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Growth factor-induced binding of dynamin to signal transduction proteins involves sorting to distinct and separate proline-rich dynamin sequences

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Dynamin, a 100 kDa GTPase, is critical for endocytosis, synaptic transmission and neurogenesis. Endocytosis accompanies receptor processing and plays an essential role in attenuating receptor tyrosine kinase signal transduction. Dynamin has been demonstrated to be involved in the endocytic processing at the cell surface and may play a general role in coupling receptor activation to endocytosis. Src homology (SH) domain dependent protein-protein interactions are important to tyrosine kinase receptor signal transduction. The C-terminus of dynamin contains two clusters of SH3 domain binding proline motifs; these motifs may interact with known SH3 domain proteins during tyrosine kinase receptor activation. We demonstrate here that SH3 domain-containing signal transduction proteins, such as phospholipase Cγ-1 (PLCγ-1), do indeed bind to dynamin in a growth factor inducible manner. The induction of PLCγ-1 binding to dynamin occurs within minutes of the addition of platelet derived growth factor (PDGF) to cells. Binding of these signal transduction proteins to dynamin involves specific sorting to individual proline motif clusters and appears to be responsible for co-immunoprecipitation of tyrosine phosphorylated PDGF receptors with dynamin following PDGF stimulation of mammalian cells. The binding of dynamin to SH3 domain-containing proteins may therefore be important for formation of the protein complex required for the endocytic processing of activated tyrosine kinase receptors.

Key words: dynamin/endocytosis/PDGF receptor/Src homology

Introduction

The activation of receptor tyrosine kinases results in various cellular responses ranging from mitogenesis to terminal differentiation, depending on the context of the cell type and the receptor (reviewed in Schlessinger and Ullrich, 1992; Fantl *et al.*, 1993). Cell activation is mediated through the induction of specific binding interactions between a number of proteins containing Src homology (SH) domains 2 and 3 and their ligands (reviewed in Koch *et al.*, 1991). As a result of receptor activation, tyrosine phosphorylation occurs on specific receptor residues, which then bind to the SH2 domains of several proteins with signal transducing functions. Binding is specific, and a single receptor such as platelet derived growth factor (PDGF) can have several discrete binding sites for different SH2 domain proteins, such as the second messenger generating proteins phospholipase Cγ-1 (PLCγ-1) and phosphatidylinositol-3′ (PI3) kinase, and the signal transduction proteins syp and GRB2 (reviewed in Pawson and Schlessinger, 1993).

GRB2 serves as an example of the protein-protein interactions made possible through SH domain interactions. The activation of ras by the signal transduction pathway can occur through a cascade of events in which GRB2, a small protein containing one SH2 and two SH3 domains (Clark *et al.*, 1992), binds through its SH2 domain to a tyrosine phosphorylated site on the receptor and then interacts with the ras activator SOS through both of its SH3 domains (Clark *et al.*, 1992; Buday and Downward, 1993; Olivier *et al.*, 1993; Simon *et al.*, 1993). Binding to SH3 domains occurs on short proline-rich sequences (Egan *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993) that approximate the proline-rich SH3 domain binding motif XPXXPPPΦXP (where P is proline, X is any residue and Φ is a hydrophobic residue) of 3BP-1 and 3BP-2, and of formins and the m4 muscarinic acetylcholine receptor (Ren *et al.*, 1993). The proline-rich SH3 domain binding motifs of 3BP-1 and 3BP-2 selectively bind specific recombinant SH3 domains (Cicchetti *et al.*, 1992; Ren *et al.*, 1993). As has been demonstrated for SH2 binding selection (reviewed in Fantl *et al.*, 1993; Pawson and Schlessinger, 1993), such target specificity is expected for proteins involved as intermediates in signal transduction.

Further permutations are made possible in the signal transduction pathway when a single protein contains more than one consensus binding sequence. Dynamin, a 100 kDa GTPase protein (Obar *et al.*, 1990; Maeda *et al.*, 1992; Shpetner and Vallee, 1992) that is critical to synaptic transmission (Ikeda *et al.*, 1976; Siddigi and Benzer, 1976), neurite and growth cone formation (Kim and Wu, 1987) and endocytosis (Herskovits *et al.*, 1993a; van der Bliek *et al.*, 1993), contains two C-terminal clusters of proline-rich sequence motifs (Gout *et al.*, 1993) and has recently been demonstrated to interact selectively with a subset of several recombinant SH3 domain fusion proteins (Gout *et al.*, 1993). The occurrence of two SH3 domain binding motifs with highly varied sequence within the same protein suggests their binding functions may be different.

Here we demonstrate that murine dynamin binds to PLCγ-1, the p85 subunit of PI3 kinase, and GRB2. Interaction of PLCγ-1 with dynamin is induced by growth factor stimulation of mammalian cells with PDGF. Stimulation by PDGF also results in the association of dynamin with the tyrosine phosphorylated PDGF receptor. We show that the two different SH3 binding clusters on dynamin are highly selective for discrete SH3 domain-

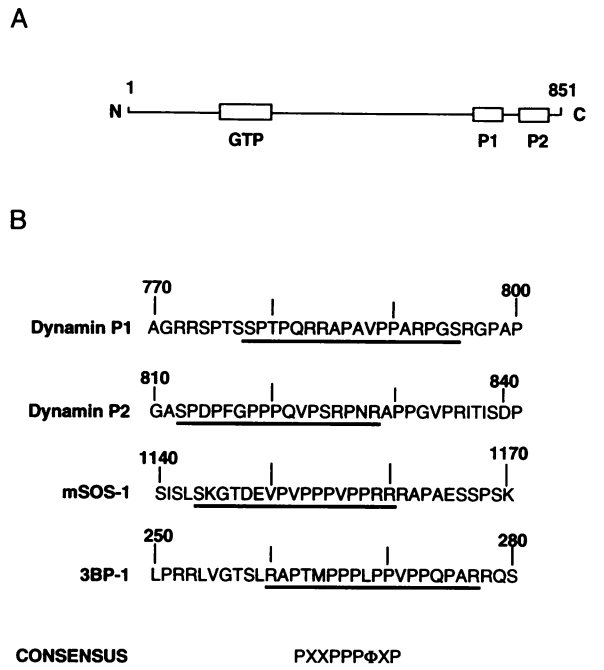

Fig. 1. Proline motif sequences of dynamin, mSOS-1 and 3BP-1. (**A**) Linear representation of the organization of the rat brain dynamin sequence (Obar *et al.*, 1990). The relative positions of the GTP binding domain, and the two proline motif clusters P1 and P2 are indicated. (**B**) Proline motif sequences of dynamin, mSOS-1 and 3BP-1 (single letter amino acid code; from Gout *et al.*, 1993). Dynamin proline motif sequences, designated P1 and P2, and proline motif sequences of mSOS-1 and 3BP-1 are underlined, and correspond to the peptides used for affinity precipitations. The corresponding SH3 domain binding proline motif consensus sequence (Ren *et al.*, 1993) is shown below.

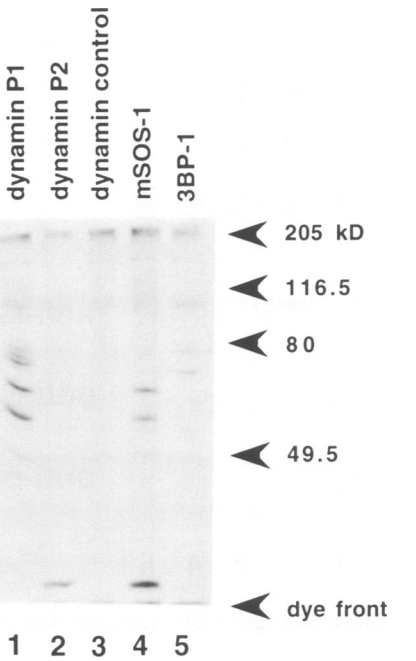

Fig. 2. Analysis of proteins affinity precipitated by dynamin, mSOS-1 and 3BP-1 proline motif peptides. PC12 cells, grown overnight in methionine- and cysteine-free medium in the presence of 5 μCi/ml ^{35}S Translabel, were lysed by Dounce homogenization. Aliquots of the 125 000 *g* supernatant of the lysate (in HNEM buffer containing protease inhibitor cocktail) were incubated with 5 μl of Actigel coupled to dynamin P1 (lane 1), dynamin P2 (lane 2), mSOS-1 (lane 4) or 3BP-1 (lane 5) proline motif peptide for 90 min at 4°C. A dynamin control peptide, not containing the SH3 domain binding proline-rich motif, was used as a negative control (lane 3). The peptide matrices were washed three times with 300 μl of HNEM buffer containing 0.1% Triton X-100 and 0.2% deoxycholic acid, and bound proteins were then fractionated by SDS–PAGE. The radiolabeled proteins were then detected by fluorographically enhanced autoradiography of the polyacrylamide gel.

containing proteins. Such selectivity may possibly generate unique associations of dynamin among different SH3 domain proteins, yielding an unexpected mode of regulation in receptor function.

Results

Interactions between proline motif peptides and SH3 domain-containing proteins

The C-terminus of dynamin contains two clusters of putative SH3 domain binding consensus sequences (Figure 1). To determine possible ligand substrates for these sequences, we have made peptides representative of these two dynamin proline-rich clusters (Figure 1B, underlined sequences).

The peptides corresponding to the two clusters of proline motifs were immobilized on Actigel and then used for affinity-precipitation of proteins from metabolically labeled cell lysates. Peptide bound proteins were then resolved by SDS–PAGE and visualized by autoradiography (Figure 2). We found that these two dynamin-derived immobilized peptides precipitated a very limited number of ^{35}S-labeled proteins from PC12 cell lysates. In contrast, a control peptide, derived from the central portion of dynamin, did not precipitate significant levels of ^{35}S-labeled proteins. As a further test of the specificity of binding, peptides corresponding to the proline motifs on the SH3 binding domains of mSOS-1 and 3BP-1 (see Figure 1B) were also tested in this assay. Each of these SH3 domain binding peptides also bound a very limited number of proteins. Immobilized dynamin peptide P1 precipitated two major proteins of 55 and 65 kDa, as well as several less abundant proteins of 70–85 kDa. Immobilized dynamin peptide P2 bound a single protein of 28 kDa, which was present at low levels in the immobilized dynamin P1 precipitate as well. The immobilized mSOS-1 peptide also precipitated a protein of 28 kDa, which may be identical to the dynamin P2 precipitated protein, as well as two proteins of 55 and 65 kDa, which appear to correspond to two of the major proteins precipitated by the dynamin P1 peptide. Immobilized 3BP-1 peptide, on the other hand, precipitated two proteins of 70 and 80 kDa. Individual proline-rich motifs therefore appear to represent specific binding sites for distinct protein subsets. Further, we conclude that the presence of two clusters of proline motifs in dynamin corresponds to the presence of two distinct binding sites on this protein, each with different ligand specificities.

The peptides used for these precipitation reactions contain proline motifs corresponding to the SH3 domain binding consensus sequence originally identified for 3BP-1 and 3BP-2 (Ren *et al.*, 1993). The few proteins selectively precipitated by these immobilized peptides therefore probably represent SH3 domain-containing proteins. In order to identify which SH3 domain-containing proteins were precipitated by these peptides, we probed the immobilized peptide precipitates by

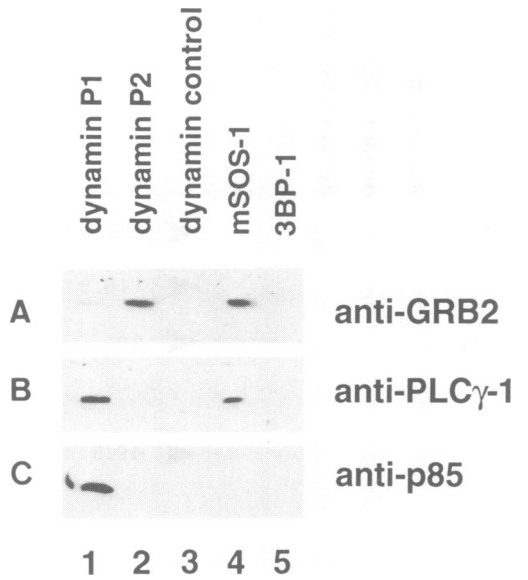

Fig. 3. Identification of specific SH3 domain proteins that bind to dynamin P1 and P2, mSOS-1, and 3BP-1 proline motif peptides. PC12 cells (**A** and **B**) and A-431 cells (**C**), grown overnight in methionine- and cysteine-free medium in the presence of 5 μCi/ml ^{35}S translabel, were lysed in HNEM buffer containing 0.5% Triton X-100 (A and B) or by Dounce homogenization (C). Aliquots of the 125 000 *g* supernatant of the lysate (in HNEM buffer containing protease inhibitor cocktail) were incubated with 5 μl of Actigel coupled to dynamin P1 (lane 1), dynamin P2 (lane 2), mSOS1 (lane 4), or 3BP-1 (lane 5) proline motif peptides for 90 min at 4°C. A dynamin control peptide, not containing the SH3 domain binding proline-rich motif, was used as a negative control (lane 3). The peptide matrices were washed three times with HNEM buffer containing 0.1% Triton X-100. The bound proteins were fractionated by SDS–PAGE, and transferred to nitrocellulose for Western blot analysis using antisera to GRB2 (A), PLCγ-1 (B) and p85 (C).

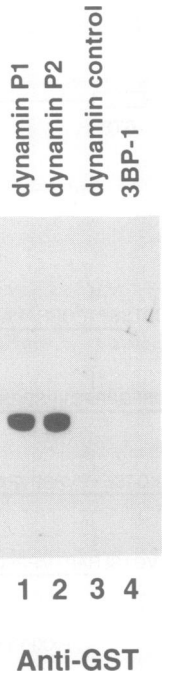

Fig. 4. Binding of recombinant PLCγ-1 SH3 domain to proline motif peptides. Aliquots of recombinant PLCγ-1 SH3 domain GST fusion protein (3.5 μg) were incubated with 5 μl of Actigel coupled to dynamin P-1 (lane 1), dynamin P-2 (lane 2), dynamin control peptide (lane 3) or 3BP-1 (lane 4) proline motif peptide, for 60 min at 4°C in the presence of 100 μl of the supernatant resulting from centrifuging the PC12 cell lysate at 12 000 *g*. The peptide matrices were washed three times with 300 μl HNEM buffer containing 0.25% Triton X-100, and bound proteins were then fractionated by SDS–PAGE. PLCγ-1 SH3 domain GST fusion protein was detected by immunoblotting with goat anti-GST serum.

Western blot analysis, using antisera to various SH3 domain-containing proteins (Figure 3).

Using antiserum to GRB2, we detected this 28 kDa protein in PC12 cell precipitates from dynamin P2, mSOS-1 and, to a much lesser extent, dynamin P1 immobilized peptide precipitates (Figure 3A). It has been demonstrated previously that mSOS binds GRB2 SH3 domains. Since this is the only ^{35}S-labeled protein detected in dynamin P2 precipitates, these results indicate that the most C-terminal proline motif in dynamin is uniquely specific for GRB2 binding. When the peptide-bound material was probed with anti-PLCγ-1 serum, we found this protein in both mSOS-1 and dynamin P1 peptide precipitates of PC12 cells (Figure 3B). In contrast, dynamin P2 peptide precipitates contained nearly undetectable levels of PLCγ-1. Antiserum to the p85 subunit of PI3 kinase showed that this protein was present only in dynamin P1 peptide precipitates of A-431 cells (Figure 3C) and PC12 cells (data not shown). Remarkably, p85 was not detected on blots of dynamin P2, mSOS-1 or 3BP-1 peptide precipitates. The immobilized dynamins P1 and P2, mSOS-1 and 3BP-1 proline motif peptides therefore appear to precipitate specifically SH3 domain-containing proteins from cell lysates. Furthermore, individual proline motif peptides, derived either from different proteins or from a single protein with two proline motifs, seem to have different specificities for SH3 domain-containing proteins. The observed specificity does not appear to be cell type specific since similar results were obtained with a variety of cell types (data not shown).

From the data in Figures 2 and 3 it is apparent that dynamin-derived peptides corresponding to SH3 domain binding motifs associate specifically with the SH3 domain-containing proteins GRB2, p85 and PLCγ-1. To confirm that this association results from the interaction of the proline motif peptide with the SH3 domains of these proteins, the immobilized proline motifs were incubated with a recombinant glutathione S-transferase (GST)–PLCγ-1 SH3 domain fusion protein, in the presence of PC12 cell lysate, and bound fractions were analyzed by immunoblotting with anti-GST (Figure 4). As anticipated, the proline motif peptides indeed bound the SH3 domain fusion proteins, indicating that the interaction between PLCγ-1 and dynamin in whole cell lysate occurs by an interaction of the dynamin P1 proline motif sequence with the SH3 domain of PLCγ-1. However, we found that, in this case, dynamin P1 and P2 proline motif peptides bound the SH3 domain fusion proteins with nearly equal efficiency, suggesting that some of the specificity of the interaction between dynamin and PLCγ-1 is determined by PLCγ-1 sequences that lie outside the SH3 domain.

Interactions between recombinant SH3 domains and dynamin

Since the proline motif sequences could be presented quite differently in the intact folded protein, we investigated

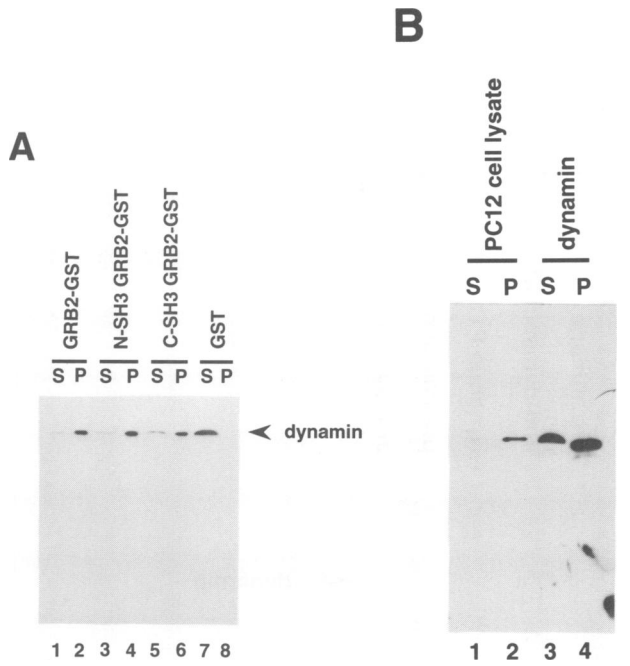

Fig. 5. Binding of recombinant GRB2 and PLCγ-1 SH3 domains to dynamin. (**A**) 1 μg of either recombinant whole GRB2-GST (lanes 1 and 2), N-terminal GRB2 SH3 domain-GST (lanes 3 and 4), C-terminal GRB2 SH3 domain-GST (lanes 5 and 6), or GST alone (lanes 7 and 8) were incubated with 15 μl of 0.5% Triton X-100 PC12 cell lysate and 10 μl of glutathione−agarose for 60 min at 4°C. The unbound fraction was removed following brief low-speed centrifugation, and the glutathione matrix was washed with HNEM buffer containing 0.25% Triton X-100. The unbound fractions (lanes 1, 3, 5 and 7) and the washed matrix fractions (lanes 2, 4, 6 and 8) were fractionated by SDS−PAGE and subjected to Western blot analysis with anti-dynamin serum. (**B**) 3.5 μg aliquots of recombinant PLCγ-1 SH3 domain were incubated with 10 μl glutathione−agarose and 20 μl of 0.5% Triton X-100 PC12 cell lysate (lanes 1 and 2) or 0.25 μg of purified dynamin (lanes 3 and 4) for 60 min at 4°C. The agarose matrix was washed with HNEM buffer containing 0.1% Triton X-100, and centrifuged. The unbound fractions (lanes 1 and 3), and the bound glutathione−agarose matrix fractions (lanes 2 and 4) were then fractionated by SDS−PAGE, and subjected to Western blot analysis with anti-dynamin serum.

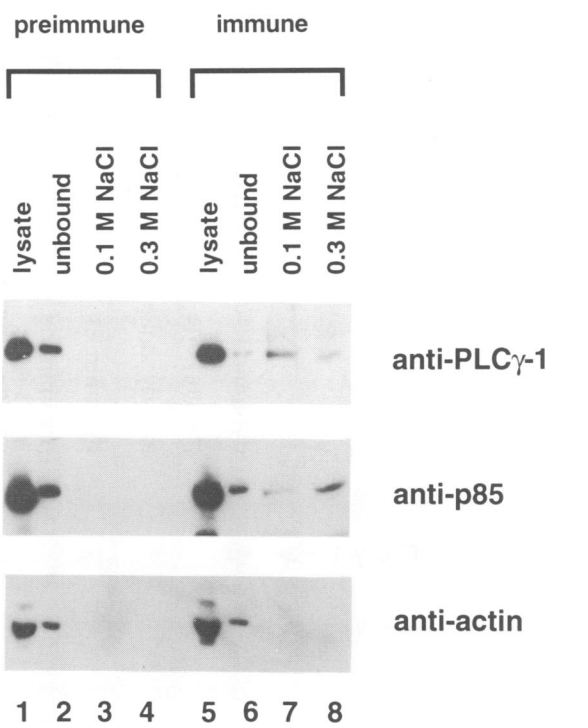

Fig. 6. Binding of PLCγ-1 and PI3 kinase p85 subunit to dynamin affinity matrix. PC12 cells from one 15 cm diameter plate were lysed by incubation for 120 min at 4°C in 1 ml of HNEM buffer containing protease inhibitor cocktail and 0.1% Triton X-100, centrifuged at 12 000 *g* for 10 min, and 500 μl aliquots of the resulting supernatant were loaded onto 0.5 ml preimmune (lanes 1−4) or immune (lanes 5−8) anti-dynamin columns equilibrated in lysis buffer containing 5 mg/ml BSA. Following elution with 20 vol. of lysis buffer, the columns were sequentially eluted with HNEM buffer containing 0.1 M and 0.3 M NaCl, and peak fractions were concentrated by TCA precipitation. Starting material (lanes 1 and 5), unbound material (lanes 2 and 6), 0.1 M NaCl eluate (lanes 3 and 7), and 0.3 M NaCl eluate (lanes 4 and 8), were subjected to SDS−PAGE and analysed by Western blot procedure using anti-PLCγ-1, anti-p85 and anti-actin sera.

whether intact (i.e. full-length) dynamin could interact with the same SH3 domain-containing proteins that bound to the dynamin-derived peptides, and whether such interactions would occur in a complex mixture of proteins such as a whole cell lysate. GRB2, expressed as a recombinant GST fusion protein and immobilized by binding to glutathione−agarose, was incubated with a clarified PC12 cell lysate. Proteins bound to GRB2-GST were precipitated from the cell lysate mixture by sedimentation of the protein−agarose complex, and resolved by SDS−PAGE. When Western blotted with anti-dynamin serum (Figure 5A), we found that the 100 kDa antigen, representing PC12 cell dynamin, was present in the GRB2-GST precipitated material. The association of dynamin with the affinity matrix is dependent on the GRB2 moiety, since GST alone, immobilized on glutathione−agarose, did not precipitate any dynamin from the cell lysate.

GRB2 is a non-catalytic molecule comprised primarily of one SH2 domain and two SH3 domains (Clark *et al.*, 1992; Lowenstein *et al.*, 1992; Matuoka *et al.*, 1992). Since we have demonstrated that one of the two proline motifs of dynamin interacts preferentially with GRB2 (see Figure 3), we wished to determine whether one or both GRB2 SH3 domains would bind to dynamin. The N-terminal and C-terminal GRB2 SH3 domains were each expressed as GST fusion proteins, then immobilized by binding to glutathione−agarose, and incubated with PC12 cell lysate. Bound and unbound fractions were resolved by SDS−PAGE, and dynamin was detected by Western blotting with anti-dynamin serum (Figure 5A). Both the N-terminal GRB2 and the C-terminal GRB2 SH-3 domain fusion proteins precipitated dynamin equally well. Binding of GRB2 to dynamin therefore appears to occur indifferently on either GRB2 SH3 domain, as was suggested by our finding that GRB2 binds selectively to only one of the two dynamin proline motif clusters.

A similar analysis was conducted on proteins that bound to the GST-PLCγ-1 SH3 domain fusion protein. The fusion protein, bound to glutathione−agarose, was mixed with PC12 cell lysate or with purified dynamin. Bound and unbound fractions were resolved by SDS−PAGE, and dynamin was detected by Western blotting with anti-dynamin serum (Figure 5B). The immobilized GST-PLCγ-1 SH3 domain fusion protein precipitated both purified dynamin and dynamin in whole cell lysate.

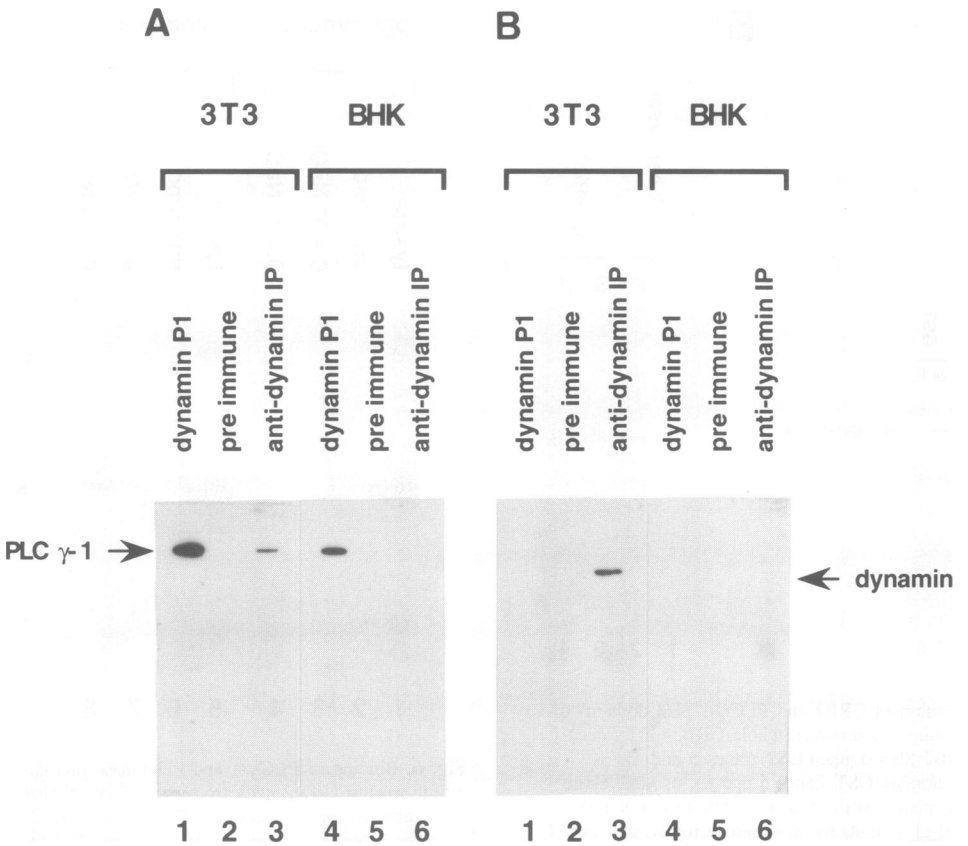

Fig. 7. Co-immunoprecipitation of PLCγ-1 with dynamin. 3T3 cells (lanes 1, 2 and 3), and BHK cells (lanes 4, 5 and 6) were lysed in HNEM buffer containing 0.5% Triton X-100 and protease inhibitor cocktail for 30 min at 4°C, centrifuged at 12 000 *g* for 10 min and then incubated at 4°C for 60 min with either 5 μl of Actigel-immobilized dynamin P1 peptide (lanes 1 and 4), 5 μl of protein A–Sepharose-coupled anti-dynamin preimmune serum (lanes 2 and 5), or 5 μl of protein A–Sepharose-coupled anti-dynamin serum (lanes 3 and 6). The peptide and protein A–antibody matrices were washed three times with HNEM buffer containing 0.5% Triton X-100, followed by a wash with HNEM buffer. Bound proteins were fractionated by SDS–PAGE and transferred to nitrocellulose for Western blot analysis using anti-PLCγ-1 (**A**) and anti-dynamin sera (**B**).

Interactions between dynamin and signal transduction proteins

To determine whether endogenous SH3 domain-containing proteins are bound to dynamin in cell lysates, we performed affinity chromatography with antidynamin antibodies coupled to protein A–Sepharose. PC12 cell lysate was loaded onto preimmune and immune anti-dynamin columns, and proteins that bound were eluted with buffers containing increasing concentrations of NaCl. Eluted material was concentrated, subjected to SDS–PAGE and probed by Western blots using antisera to p85 and PLCγ-1 (Figure 6). We found that both these proteins bound to the dynamin antibody column, but not to the preimmune column. PLCγ-1 eluted from the immune column with 0.1 M NaCl-containing buffer, while p85 eluted with 0.3 M NaCl-containing buffer. As a negative control, the column fractions were also screened for the presence of an abundant test protein, actin, that does not contain SH3 domains. In contrast with PLCγ-1 and p85, the dynamin antibody column did not bind detectable levels of actin (Figure 6).

PLCγ-1 could also be detected, though only faintly, in dynamin immunoprecipitates from 3T3 cells (Figure 7) and from PC12 cells (data not shown). BHK cells do not contain appreciable levels of dynamin. Correspondingly, dynamin immunoprecipitates from these cells did not contain detectable levels of PLCγ-1, although dynamin proline motif peptide precipitates from these cells contained substantial levels of PLCγ-1 (Figure 7). The recovery of PLCγ-1 in the 3T3 and PC12 cell dynamin immunoprecipitates is therefore not due to interaction of PLCγ-1 with the antibody affinity matrix, but rather appears to be due to a direct interaction with dynamin.

PDGF-induced association of signal transduction proteins with dynamin

Since PLCγ-1 is involved in receptor tyrosine kinase signal transduction (reviewed in Rhee and Choi, 1992), we assayed for changes in the association of dynamin with this SH3 domain-containing protein, following growth factor activation of receptor tyrosine kinases. Following stimulation of 3T3 cells with PDGF for various periods of time, the cells were lysed and dynamin immunoprecipitated by anti-dynamin antibodies that had been coupled to protein A–Sepharose. Precipitated proteins were resolved by SDS–PAGE, and PLCγ-1 detected by Western blotting (Figure 8). Brief exposure of the 3T3 cells to PDGF greatly increased the association of PLCγ-1 with dynamin in immunoprecipitates. The PDGF-induced association of PLCγ-1 with dynamin occurs very rapidly, peaking within 3 min of growth factor addition to the cells. The association is transient, and returns to levels comparable to those seen in untreated cells at 120 min after PDGF addition.

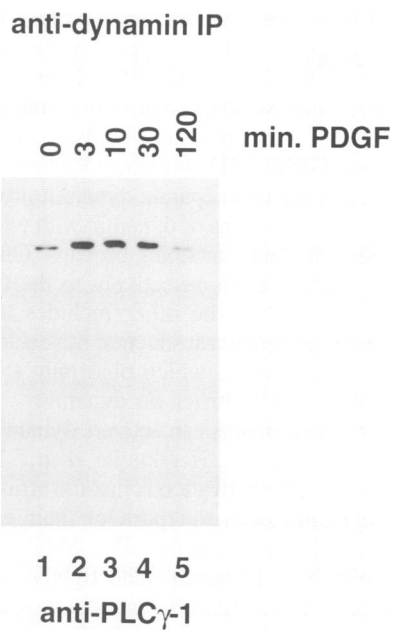

Fig. 8. PDGF-induced association of PLCγ-1 with dynamin. 3T3 cells, placed in RPMI containing 0.2% FBS for 48 h were lysed at 4°C for 60 min in HNEM buffer containing 0.5% Triton X-100, protease inhibitor cocktail and 300 μM orthovanadate following addition of 25 ng/ml PDGF to the culture medium for either 0 (lane 1), 3 (lane 2), 10 (lane 3), 30 (lane 4) or 120 min (lane 5). The cell lysates were then centrifuged at 12 000 *g* for 10 min, and the resulting supernatant was incubated at 4°C for 60 min with 5 μl of protein A-coupled anti-dynamin serum. The protein A−antibody matrices were washed with HNEM buffer containing 0.25% Triton X-100 and 150 μM orthovanadate. Bound proteins were fractionated by SDS−PAGE and transferred to nitrocellulose for Western blot analysis using anti-PLCγ-1 serum.

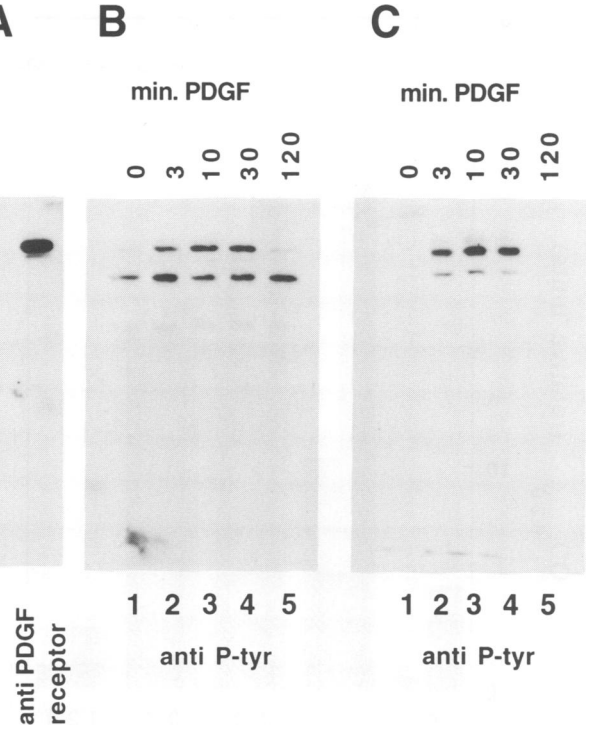

Fig. 9. Tyrosine phosphorylation and co-immunoprecipitation of PDGF receptor with dynamin. 3T3 cells, placed in RPMI containing 0.2% FBS for 48 h, were lysed at 4°C for 60 min in HNEM buffer containing 0.5% Triton X-100, protease inhibitor cocktail and 300 μM orthovanadate following addition of 25 ng/ml PDGF to the culture medium for either 0 (lane 1), 3 (lane 2), 10 (lane 3), 30 (lane 4) or 120 min (lane 5). The cell lysates were then centrifuged at 12 000 *g* for 10 min, and the resulting supernatants were fractionated by SDS−PAGE and transferred to nitrocellulose for Western blot analysis using either anti-PDGF receptor serum (**A**), or anti-phosphotyrosine serum (**B**). Alternatively, the resulting supernatants were incubated at 4°C for 60 min with 5 μl of protein A-coupled anti-dynamin serum. The protein A−antibody matrices were washed with HNEM buffer containing 0.25% Triton X-100 and 150 μM orthovanadate. Bound proteins were fractionated by SDS−PAGE, and transferred to nitrocellulose for Western blot analysis using anti-phosphotyrosine serum (**C**).

Binding of signal transduction molecules such as PLCγ-1 to tyrosine phosphorylated growth factor receptors for PDGF occurs through interaction of an SH2 domain with specific receptor phosphotyrosine residues (Ronnstrand *et al.*, 1992; Valius *et al.*, 1993). We therefore tested whether tyrosine phosphorylated PDGF receptor associated with dynamin-bound SH3 domain-containing proteins, such as PLCγ-1, upon PDGF stimulation of the cells. After stimulating 3T3 cells with PDGF for various periods of time, we immunoblotted their lysates with both anti-PDGF-receptor (Figure 9A), and anti-phosphotyrosine sera (Figure 9B). From this comparative analysis, we determined that tyrosine phosphorylated PDGF receptor can be detected between 3 and 30 min of PDGF stimulation of the cells. Using anti-dynamin antibodies that had been coupled to protein A−Sepharose, dynamin was immunoprecipitated from lysates of 3T3 cells that had been stimulated for up to 120 min with PDGF. Precipitated proteins were resolved by SDS−PAGE, and tyrosine phosphorylated PDGF receptor identified by Western blotting with anti-phosphotyrosine serum (Figure 9C). Exposure of the 3T3 cells to PDGF resulted in a co-immunoprecipitation of tyrosine phosphorylated PDGF receptor with dynamin. The co-immunoprecipitation of tyrosine phosphorylated PDGF receptor with dynamin occurred as a rapid and transient response to PDGF exposure of the 3T3 cells.

The time courses of the PDGF-induced association of PLCγ-1 with dynamin, and the PDGF-induced tyrosine phosphorylation of the PDGF receptor were determined by quantitative densitometric analysis of the anti-PLCγ-1, and anti-phosphotyrosine blot signals shown in Figures 8 and 9B, respectively (Figure 10). The time courses of these two events closely parallel each other, suggesting a dependence of the dynamin−PLCγ-1 association on PDGF receptor tyrosine phosphorylation.

Discussion

Initial stages of receptor tyrosine kinase mediated signal transduction involve binding of SH2 domain-containing proteins to specific receptor phosphotyrosine residues (reviewed in Schlessinger and Ullrich, 1992; Fantl *et al.*, 1993). SH2 domain-containing proteins can in turn link to downstream effectors through their SH3 domains, which recognize specific proline motifs, such as those of the ras activating protein SOS (Egan *et al.*, 1993; Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993).

We found that dynamin-derived proline motifs bind very specifically to SH3 domain-containing signal transduction

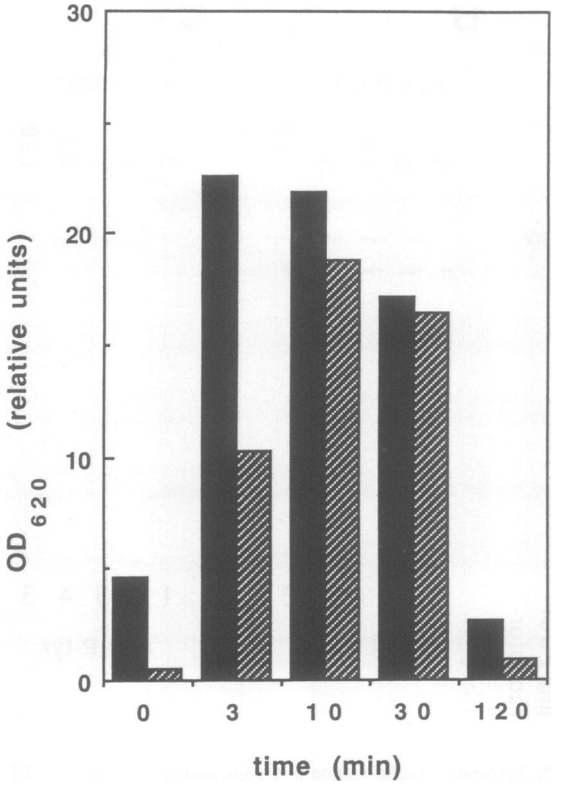

Fig. 10. Time course of PDGF-induced association of PLCγ-1 with dynamin, and PDGF-induced receptor tyrosine phosphorylation. To determine the level of PLCγ-1 associated with dynamin after PDGF stimulation of 3T3 cells, PLCγ-1 was immunoprecipitated with anti-dynamin antibodies following PDGF addition, and the resulting Western blots were quantified by optical scanning densitometry at 620 nm (solid bars). The data shown here were obtained from the blot shown in Figure 8. Similarly, PDGF receptor tyrosine phosphorylation was quantitated by scanning anti-phosphotyrosine blots of PDGF stimulated 3T3 cell lysates (hatched bars). The data shown here were obtained from the blot shown in Figure 9B.

enzymes. Binding of dynamin to one of these enzymes, PLCγ-1, is greatly induced by cell activation with PDGF. Furthermore, we detected a PDGF dependent association of dynamin with tyrosine phosphorylated PDGF receptor. Our results therefore indicate a role for dynamin SH3 domain interactions in PDGF signal transduction and growth factor down-regulation by endocytosis, as recently suggested (Trowbridge, 1993).

Binding of SH3 domain-containing proteins to proline motif peptides

Our data indicate that individual proline motifs represent binding sites for distinct SH3 domains since peptides corresponding to the 3BP-1 and mSOS-1 SH3 domain binding proline motifs bind selectively to different subsets of proteins in cell lysates. Dynamin contains several putative SH3 domain binding proline motifs, clustered at two C-terminal sites (Gout *et al.*, 1993). We have shown that peptides corresponding to these two sites represent specific binding sites for different well defined SH3 domain-containing proteins. The binding of these molecules to the proline motif peptides occurs by direct interaction with their SH3 domains, since recombinant SH3 domains bind quantitatively to these peptides.

The presence of these two sites in dynamin does not appear to be important for cooperative binding to proteins containing more than one SH3 domain, as appears to be the case for GRB2 binding to SOS (Clark *et al.*, 1993; Egan *et al.*, 1993; Li *et al.*, 1993), since we found that only a single dynamin SH3 binding motif binds GRB2, but binds quantitatively to either of the two GRB2 SH3 domains. Rather, it appears that the presence of the two separate dynamin proline motif clusters allows for interactions of dynamin with two different subclasses of SH3 domain-containing proteins. One subclass includes GRB2, which binds selectively to the C-terminal proline motif cluster, and the other includes the second messenger-generating signal transduction molecules PLCγ-1 and PI3 kinase p85 subunit, which bind only to the more N-terminal proline motif cluster on dynamin.

Recombinant SH3 domains can activate dynamin GTPase activity (Gout *et al.*, 1993; Herskovits *et al.*, 1993b). A remarkable aspect of this activation is that the affinity of SH3 domains for dynamin does not parallel their capacity to induce dynamin GTPase activity. The SH3 domains of PLCγ-1 and p85 bind dynamin quite tightly, yet do not appreciably stimulate dynamin GTPase activity while, on the other hand, the SH3 domains of GRB2 and c-src yield substantial GTPase activation. Such a differential effect is probably a reflection of the presence of the two distinct and separate SH3 domain binding sites on dynamin, and suggests that only binding to the C-terminal site can activate GTPase activity.

Interaction of dynamin with SH3 domain-containing proteins in vitro

Using affinity chromatography, two proteins that bind to the proline motifs of dynamin, PLCγ-1 and the p85 subunit of PI3 kinase, were also shown to bind selectively to dynamin in whole cell lysates. PLCγ-1 and PI3 kinase are important components of receptor tyrosine kinase signal transduction pathways (reviewed in Cantley *et al.*, 1991), and following activation of the PDGF receptor, PLCγ-1 undergoes phosphorylation on both serine and tyrosine residues (reviewed in Rhee and Choi, 1993). We found that association of dynamin with PLCγ-1 is greatly induced by brief exposure of the 3T3 cells to PDGF. While PLCγ-1 phosphorylation may play a role in the PDGF-induced association with dynamin, it is also possible that conformational changes induced by the interaction of PLCγ-1 SH2 domains with PDGF receptor phosphotyrosine residues increase the affinity of PLCγ-1 SH3 domains for dynamin. Similarly, an induction of mSOS binding to GRB2 has been observed following EGF stimulation of cells (Buday and Downward, 1993; Li *et al.*, 1993), although mSOS and GRB2 are not known to undergo receptor tyrosine kinase dependent phosphorylation.

Interaction of dynamin with tyrosine kinase receptor

Dynamin is clearly involved in endocytosis mechanisms. Mutation in the *Drosophila* homolog of dynamin, *shibire*, has been directly linked to failure of endocytosis (Poodry and Edgar, 1979; Kosaka and Ikeda, 1983). Furthermore, transfection of mammalian cells with plasmids expressing mutated dynamin has suggested a role of dynamin in a GTP dependent process by which the neck of the budding coated vesicle is pinched off from the cell surface (van der Bliek *et al.*, 1993). Growth factor receptor must induce endocytosis as an important part of growth factor response

attenuation (Wells *et al.*, 1990). Analogously, the interaction of dynamin with the SH3 domain of PLCγ-1, p85 or GRB2, may provide a molecular link between dynamin and PDGF receptors. As our evidence demonstrates that dynamin is recruited to the stimulated growth factor receptor, and that it in turn binds several of the factors that are requisite for signal induction, it is quite possible that the interaction of these factors with dynamin serves to induce endocytosis in a coordinate response at the site of growth factor receptor induction. Specifically, receptor tyrosine kinase dependent interaction of dynamin with a receptor bound SH3 domain of PLCγ-1, p85, or GRB2, may provide the molecular signal for pinching off of endocytic vesicles, by induction of dynamin GTPase activity. Early stages of PDGF receptor endocytosis are indeed disrupted by mutation of the receptor binding sites for p85 (Joly *et al.*, 1994). It will be interesting to determine whether this effect on receptor trafficking reflects a p85-mediated interaction of PDGF receptor with dynamin.

Since dynamin is substantially a neuronal protein (Scaife and Margolis, 1990; Nakata *et al.*, 1993), it might be argued that such dynamin dependent coupling processes might occur only in neurons. Recently, however, a dynamin homolog, dynamin 2, has been discovered (Cook *et al.*, 1994) which has widespread tissue distribution, so it is now possible to imagine similar functions occurring in a variety of tissues. A general role for dynamin in receptor tyrosine kinase function is further indicated by the recent finding that dynamin also interacts with insulin receptors substrate (IRS-1) upon insulin stimulation (Endo and Kasuga, personal communication).

Materials and Methods

All reagents (except primary antibodies) were purchased from Sigma, unless otherwise stated. Dynamin was purified as described by Gout *et al.* (1993). The radioactivity on Hyperfilm-MP (Amersham) was quantified by optical density scanning at 620 nm, using a Shimadzu CS-9000 scanner.

Cell culture and cell lysis

PC12 cells were propagated in RPMI medium (Gibco) containing 5% fetal bovine serum (FBS; Hyclone) and 5% heat-inactivated horse serum (Hyclone). NIH-3T3, BHK and A-431 cells were propagated in RPMI medium containing 10% FBS. For metabolic labeling experiments, cells were placed overnight in methionine- and cysteine-free RPMI (ICN) containing serum and 5 μCi/ml ^{35}S Translabel (ICN). Following a wash in phosphate-buffered saline (PBS; 1.4 ml/10 cm diameter dish), cells were lysed in hypotonic buffer essentially as described by Harlow and Lane (1988) by 40 strokes in a type B Dounce homogenizer, following a 15 min incubation in 5 mM HEPES pH 7.4 buffer (300 μl/10 cm diameter dish) containing protease inhibitor cocktail [0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 μg/ml pepstatin, 1 μg/ml TPCK, 2 μg/ml leupeptin (Boehringer), 18 mg/ml benzamidine, and 0.05 μg/ml aprotinin (Boehringer)], after which 1/20 vol. of 100 mM HEPES pH 7.4, 1 M NaCl, 2 mM EGTA, 2 mM $MgCl_2$ was added. Alternatively, cells were lysed by incubation for 30 min at 4°C in HNEM buffer (5 mM HEPES pH 7.4, 50 mM NaCl, 0.1 mM EGTA, 0.1 mM $MgCl_2$) containing protease inhibitor cocktail (as above) and Triton X-100, as indicated in figure legends (1.2 ml/10 cm dish).

Electrophoresis and Western blotting

SDS–PAGE on 7.5% or 10% polyacrylamide gels (Bio-Rad) was performed as described (Laemmli, 1970), and ^{35}S signal was detected by autoradiography following fluorographic enhancement with Entensify (NEN). Western blotting was done as described (Khyse-Anderson, 1984), and after electrophoretic transfer, nitrocellulose sheets (Amersham) were blocked with 10% non-fat milk (Carnation) for 60 min and incubated for either 60 min at room temperature, or overnight at 4°C, with primary antisera diluted into PBS containing 0.05% Tween 20 and 1 mg/ml BSA. Antibodies were used at the following dilutions: monoclonal anti-PLCγ-1, monoclonal anti-p85α, anti-phosphotyrosine (UBI) and monoclonal anti-actin (Sigma), 1/1000; polyclonal anti-GRB2 and polyclonal anti-PDGF receptor (a generous gift of Heldin), 1/500; polyclonal goat anti-GST (Pharmacia), 1/200. Purified polyclonal anti-dynamin immunoglobulin was used at 10 μg/ml. The nitrocellulose sheets were then washed with PBS containing 0.05% Tween 20, incubated for 60 min at room temperature with 10 000-fold diluted secondary antibody [horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and IgM, or HRP-conjugated goat anti-rabbit IgG and IgM, Tago], and bound antibody was visualized using enhanced chemiluminescence (ECL, Amersham).

Affinity purifications

Recombinant PLCγ-1 and GRB2 SH3 domains were expressed and purified as GST fusion proteins as described (Gout *et al.*, 1993). HPLC purified dynamin P1, dynamin P2, mSOS1 and 3BP-1 peptides used for affinity precipitation reactions correspond to the sequences SPTPQRRAPAVPPAR-PGS, SPDPFGPPPQVPSRPNR, SKGTDEVPVPPPVPPRR and RAP-TMPPPLPPVPPQPAR, respectively, and were coupled to Actigel (Sterogene) following the manufacturer's recommended procedure. A control dynamin peptide was also used, corresponding to dynamin amino acids C^{629}RVGDKEKASETEENGSD^{645}. Antidynamin preimmune and immune resins were generated by dimethyl-pimelimidate chemical crosslinkage of 20–50% ammonium sulfate precipitates of preimmune and immune anti-dynamin sera to protein A–Sepharose (Harlow and Lane, 1988). Prior to use, nonspecific binding sites on the antibody, peptide and glutathione affinity matrices were blocked by incubation with HNEM buffer containing 5 mg/ml BSA, at ambient temperature. Anti-dynamin sera were generated by immunization of rabbits with keyhole limpet hemocyanin (KLH)-coupled peptide corresponding to rat brain dynamin amino acids 629–645. Coupling of this peptide to maleimide activated KLH (Pierce) was performed according to the manufacturer's recommendations, using a peptide containing an additional N-terminal cysteine residue.

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