SH3 domain or its peptide that interacts with dynamin results in blockage of synaptic vesicle recycling (*31*).

The N-terminal portion of amphiphysin contains a BAR (BIN/Amphiphysin/Rvs) domain, which mediates homo- and hetero-dimerization (*32*, *33*) as well as binding

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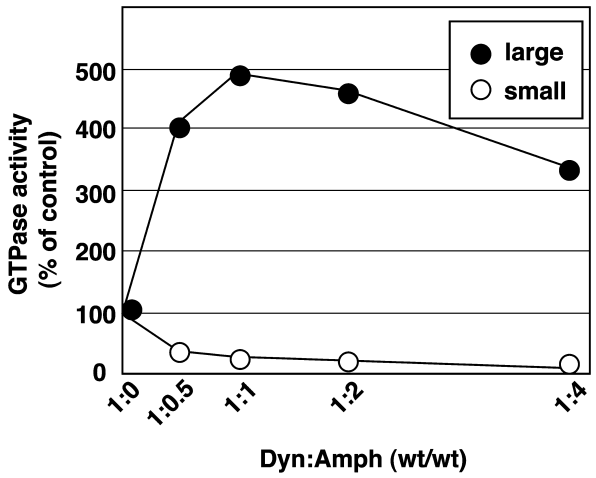

Fig. 2. **Stimulation of dynamin GTPase activity by amphiphysin 1.** Dynamin 1 and increasing amounts of amphiphysin 1 were incubated either with large unilamellar liposomes (closed circles) or with sonicated small liposomes (open circles), in the presence of GTP. Composition of liposomes is 74% Folch Fraction, 20% cholesterol and 6% PtdIns(4,5)P_2 (modified from Ref. *28*).

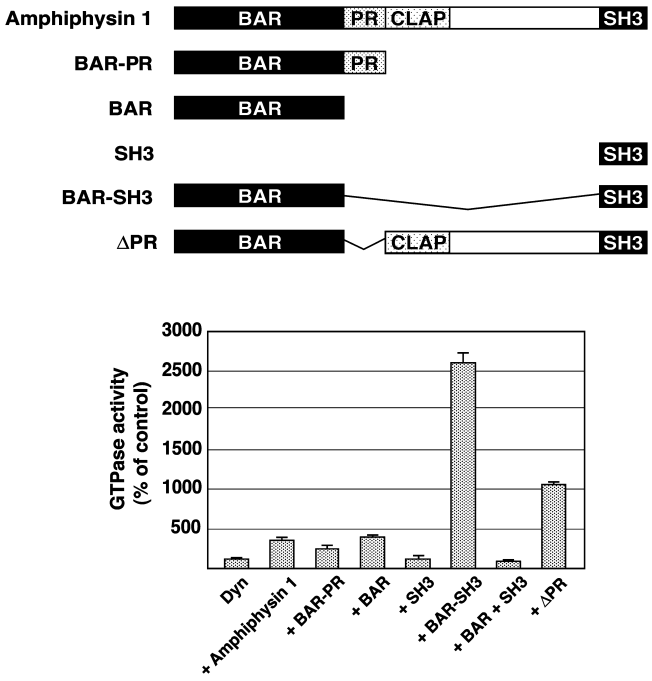

Fig. 3. **Amphiphysin 1 domains required for stimulation of dynamin GTPase activity.** Constructs used for assays and dynamin GTPase activity in the presence of each construct are shown. All incubations were carried out in the presence of large unilamellar liposomes containing 74% Folch Fraction, 20% cholesterol and 6% PtdIns(4,5)P_2. Dyn:Amph = 1:2 (mol/mol) (modified from Ref. *28*).

to acidic phospholipids (*34*). Amphiphysin, like dynamin, binds to and tubulates liposomes (*34*) and can also generate narrow plasma membrane tubules in living cells when overexpressed (*35*). The BAR domain is responsible for these membrane-tubulating activities. The three-dimensional structure of the BAR domain of amphiphysin has recently been determined by X-ray crystallographic analysis: it was found to have a crescent-like shape and consist of a dimer of triple-helices arranged in anti-parallel (*35*). This configuration represents a module conserved in a variety of proteins (*36*). The concave portion of this module is proposed to mediate interaction with membrane lipid (*35*), in cooperation with an amphipathic N-terminal helix (*37*). The BAR domain preferentially binds to relatively small liposomes, suggesting an additional role of the BAR domain as a curvature-sensor (*35*). In view of the drastic change of plasma membrane curvature during coated pit formation and the following fission, curvature-sensing properties of the BAR domain may play a role in sequential recruitment and dissociation of cytosolic factors during vesicle formation (*38*).

Dynamin-dependent vesicle formation can be reconstituted *in vitro* by incubating large unilamellar liposomes (≥1 μm in diameter) with brain cytosol in the presence of ATP and GTP (*19*). Dynamin-dependent membrane fission can also be reconstituted in a simple experimental system, in which liposomes are incubated with purified dynamin and GTP (*7*, *8*). Using these systems, the function of amphiphysin in dynamin-dependent endocytosis has recently been examined, and amphiphysin has been found to enhance the vesicle formation. Further analysis has revealed that the dynamin GTPase activity is strongly enhanced by amphiphysin, and both its BAR and SH3 domains are required for this effect. Interestingly, this stimulatory effect of amphiphysin is highly dependent on the presence of large liposomes (Fig. 2) (*30*). Dynamin and amphiphysin co-assemble into rings even without liposomes (*34*), while dynamin alone does not form rings in the same physiological buffer conditions. Formation of the dynamin/amphiphysin ring also required both BAR and SH3 domains. Then how are the dynamin/amphiphysin ring formation and the stimulatory effect of amphiphysin on dynamin GTPase activity coupled? Dynamin molecules are polymerized in the rings and in close enough proximity for the GED domain of one molecule to make contact with the GTPase domain of a neighboring molecule, resulting in stimulation of dynamin GTPase activity (*10*). The presence of large liposomes would allow formation of lipid tubes surrounded by dynamin/amphiphysin rings, on which the dynamin GTPase activity is optimized.

Between the BAR and SH3 domains, there are a proline-rich stretch (PR) and a clathrin- and AP-2–binding domain (CLAP) (*37*, *39*). PR is proposed to mediate intramolecular binding to the C-terminal SH3 domain, and to indirectly regulate binding between the dynamin PRD and the amphiphysin SH3 domain (*37*). The PR region contains five phosphorylation sites for cyclin-dependent kinase 5 (Cdk5) (*40*), and therefore the phosphorylation might regulate the intramolecular interaction in amphiphysin and the dynamin-amphiphysin interaction. PR is followed by binding sites for clathrin and for the AP-2, in a region termed CLAP (*39*, *41*, *42*). Deletion of PR or the whole middle domain containing PR and CLAP from amphiphysin dramatically potentiates its stimulatory effect on the dynamin GTPase activity (Fig. 3) (*30*). The dynamin GTP activity might be negatively regulated by interacting molecules on these domains.

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Amphiphysin 1 knockout mice demonstrate relatively mild defects in synaptic vesicle recycling (*27*), and amphiphysin 1 is not enriched in *Drosophila* neurons (*43–45*). These reports question the significance of amphiphysin 1 in the regulation of dynamin's activity. It is possible that other dynamin-binding molecules containing both BAR and SH3 domains, such as amphiphysin 2, endophilins, syndapin/pacsin, and sorting nexin 9 (*46–50*), function in place of amphiphysin 1. Neuronal isoforms of these proteins are often enriched in the synapse. A muscle isoform of amphiphysin 2, BIN1, stimulates dynamin 2–dependent endocytosis, suggesting that regulation of dynamin by amphiphysin is not specific to the synapse. However, direct effects of most of these proteins on dynamin remain to be elucidated.

Regulation of dynamin-dependent vesicle formation by phosphorylation

Several synaptic proteins undergo dephosphorylation upon depolarization of the synapses, and such proteins are collectively termed dephosphins (*51*). The dephosphins lack structural similarity to one another but are similar in their function in endocytosis. Two of the dephosphins have turned out to be dynamin 1 and amphiphysin 1. Although several kinases for dynamin have been reported based on *in vitro* studies, Cdk5 has recently been reported to phosphorylate both dynamin 1 and amphiphysin 1 both *in vivo* and *in vitro*, and the phosphorylation sites have been determined (*40*, *52*, *53*).

Cdk5 phosphorylates threonine 780 in PRD of dynamin 1, and serine residues 261, 272, 276, and 285 and threonine 310 in amphiphysin 1 (Fig. 1) (*40*). The phosphorylation of dynamin PRD reduces its ability to interact with the amphiphysin 1 SH3 domain. Due to the reduced binding, phosphorylated dynamin and amphiphysin form fewer rings and generate many fewer vesicles *in vitro*. Conversely, synaptic vesicle recycling is enhanced in cultured neurons treated with a specific inhibitor for Cdk5, and in neurons from mice deficient in p35, an activator for Cdk5. Thus, Cdk5-dependent phosphorylation negatively regulates dynamin-dependent endocytosis (*40*). Phosphorylation sites in amphiphysin 1 are all located in PR, which has been proposed to be implicated in intramolecular interaction with the SH3 domain. Therefore, it is possible that the phosphorylation directly regulates the intramolecular interaction in amphiphysin, which in turn regulates interaction with dynamin.

In conclusion, the activity of dynamin is regulated by the binding of molecules such as PtdIns(4,5)P_2 and amphiphysin. Cdk5-dependent phosphorylation of dynamin and amphiphysin is also a regulatory factor for dynamin, as the phosphorylation modulates the interaction between these molecules. Since several other proteins that contain an SH3 domain and/or are phosphorylated by Cdk5 function in endocytosis, the complete scheme of the regulatory mechanism of dynamin would be much more complex.

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