Role of the Basic, Proline-rich Region of Dynamin in Src Homology 3 Domain Binding and Endocytosis

(Received for publication, August 26, 1996, and in revised form, January 6, 1997)

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The GTPase dynamin has been implicated in the regulation of the scission of coated and noncoated pits during the early stages of endocytosis. Various macromolecules including microtubules, acidic phospholipids, and Src homology 3 (SH3) domains have been shown to interact with the basic, proline-rich region of dynamin and act as effectors of its GTPase activity. The interaction of dynamin with SH3 domain-containing proteins is of particular interest since SH3 domains are known to mediate protein-protein interactions in signal transducing complexes. In this study, we have systematically defined three distinct SH3 binding regions within the dynamin proline-rich C terminus. These binding regions conform to either the Class I or II SH3 binding consensus sequence, and their location coincides with a region previously shown to be important in the colocalization of dynamin with clathrin-coated pits. Two of these SH3 binding regions are well conserved among four dynamin isoforms, and we show that the overall binding pattern for SH3 domains is comparable among the isoforms. We also demonstrate that neither transferrin nor platelet-derived growth factor receptor uptake is restored upon removal of the basic, proline-rich region in a dominant negative dynamin GTP binding mutant. Together with earlier evidence from our laboratory, these findings suggest that SH3 domains may serve to target dynamin to coated pits and are not the direct targets of dominant inhibitory mutants of dynamin.

Dynamin is a 100-kDa GTPase responsible for controlling the initial stages of endocytosis (1–4). It has been found to assemble into helical collars around the necks of clathrin-coated pits (5) and is thought to regulate the formation of clathrin-coated and possibly other endocytic vesicles (5–7).

Dynamin belongs to a functionally diverse family of high molecular weight GTPases, some of which are involved in other aspects of membrane sorting (8, 9). In the yeast Saccharomyces cerevisiae three different dynamin-related genes have been identified, VPS1 (8, 10), MGM1 (11), and DNMI (9), none of which appear to be functionally equivalent to dynamin. Homologues of these genes have not yet been identified in higher organisms. Instead, three dynamin genes have so far been found in mammals, which encode a neuron-specific form (the originally described form of dynamin, now termed dynamin-1), a ubiquitously expressed form (dynamin-2) (12, 13), and a testis-enriched form (dynamin-3) (14). The products of the three dynamin genes share significant primary sequence homology (~70% identity), with the greatest divergence toward the C terminus, suggesting that this region of the molecule may be important in differentiating isoform function. Whether the three dynamin genes have common or distinct functions is uncertain. Apparent alternate splicing is responsible for further dynamin complexity, such as the three alternative C-terminal sequences for dynamin-1 (4).

A variety of macromolecules have been identified that bind to dynamin and stimulate its GTPase activity, including microtubules (15), acidic phospholipids (16), and various Src homology 3 (SH3) domains (15, 17–20), which are known to mediate protein-protein interactions involved in signaling processes (21, 22). Considerable interest has been focused on the interaction with SH3 domains because of the involvement of both dynamin and a number of SH3 domain-containing proteins in cell surface receptor regulation (see, for example, Ref. 23). Which of the several dynamin interactions that have been identified occur in vivo remains unresolved. A recently identified SH3 domain-containing protein, amphiphysin, has been shown to copurify and to colocalize at the cellular level with dynamin, supporting a role for amphiphysin in particular in dynamin function in neuronal cells (24).

A number of structural domains can be identified within the dynamin primary sequence (25). In addition to the ~300-amino acid (a.a.) GTPase domain at the N terminus (1), dynamin contains a central pleckstrin homology domain (26–30) implicated in phosphotyrosylinositol 4,5-bisphosphate (31, 32) and heterotrimeric G-protein g subunit binding (33, 34). Downstream lie two short predicted coiled coil domains (25). The C-terminal ~100 a.a. represent a distinct domain with a very high pl (12.3–12.5) and proline content (32–33%) (1). Earlier work from our laboratory implicated the latter region in SH3 domain binding and GTPase regulation (15). Exposure of dynamin to papain released a 7–9-kDa C-terminal fragment, resulting in the loss of both binding of microtubules and SH3 domains and their ability to stimulate the dynamin GTPase activity. Recombinant SH3 domains from a number of proteins, including Grb2, phospholipase Cγ (PLCγ), and the p85 subunit of phosphotydirinositol 3-kinase, have also been found to bind prolinerich synthetic peptides corresponding to regions within the dynamin C-terminal domain. This analysis has not been done systematically, however, and the selection of sequences predated the full analysis of SH3 binding consensus sequence elements (17–20).

More recently, immunocytchemoanalysis of the distribu-

* This work was supported by National Institutes of Health Grant GM26701 (to R. B. V.) and postdoctoral training Grant HD07312-10 (to P. M. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: SH3, Src homology 3; a.a., amino acid(s); PLCγ, phospholipase Cγ; PIPES, 1,4-piperazinediethanesulfonic acid; PDGFR, platelet-derived growth factor receptor; GST, glutathione S-transferase.
tion of full-length and truncated forms of dynamin have indicated that the basic, proline-rich region is required for targeting dynamin to coated pits, thereby raising the possibility that targeting is mediated by SH3 domain-containing proteins (35). Our studies have also indirectly implicated the C-terminal domain in endocytosis; the effect of dominant inhibitory mutations in the GTPase domain of dynamin could be reversed by truncation of 188 amino acids from the dynamin C terminus (3). Although this region encompasses more than the basic proline-rich region of the molecule, these results were consistent with a role for sites near the C terminus of dynamin in “poisoning” interactions with other components of the endocytic pathway.

In the current study, we sought to define further the role of the proline-rich region in dynamin function, with a focus on the interaction with SH3 domains. We sought to identify the sites for SH3 domain binding in a systematic manner, to assess functional differences among dynamin isoforms, and to define distinct SH3 domain binding sites, at least two of which are shared among dynamin isoforms, and suggest that SH3 domains may be important for targeting dynamin to clathrin-coated pits.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Construction of the dominant negative K44E dynamin-1 mutant and the N272 dynamin-1 truncation mutant have been described previously (3). Mutagenesis by polymerase chain reaction was used to construct C-terminal deletions of the wild-type, K44E, and N272 dynamin-1 cDNAs. For the SH3 binding studies, polymerase chain reaction was also used to tag the C-terminal dynamin truncation mutants with the myc epitope (36) at the 5′-end. All mutant and epitope-tagged dynamin constructs were cloned into the mammalian expression vector pSVal (Pharmacia Biotech Inc.) and sequenced to verify the deletion sites and myc tag. The C-terminal alternate splice variant of dynamin-1 was cloned from a rat brain cDNA library (Stratagene). A partial dynamin-2 cDNA fragment, which was amplified from rat pancreas mRNA via reverse transcriptase-polymerase chain reaction with degenerate primers, was used as a probe to clone the entire dynamin-2 cDNA from a rat testis cDNA library (Stratagene). For the expression studies, the dynamin-2 cDNA was subcloned into pSVal.

Antibodies and Immunological Methods—The anti-myc monoclonal antibody was produced from the 9E10 hybridoma cell line (American Type Culture Collection). Isoform-specific rabbit polyclonal antibodies to dynamin-2, dynamin-3, and a C-terminal splicing variant of dynamin-1 were raised against peptides that corresponded to a.a. 858–870, a.a. 825–839, and a.a. 845–864, respectively (Research Genetics, Inc.) (4, 12, 14). Generation of an isoform-specific polyclonal antibody against the C terminus of the originally identified dynamin-1 protein (1) has been described elsewhere (referred to as antibody RA in Ref. 3). Generation and affinity purification of the R2 polyclonal antibody against a dynamin-1 fusion protein have also been reported (3). Horse radish peroxidase-conjugated donkey anti-mouse and anti-rabbit antibodies were from Pierce.

Western blots were typically blocked overnight in 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). All primary antibodies were diluted in 1% nonfat milk in TBST, and subsequent development of the blots was by chemiluminescence. Densitometric analysis was performed using a PDI model DNA 35 scanner interfaced to the Quantity One program (version 2.2) of the Protein-DNA Imagerway system (PDI, Inc.).

Preparation of Rat Tissue and COS-7 Cytosolic Extracts—Brain, heart, kidney, liver, lung, spleen, and testis were dissected from young adult male rats (46–48 weeks old; Charles River Laboratories) and homogenized (1:1, w/v) in extraction buffer (80 mM PIPES, pH 6.9, 5 mM EDTA, 10 mM MgCl₂, 5 mM EGTA, 0.25 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin) via three passes at 2000 rpm using a Potter-Elvejem Teflon pestle homogenizer. To obtain cytosolic extracts, the homogenates were first centrifuged at 3500 rpm at 4°C for 20 min to remove nuclei and cellular debris followed by clarification of the supernatants at 45,000 rpm at 4°C for 60 min. Protein concentrations were determined using the BCA assay (Pierce).

COS-7 cells were transiently transfected with the dynamin plasmids using lipofectamine according to the manufacturer’s instructions (Life Technologies, Inc.). After 36–48 h post-transfection, the cells were washed on ice with cold Dulbecco’s phosphate-buffered saline and then lysed in RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet 40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin). The lysates were centrifuged at 30,000 × g for 30 min at 4°C. The resulting cytosolic extracts were immediately used in coprecipitation assays. 

Coprecipitation Assays—The GST and GST fusion proteins consisting of the SH3 domains of Lyn, Csk, c-Src, and p85 or the entire Grb2 protein were expressed in bacteria by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 1 h at 37°C. Cell lysis was in STE buffer (10 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin) by two passes in a French press (American Instrument Co., Inc.) at 750 psi. Alternatively, cells expressing the GST-Grb2 fusion protein were resuspended in buffer consisting of 50 mM Tris, pH 7.5, 0.5 mM EDTA, 0.3 mM NaCl, 0.05% Triton X-100, and 0.1 mg/ml lysozyme and lysed by quickly freezing the suspension in liquid nitrogen followed by a slow thaw in a room-temperature water bath after which the lysate was exposed to DNase I. With either lysis method, the resulting lysates were centrifuged at 12,000 rpm at 4°C for 30 min to remove cellular debris. The proteins were purified using Sepharose 4B glutathione beads following the instructions (Pharmacia Biotech Inc.) (3). Purification of the fusion proteins was determined using the BCA assay. The GST-SH3 PLCγ fusion protein was from Upstate Biotechnology, Inc. The GST-SH3 amphiphysin protein was kindly provided by C. David and P. De Camilli (Howard Hughes Medical Institute, Yale University).

Recombinant GST-SH3 fusion protein or the GST protein were immobilized on Sepharose 4B glutathione beads in RIPA buffer (see above) at 4°C. Cytosolic extract from either rat brain tissue, rat testis tissue, or transfected COS-7 cells was then added to the protein-bound beads. Coprecipitation assays were carried out for 12 h at 4°C with end-over-end rotation, after which the beads were washed several times with cold RIPA buffer and boiled in Laemmlı SDS-sample buffer. The samples were resolved in 8% polyacrylamide-SDS gels, and dynamin was assayed by immunoblotting. Coprecipitation was quantitated by densitometry of the range of SH3 domain concentrations. For amphiphysin-SH3, binding of dynamin-1 and dynamin-2 was half-maximal at 3.5 μM and appeared to be saturated by 30 μM; 52% of dynamin-1 and 37% of dynamin-2 coprecipitated at the highest amphiphysin-SH3 concentration. In the case of Grb2, binding of dynamin-1 and dynamin-2 was half-maximal at 4.5 μM and appeared to be saturated at 20 μM; 32% of dynamin-1 and 51% of dynamin-2 coprecipitated at the highest Grb2 concentration. Routine binding assays were conducted well below saturation, typically using 2 μg of the SH3 domain-containing protein.

Endocytosis Assays—Endocytosis of fluorescein isothiocyanate-labeled transferrin in COS-7 cells was assayed as described previously (3). Platelet-derived growth factor receptor (PDGFR) internalization in human Hep G2 cells was also assayed using a monoclonal antibody to PDGFR (PDGFR-B2, Oncogene Sciences) according to previously reported procedures (37). A Zeiss Axioskop microscope was used in the immunofluorescence images.

RESULTS

Mapping of the SH3 Domain Binding Sites within Dynamin—To define the SH3 interaction sites within dynamin-1, a series of dynamin C-terminal truncation mutants were assayed for coprecipitation with recombinant GST-SH3 fusion proteins. These included the SH3 domains of c-Src, Lyn, the p55 subunit of phosphotyidylinositol 3-kinase, amphiphysin, Csk, and PLCγ as well as the entire Grb2 polypeptide. All of the SH3 domains bound to the full-length dynamin-1 protein (Fig. 1, lane C851) with the exception of Csk-SH3 (see Fig. 3). Deletion of the entire basic, proline-rich region completely abolished binding of all the SH3 domains to dynamin-1 (Fig. 1, lane C750), demonstrating that this region of the molecule was essential for its interaction with SH3 domains.

To identify specific interacting sites within the C-terminal 100 a.a. for SH3 domains, a series of C-terminal deletion mutations of dynamin-1 were evaluated for SH3 binding. Three different patterns were identified. Deletion of the last 12 a.a. of the dynamin-1 C terminus to a.a. 839, which partially removes a Class I SH3 binding consensus sequence (see Fig. 7A), did not
affect interactions between dynamin and SH3 domains (Fig. 1). However, deletion of the next 13 a.a. (to a.a. 827) abolished the binding of both Grb2 and amphiphysin to dynamin-1 (Fig. 1A, lane C826). Truncation of the dynamin C terminus to a.a. 808 resulted in loss of binding of PLC\(_{\gamma}\), p85, and Lyn (Fig. 1A, lane C808), and further deletions uncovered yet another binding region for the SH3 domains of c-Src and Fyn (Fig. 1B, lane C785). Thus, multiple distinct SH3 domain binding sites within the last 66 a.a. of the proline-rich domain could be discerned by this analysis.

**Binding of Dynamin Isoforms to SH3 Domains**—These findings clearly indicate that SH3 domains bind to dynamin-1 in a sequence-specific manner. However, comparison of the C-terminal sequences of four dynamin isoforms revealed that the binding sites were not equally well conserved (see Fig. 7B). Therefore, we tested the isoform specificity of SH3 domain binding.

For these experiments, antibodies were raised specifically against the C termini of dynamin-2, dynamin-3, and two alternate splicing variants of dynamin-1. As expected from the lack of homology between the antigenic sequences, each of the antibodies was isoform-specific as judged by Western analysis (Fig. 2). Coprecipitation of the dynamin isoforms from rat brain lysate blot. Averages of the densitometric measurements are shown in each graph, and the S.E. is represented as a function of the range of the measurements per data point.

**Fig. 2. Specificity of anti-dynamin isoform antibodies.** Polyclonal antibodies were raised against peptides unique to the C termini of two dynamin-1 isoforms, dynamin-2, and dynamin-3. Either various rat tissue cytosolic extracts (A) or cytosolic extracts of COS cells expressing the dynamin isoforms (B) were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting for the dynamin isoforms shown. Detection was by chemiluminescence. dynamin-1 refers to the originally identified dynamin-1 isoform with the C-terminal sequence \(\text{RITISDP} \) (1); dynamin-1* refers to the dynamin-1 splicing variant with the C-terminal sequence \(\text{SRSGASPSRPEFSPFPDL} \) (4).
Coprecipitation assays of dynamin isoforms with SH3 domains. GST-SH3 fusion proteins were immobilized on Sepharose 4B glutathione beads and incubated with either rat brain cytosolic extract (A) or transiently expressed dynamin-1 or dynamin-2 cytosolic extract (B). The precipitants were separated by 8% polyacrylamide-SDS gels and immunoblotted with the isoform-specific polyclonal antibodies. dynamin-1 and dynamin-1* refer to the same dynamin-1 C-terminal splicing variants as in Fig. 2.

Specificity of GST-Grb2 and GST-SH3 amphiphysin for dynamin-1 and dynamin-2 isoforms expressed in COS-7 cells. Cytosolic extract consisting of either dynamin-1 (A) or dynamin-2 (B) expressed in COS-7 was coprecipitated with increasing concentrations of either GST-SH3 amphiphysin or GST-Grb2 protein that had been immobilized on Sepharose 4B glutathione beads. The precipitants were separated in 10% polyacrylamide-SDS gels and immunoblotted with the isoform-specific polyclonal antibodies. dynamin-1 and dynamin-1* refer to the same dynamin-1 C-terminal splicing variants as in Fig. 2.

Effect on transferrin uptake of C-terminal deletions of a dominant inhibitory dynamin mutant. Transferrin uptake was assayed in COS-7 cells that had been transiently transfected with either the K44E/C746 or K44E/C733 C-terminal deletion constructs of the dominant negative K44E dynamin-1 mutant. Cells were double labeled using the affinity-purified R2 polyclonal antibody (3) for dynamin and fluorescein isothiocyanate-labelled transferrin. The K44E/C746 mutant lacks the basic, proline-rich region only, whereas the K44E/C733 mutant lacks both the basic, proline-rich region and part of a putative coiled coil domain.

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Role of the Dynamin Proline-rich Region in Endocytosis—To map the region of the dynamin C-terminal domain required for physiological binding partner of dynamin (24).

To confirm the specificity of dynamin binding in these experiments, we examined the precipitants by staining for total protein. COS-7 cytosolic extracts containing overexpressed dynamin-1 and dynamin-2 were used in these assays. As shown in Fig. 4, we observed coprecipitation of both dynamin-1 and dynamin-2 with the GST-SH3 proteins in Coomassie Blue-stained gels. Coprecipitation of other peptides was limited and, in general, independent of the concentration of SH3 fusion protein used. We did notice, however, several low molecular mass polypeptides (30, 32, and 38 kDa; Fig. 4) that coprecipitated with Grb2 and amphiphysin. Western analysis revealed that these were unlikely to represent dynamin degradation products (data not shown). Further investigation will be needed to determine the significance of these proteins. Coprecipitation of other known Grb2 binding proteins such as SOS was not observed, probably due to their low levels relative to dynamin in these extracts.

COS-7 cells were transfected with the dynamin-1 construct indicated and were assayed for transferrin uptake (as in Fig. 6). C-terminal a.a. number is indicated after the slash (/) for truncation mutant construction. (C-terminal a.a. for full-length dynamin-1 is 851.)

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the dominant inhibitory mutant phenotype, a series of C-terminal truncations of both the K44E point mutant construct and the N272 N-terminal deletion mutant construct were expressed in COS-7 cells. In previous work both the K44E and the N272 mutations blocked transferrin uptake (3). A short C-terminal deletion (58 a.a.) had no effect on the inhibitory phenotype, but a longer deletion (188 a.a.) reversed it (3). As shown in Table I and Fig. 5, we now find that the inhibitory phenotype is unaffected by removal of the C-terminal 66 a.a., the region we find to be involved in SH3 binding, or the entire proline-rich domain (K44E/C746; Fig. 5). Furthermore, we find that deletion just beyond this region (to a.a. 733) reversed the phenotype (K44E/C733; Fig. 5).

Because involvement of SH3 domain-containing proteins has not been demonstrated for transferrin internalization, we reasoned that transferrin uptake might be uniquely insensitive to C-terminal deletions of dynamin. The transferrin receptor has not been found to require the involvement of SH3 proteins for its internalization. We reasoned, therefore, that removal of the C terminus might be more likely to affect other types of receptors. To test this possibility, we assayed for endocytosis of the PDGFR. As has been found for transferrin and epidermal growth factor receptor (6), PDGFR endocytosis was blocked in cells overexpressing dynamin-1 mutated at K44 (K44E/C746; Fig. 6). As we have now found for transferrin, truncation of the C-terminal region down to a.a. 746 had no effect on the inhibitory mutant phenotype, which, however, was abolished by truncation to a.a. 733 (K44E/C733; Fig. 6).

**DISCUSSION**

In this study, we have demonstrated that SH3 domains interact with dynamin in a sequence-specific manner and that the binding sites are localized within a 66-amino acid subregion of the dynamin proline-rich domain. These interactions have been found to occur with four different dynamin isoforms. Despite these observations, the dominant inhibitory mutant dynamin phenotype is unaffected by removal of the proline-rich region.

**SH3 Domain Binding Sites**—Previous efforts to map potential SH3 binding sites have made use of synthetic peptides corresponding to selected sequences within the dynamin C-terminal domain (17–20). Some results have been contradictory; for example, Grb2 was reported to bind to a.a. 778–795 in one study (17) but to a.a. 791–818 in another (19). Furthermore, much of the dynamin C-terminal region was unexamined in these studies. By systematically deleting short segments from the proline-rich domain, we have surveyed this region comprehensively and identified limited, specific binding sites for each SH3 domain-containing polypeptide examined (Fig. 7).

Our results identify three distinct proline-rich sequences within the dynamin-1 C terminus important for SH3 domain binding (Fig. 7B): a.a. 785–794 (region I, c-Src and Fyn binding); a.a. 809–826 (region II, PLCγ and p85 binding); and a.a. 827–839 (region III, Grb2 and amphiphysin binding). No SH3 interactions were detected with other proline-rich sequences farther upstream. The binding region defined in this study for PLCγ-SH3 is consistent with the previously reported binding to an a.a. 812–820 peptide (19) but not to an a.a. 778–785 peptide (17, 18). Similarly, in the case of Grb2, our results are consist-

![Fig. 6. Effect on PDGFR endocytosis of C-terminal deletions of a dominant inhibitory dynamin mutant.](https://example.com/fig6)

**Fig. 6.** Effect on PDGFR endocytosis of C-terminal deletions of a dominant inhibitory dynamin mutant. Human Hep G2 cells were transfected with the dynamin mutants K44E/C746 or K44E/C733 and assayed for internalization of the PDGFR using an anti-PDGFR monoclonal antibody. Cells expressing dynamin were detected with the R2 polyclonal antibody. As with transferrin uptake, deletion of the basic, proline-rich region alone did not reverse the dominant inhibitory effect of the K44E mutant. However, partial deletion of a putative coiled coil region was found to suppress the K44E phenotype.

![Fig. 7. Schematic summary of the SH3 binding regions in dynamin.](https://example.com/fig7)

**Fig. 7.** Schematic summary of the SH3 binding regions in dynamin. A, the partial sequence of the coiled coil region and the entire basic, proline-rich domain with the three SH3 binding regions (labeled I, II, and III) that we have identified in this study. Proline-rich regions that conform to the Class I consensus sequence for SH3 binding are underlined with an *open bar* and highlighted in *bold*, whereas those that fit the consensus for a Class II site are underlined with a *black bar* and also highlighted in *bold*. *Small arrows* indicate the positions of the deletion sites that were used in the current studies. Regions important for the dominant inhibitory endocytic phenotype, SH3 binding, and clathrin colocalization precede the *large arrows*. B, sequence alignment of the basic, proline-rich regions of dynamin-1, dynamin-2, and dynamin-3 was done using the Genetics Computer Group program PILEUP. Amino acids in *bold* indicate identical amino acid matches within the SH3 binding sequences of the isoforms.
ent with binding to an a.a. 827–838 peptide (20) but not to an a.a. 791–818 peptide (19) or an a.a. 778–795 peptide (17, 38). Finally, in the case of p85, our results are consistent with a previous finding that p85 binds to the same region in dynamin as PLCγ (18), which, however, is distinct from the a.a. 778–785 region as previously reported (17, 18). Peptide binding analysis has not been reported for amphiphysin.

We note that a Class I consensus sequence for SH3 domain binding (22, 39), RXQPXR (where q may be any hydrophobic amino acid and X is any amino acid), is located within region I (except for the Arg, which is just upstream of the truncation site; see Fig. 7B). An additional Class I consensus sequence is located between a.a. 838 and 844, outside of the regions found in this study to be important for SH3 domain binding (see Fig. 7B). A Class II consensus sequence (22, 39), qPXqPXR, is located within regions II and III. Based on previous structural analysis of SH3 interactions, SH3 domains may be predicted to bind to the site within region I in the “plus” orientation and to the sites within regions II and III in the “minus” orientation (40).

An important implication of our study is that multiple SH3 domain-containing proteins may be able to bind dynamin simultaneously. We also note that, since the SH3 domains of Fyn, Lyn, c-Src, Grb2, PLCγ, and p85 are known to stimulate the dynamin GTPase (15, 17), we can now conclude that three distinct sites are active in this process.

We also note that the region responsible for Grb2 and amphiphysin-SH3 binding to dynamin was more sharply delimited than the other SH3 binding regions identified in this study. This observation may be interpreted as evidence of binding specificity, although it could also reflect the incomplete correspondence between the deletion sites we chose and the SH3 binding consensus sequences (for example, see region I).

We found very limited variation in SH3 domain binding among dynamin isoforms. Consistent with this observation, regions II and III are well conserved among all four of the dynamin isoforms tested, and the SH3 domain binding consensus elements within these regions are themselves well conserved (Fig. 7B). In contrast, region I is poorly conserved among the isoforms (Fig. 7B). This observation may explain the differences in Fyn-SH3 binding among dynamin isoforms (Fig. 3) but makes the constancy of c-Src-SH3 binding somewhat puzzling. Conceivably, c-Src-SH3 may bind to region II or III in the dynamin-2 and dynamin-3 isoforms.

Role of Dynamin Proline-rich Domain in Clathrin Colocalization and Endocytosis—Recently, our laboratory reported that deletion of the entire proline-rich region abolishes colocalization of dynamin with clathrin, although dynamin staining was still observed on membrane fragments. The region for clathrin colocalization was found to be within amino acids 786–851, which we find here to coincide with the region for SH3 binding. Taken together, these data suggest that the SH3 domain binding region in dynamin is important for colocalization with clathrin. Therefore, it is appealing to speculate that dynamin is directed to clathrin-coated pits via binding to SH3 domain-containing proteins that, directly or indirectly, interact with receptors in the pits (25), although additional work will be needed to resolve this issue.

We also find that the inhibition of endocytosis produced by mutations in the GTPase domain of dynamin persists when the proline-rich domain is removed (Table I, Figs. 5 and 6). This result indicates that SH3 binding is not involved in producing the inhibitory phenotype, although it does not argue against the functional importance of SH3 binding. We also tested the effect of the truncated inhibitory mutant dynamin construct K44E/C746 on PDGFR internalization because of the known role of SH3 domain-containing proteins in the endocytosis of receptor tyrosine kinases (23). As in the case of transferrin internalization, removal of the proline-rich domain failed to reverse the inhibitory phenotype.

Removal of an additional 13 a.a. did, however, abolish the effect. The region involved, a.a. 733–746, is predicted to participate in coiled coil formation (25). We speculate that removal of a portion of the coiled coil domain may affect the self-association of dynamin into dimers or the higher order helical polymers that have been observed by electron microscopy (5, 41, 42). We have, in fact, observed a loss in dynamin self-association using the same deletion mutant constructs.2 Thus, it now seems likely that inhibition of endocytosis by dynamin mutants may involve poisoning of dynamin-dynamin interactions rather than interactions with proteins containing SH3 domains.

We note that mutant GTPase constructs lacking the entire proline-rich domain localize to spots on the plasma membrane, which, however, are distinct from clathrin-coated pits (35). Although the function of the nonclathrin-containing structures is uncertain, our results suggest that they are loci within which mutant dynamin interacts with endogenous dynamin or other proteins involved in the endocytic pathway. That the presence of the SH3 binding region defined in the present study correlates well with clathrin colocalization (35) suggests that SH3 domains are responsible for recruiting dynamin to the coated pit. Thus, we envision a model in which dynamin-dynamin interactions are initiated at the plasma membrane followed by coated pit recruitment mediated by SH3 domain-containing proteins. Which SH3 domain-containing proteins are involved in this step of the dynamin pathway in vivo remains an important issue for further research.

Acknowledgments—We thank J. Taylor and C. Seidel-Dugan (ARIAD Pharmaceuticals, Cambridge, MA) for the GST-SH3 fusion constructs of Fyn, Lyn, Csk, c-Src, Grb2, and p85. We are also grateful to C. David and P. De Camilli (Howard Hughes Medical Institute, Yale University) for kindly providing us with the GST-amphiphysin SH3 protein and S. Corvera (University of Massachusetts Medical School) for help with the PDGFR endocytosis assays. We thank P. McNulty for his technical assistance in the preparation of the manuscript and figures, and M. Gee, K. Vaughan, S. Tynan, and C. Echeverri for helpful discussions.

References


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2. P. M. Okamoto and R. B. Vallee, unpublished observations.
Role of the Basic, Proline-rich Region of Dynamin in Src Homology 3 Domain Binding and Endocytosis
Patricia M. Okamoto, Jonathan S. Herskovits and Richard B. Vallee

doi: 10.1074/jbc.272.17.11629

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