Synaptojanin 1: localization on coated endocytic intermediates in nerve terminals and interaction of its 170 kDa isoform with Eps15

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Abstract Synaptojanin 1 is an inositol 5-phosphatase with a putative role in clathrin-mediated endocytosis. Goal of this study was to provide new evidence for this hypothesis. We show that synaptojanin 1 is concentrated at clathrin-coated endocytic intermediates in nerve terminals. Furthermore, we report that synaptojanin-170, an alternatively spliced isoform of synaptojanin 1, binds Eps15, a clathrin coat-associated protein. Binding is mediated by the COOH-terminal region of synaptojanin-170 which we show here to be poorly conserved from rat to humans, but to contain in both species three asparagine-proline-phenylalanine (NPF) repeats. This motif has been found to be the core of the binding site for the EH domains of Eps15. Together with previous data, our results suggest that synaptojanin 1 can be recruited to clathrin-coated pits via a multiplicity of interactions.

Key words: Endocytosis; Inositol 5-phosphatase; Dynamin; Synaptic vesicle; Synapse

1. Introduction

Synaptojanin 1 (formerly referred to as synaptojanin, [1]) was originally identified as one of the major Grb2-binding proteins in synaptic membranes [2]. Immunofluorescence as well as biochemical evidence suggests that it participates in synaptic vesicle endocytosis at a step closely related to dynamin's site of action [3]. Cloning of rat synaptojanin 1 revealed a NH_2-terminal domain homologous to the cytoplasmic portion of the yeast protein Sac1p, a central inositol 5-phosphatase domain and a COOH-terminal proline-rich region [4]. This proline-rich region mediates the binding of synaptojanin 1 to a variety of SH3 domain-containing proteins including amphiphysin [5,6], SH3p4, SH3p8, SH3p13 [7,8] and Grb2 [2]. Synaptojanin 2, a recently identified synaptojanin homolog, is very similar to synaptojanin 1 in the Sac1 homology and inositol 5-phosphatase domains, but differs substantially in the proline-rich region. Accordingly, synaptojanin 1 and 2 have different interaction partners and are likely to have different biological functions [1].

Two synaptojanin 1 isoforms of 145 kDa (synaptojanin-145) and 170 kDa (synaptojanin-170) are generated by an alternative splicing mechanism [9]. Synaptojanin-170 differs from synaptojanin-145 by a 30 kDa extension of its proline-rich COOH-terminal domain. Both synaptojanin-145 and synaptojanin-170 are expressed at low concentrations in a variety of tissues [9], but synaptojanin-145 is very abundant in nerve terminals, consistent with the hypothesis that it may play a key role in the clathrin-mediated endocytosis of synaptic vesicles [4,10].

A protein that has recently been implicated in clathrin-mediated endocytosis is Eps15, a ubiquitously expressed 150 kDa protein first identified as a major substrate for the tyrosine kinase activity of the EGF receptor [11]. Several lines of evidence strongly suggest the involvement of Eps15 in the generation of clathrin-coated vesicles from the plasma membrane. First, Eps15 was shown to bind to the α-subunit (α-adaptin) of the clathrin adaptor protein AP2 [12,13]. Second, an electron microscopy immunocytochemical study demonstrated a concentration of Eps15 at the neck of clathrin-coated pits [14]. Third, the NH_2-terminal domain of Esp15 contains three conserved repeats of about 70 amino acids referred to as EH (Eps15 homology) domains [15]. EH domains are protein-protein interaction modules which are present in a variety of proteins including two yeast proteins with a putative role in endocytosis and actin function, End3 [16] and Pan1 [17]. A recent analysis of the EH domains of Eps15 performed by a phage display library identified the amino acid sequence asparagine-proline-phenylalanine (NPF) as the core of the EH domain-binding site [18]. Three NPF motifs are present in the COOH-terminal region unique to rat synaptojanin-170, suggesting that this protein is a potential binding partner for Eps15.

In this paper we show the localization of synaptojanin 1 at clathrin-coated endocytic intermediates and demonstrate an interaction between synaptojanin-170 and Eps15 providing further evidence for the role of synaptojanin 1 in clathrin-mediated endocytosis.

2. Materials and methods

2.1. Antibodies

Antibodies against Eps15 were previously described [18]. Rabbit and mouse polyclonal antibodies specific for synaptojanin 1 were raised against the proline-rich region of rat synaptojanin-145 using a fusion protein comprising amino acids 1156–1286 as the immunogen. A mouse monoclonal antibody (AC1) directed against the same region of synaptojanin 1 was raised by standard methods. This antibody recognizes human synaptojanin 1 only very weakly. A rabbit antibody specific for synaptojanin-170 was previously described [4].

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2.2. Immunogold electron microscopy

Gently homogenized rat retinas or hypotonically lysed brain synaptosomes were incubated in the presence of brain cytosol, ATP and GTPγS to enhance formation of coated endocytic intermediates and embedded in agarose [19]. Immunogold labeling was performed as described using anti-dynamin monoclonal antibodies Hudy-1 (Upstate Biotechnology, Lake Placid, NY, USA) and mouse anti-synaptojanin 1 polyclonal antibodies.

2.3. Cloning of human synaptojanin 1

Rat synaptojanin probes were used to isolate from a human cerebellar cDNA library (Stratagene) two overlapping clones encompassing the complete coding region of human synaptojanin 1, including the stop codon at position 1311 and the second open reading frame [4]. A full-length clone was constructed and sequenced on both strands. The presence of an alternatively spliced human synaptojanin 1 mRNA encoding synaptojanin-170 was demonstrated by amplifying the region around the stop codon at amino acid 1311 from human peripheral lymphocyte cDNA using the primers 5′-CGGCGGAATTCGGGCATCTGCTGGAAGACTGACT-3′ and 5′-CGGCGTCTAGAAGCTGGAATTGGAGGCATTGTTG-3′.

2.4. Recombinant proteins

The NH_2-terminal region of Eps15 (amino acids 2–330) including the three EH domains was expressed as a GST fusion protein (GST-EH) and purified on GST-sepharose [18]. The COOH-terminal region unique to rat synaptojanin-170 (amino acids 1309–1576) (SJ170C) containing the three NPF motifs was amplified using the primers 5′-AAACCGGAATTCCAGCAGGTAAAAATAAATGG-3′ and 5′-AAACGCATCGACTCTTTCTGTGAAGTCCAATG-3′, subcloned into pGEX (Pharmacia), sequenced and expressed as a GST fusion protein. SJ170C subcloned into pCDNA3.1 (Invitrogen) was transiently expressed in CHO cells using the lipofection method (Gibco BRL).

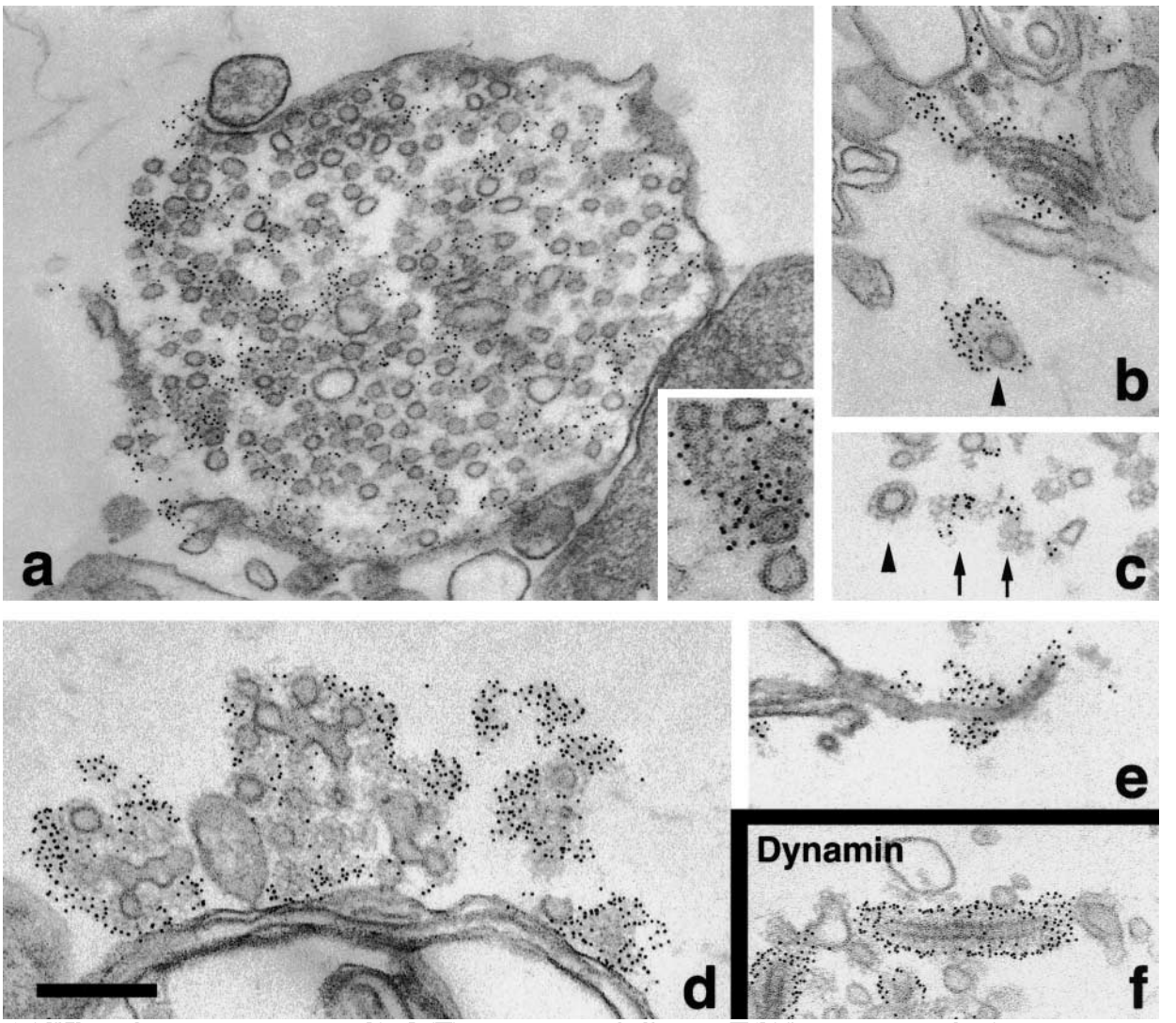

Fig. 1. Localization of synaptojanin in nerve terminal membranes revealed by immunogold electron microscopy. Gently homogenized rat retinas (a, b, d, e) or hypotonically lysed brain synaptosomes (c and f) were incubated in the presence of brain cytosol, ATP and GTPγS to enhance formation of coated endocytic intermediates, embedded in agarose and labeled by immunogold as described [19]. Note in (a) the concentration of anti-synaptojanin immunogold on matrix material interspersed among synaptic vesicles. The inset in (a) shows at a higher magnification an area of the main field demonstrating the presence of a clathrin-like lattice in correspondence to a cluster of immunogold. Arrowheads in (b) and (c) point to a labeled and an unlabeled clathrin-coated profile respectively. Arrows in (c) indicate grazing sections through tangentially cut clathrin coats. A complex membrane invagination ending in multiple clathrin-coated buds is visible in (d) and is heavily labeled by immunogold. Dynamin-coated tubules decorated by scattered anti-synaptojanin immunogold are visible in (b) and (e). The labeling was performed by mouse anti-synaptojanin polyclonal antibodies and anti-dynamin monoclonal antibodies Hudy-1 (Upstate Biotechnology, Lake Placid, NY, USA) folowed by rabbit anti-mouse IgGs and protein A gold. The same type of tubules are heavily and homogeneously decorated by anti-dynamin immunogold (f). Bar: a = 250 nm (inset 500 nm); b–f = 200 nm.

2.5. Affinity chromatography

Triton X-100 extracts of 19 days embryonic rat brain were prepared as described [9], incubated for 4 h with GST-EH or GST-SJ170C prebound to glutathione sepharose, and eluted with SDS sample buffer. Triton X-100 extracts of SJ170-expressing CHO cells were prepared as described [3] and either incubated with GST-EH or separated by SDS-PAGE for overlay experiments.

2.6. Miscellaneous procedures

Overlay assays, SDS-PAGE and Western blotting were performed as described [5].

3. Results

Immunofluorescence and subcellular fractionation data have demonstrated a concentration of synaptojanin 1 in nerve terminals [3]. However, its distribution was not investigated so far by electron microscopy. To determine the subcellular localization of synaptojanin 1 at the ultrastructural level, nerve terminal membranes from rat brain and retinas (synaptojanin 1 is highly concentrated in retinal synapses) were incubated with rat brain cytosol, ATP and GTPγS, i.e. under conditions which greatly enhance the formation of coated endocytic intermediates [19]. After fixation, these membranes were labeled by immunogold for synaptojanin 1 (using an antibody that recognizes both synaptojanin 1 isoforms) and for dynamin as a control. Fig. 1a shows that in nerve terminals synaptojanin 1 immunoreactivity is concentrated at 'hot spots' interspersed among the synaptic vesicles. The vesicles themselves were not labeled in agreement with previous subcellular fractionation data [3]. The 'hot spots' generally corresponded to matrix material and often included portions of clathrin coats recognizable by their characteristic icosahedral morphology (Fig. 1a–c). Complex membrane invaginations ending in multiple clathrin-coated buds were often visible in retinal synapses, and synaptojanin 1 was highly concentrated around their coats (Fig. 1d). Synaptojanin 1 immunolabeling, however, was not homogeneous on clathrin coats (Fig. 1d), and some

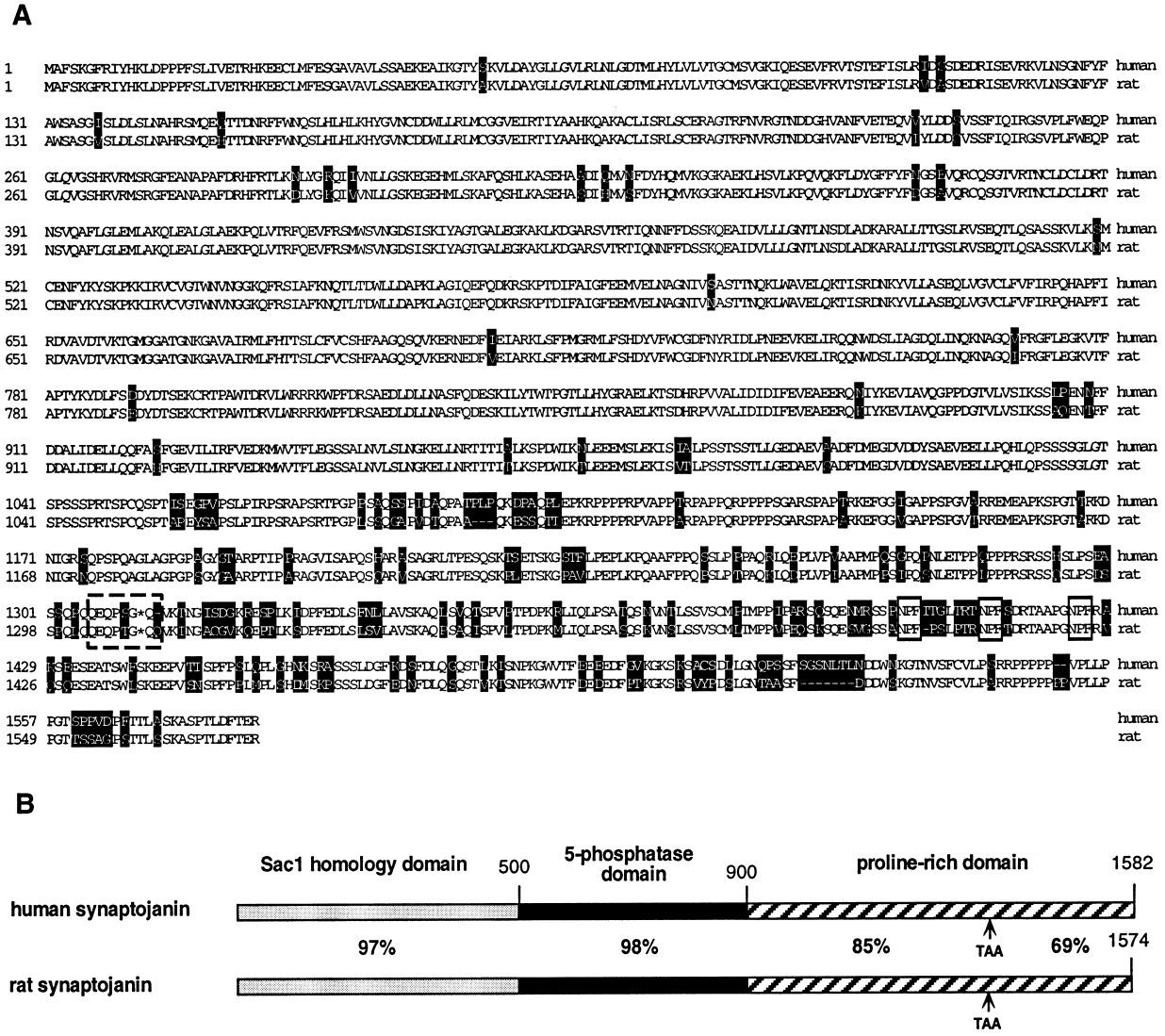

Fig. 2. Comparison between the amino acid sequences of rat [4] and human (accession numbers AF009039 and AF009040) synaptojanin. A: Alignment of the two sequences (non-identical amino acid residues are highlighted). The sequence enclosed by a discontinuous line around the stop codon at position 1311 of human synaptojanin (asterisk) corresponds to an exon which is omitted in the mRNA encoding synaptojanin-170, but included in the mRNA encoding synaptojanin-145. This alternative splicing mechanism generates the 170 and the 145 kDa isoform respectively. The three NPF motifs in the COOH-terminal region of synaptojanin-170 are outlined. B: Domain cartoon of synaptojanin and percent identity between the human and rat protein for each of the domains.

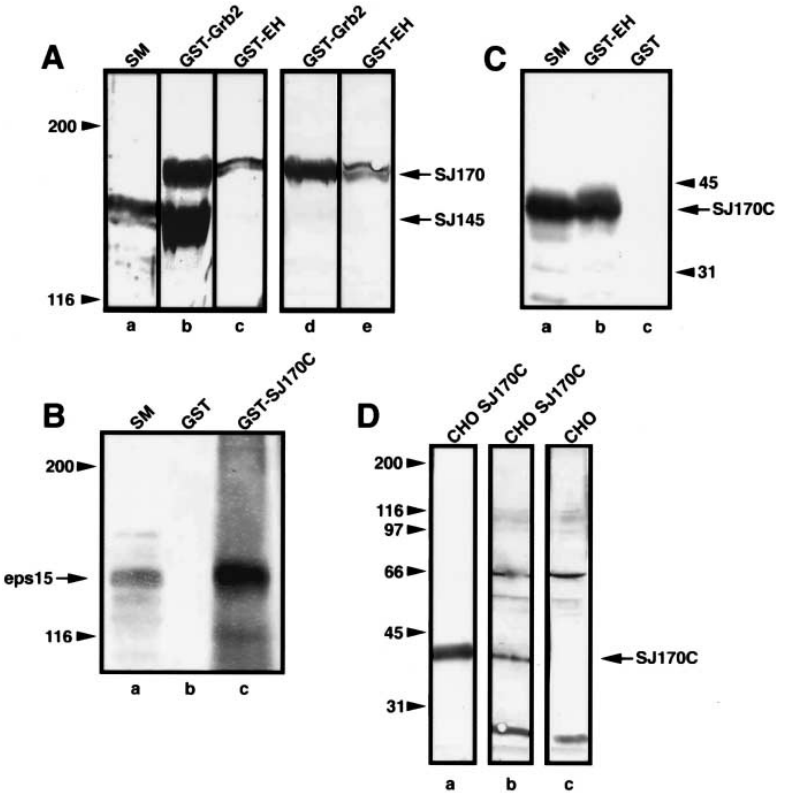

Fig. 3. Interaction between Eps15 and synaptojanin-170. A: Affinity purification of a Triton X-100 extract of 19 days embryonic rat brain on GST fusion proteins of either Grb2 or the NH_2-terminal region of human Eps15 (amino acids 2–330) which comprises its three EH domains (GST-EH) [18]. The figure shows Western blots probed with an antibody directed against the proline-rich region of synaptojanin-145 (lanes a–c) or against the C-terminal region specific to synaptojanin-170 (lanes d and e). SM, starting material (detergent extract); lanes b–e, material bound to the GST proteins immobilized on glutathione-agarose beads. B: Affinity purification of a detergent extract of adult rat brain on a GST fusion protein consisting of the COOH-terminal region of rat synaptojanin-170 (SJ170C) (amino acids 1309–1576). The starting material (lane a) and the material bound to the fusion protein or to GST alone (lanes b and c) were detected by Western blotting with antibodies directed against Eps15. C: Affinity purification on GST-EH of a detergent extract of CHO cells expressing the COOH-terminal region of synaptojanin-170 (SJ170C). The starting material (a) and the material bound to GST-EH or to GST alone (lanes b and c) were probed by Western blotting with antibodies directed against SJ170C. D: SDS gels of total homogenates of untransfected CHO cells (lane c) and CHO cells transfected with SJ170C (lanes a and b) were probed with either antibodies directed against SJ170C (lane a) or the GST-EH fusion protein followed by anti-GST antibodies (lanes b and c). The GST-EH fusion protein binds to several bands (see also [15]), but only in transfected cells does it detect a protein band with the same motility as SJ170C.

clathrin-coated membrane profiles were completely unlabeled (Fig. 1c), indicating that synaptojanin 1 is not an intrinsic component of the coat. Similar results were previously obtained for amphiphysin [20] and dynamin [21]. Clusters of synaptojanin 1 immunogold were also present on the dynamin-coated tubules which form in the presence of GTPγS, but the uneven localization of anti-synaptojanin 1 immunogold on these structures (Fig. 1b,e) clearly differed from the heavy, continuous labeling produced by anti-dynamin immunogold (Fig. 1f). These data further support an involvement of synaptojanin 1 in clathrin-mediated endocytosis.

Targeting of both synaptojanin 1 isoforms to clathrin-coated membrane areas may be mediated by SH3 domain-containing proteins, including amphiphysin [5], as previously shown for dynamin [22,23]. Amphiphysin binds to synaptojanin 1 and dynamin 1 via its SH3 domain [5,24], whereas a different domain is responsible for its binding to clathrin [6,25] and to the appendage domain of the α-subunit (α-adaptin) of the clathrin adaptor AP2 [5,26]. The possibility of an alternative targeting mechanism selective for synaptojanin-170 was raised by the recent discovery of NPF motifs as the core sequence of ligands for the EH domains of Eps15 [18]. Via a distinct domain Eps15, like amphiphysin, binds α-adaptin [12,13]. Three NPF motifs are present in the COOH-terminal domain of synaptojanin-170. We have now cloned both isoforms of human synaptojanin 1 and found that the alternative splicing mechanism generating these isoforms is conserved (Fig. 2). Rat and human synaptojanin 1 have highly conserved Sac1 homology and inositol 5-phosphatase domains but differ substantially in their COOH-terminal proline-rich tails. However, the three NPF motifs in the COOH-terminal domain unique to synaptojanin-170 are conserved, indicating their functional importance.

To investigate whether synaptojanin-170 and Eps15 can interact, a GST fusion protein of the NH_2-terminal domain of Eps15, containing its three EH domains (GST-EH) [18], was used to affinity purify a non-ionic detergent extract of embryonic brain, which is enriched in synaptojanin-170 [4]. As shown in Fig. 3A, synaptojanin-170 was present in the starting material only at the limit of detectability under the Western blotting conditions used, but was highly enriched in the eluate of the GST-EH affinity column. A Grb2 affinity column retained both synaptojanin isoforms as previously described [2] (Fig. 3A). Conversely, Eps15 present in an adult rat brain

extract was selectively affinity purified by a GST fusion protein comprising the COOH-terminal region of rat synaptojanin-170 (SJ170C) (Fig. 3B).

To further confirm that this interaction is mediated by the NPF-containing region of synaptojanin-170, the COOH-terminal fragment unique to synaptojanin-170 was expressed in CHO cells and detergent extracts of the cells were affinity purified on the GST-EH affinity column. As shown by Fig. 3C, the synaptojanin 1 fragment was specifically retained by the column. Finally, the COOH-terminal fragment of synaptojanin-170 expressed in CHO cells was recognized by the GST-EH fusion protein in a gel overlay assay (Fig. 3D), thus demonstrating that the interaction is direct.

4. Discussion

The results reported in this study provide new evidence supporting a role of synaptojanin 1 in endocytosis. We have shown that in nerve terminal membranes synaptojanin 1 is concentrated on endocytic intermediates. Its localization on clathrin-coated membrane invaginations was similar to that of amphiphysin 1 [20] and dynamin ([21] and this study). On dynamin-coated tubules, synaptojanin 1 had a scattered distribution similar to that of amphiphysin 1 [20]. These data strongly support the hypothesis that the functions of dynamin and synaptojanin 1 are closely interrelated and that amphiphysin is a physiological binding partner for both proteins [10]. Synaptojanin 1 was also recruited to clathrin- and dynamin-coated intermediates when brain cytosol was incubated with fibroblastic membranes (our unpublished observations). Although our anti-synaptojanin 1 antibodies did not produce a detectable fluorescent signal outside the nervous system (probably due to the much lower concentration of synaptojanin 1 in non-neuronal tissue), synaptojanin 1 may play a conserved function in nerve terminals and non-neuronal cells. This possibility is supported by the biochemical detectability of both isoforms of synaptojanin 1 in a variety of tissues and by the presence of synaptojanin homologs in *S. cerevisae* [27].

Additional evidence for an involvement of synaptojanin 1 in endocytosis comes from the identification of its 170 kDa isoform as an Eps15-binding protein. Eps15, first discovered as a component of the EGF receptor signal transduction machinery [11], is now recognized as an important protein of endocytic clathrin coats [12–15]. Binding of the EH domain-containing region of Eps15 to the NPF-containing region of synaptojanin 1 could be demonstrated by a variety of in vitro assays, although not by in vivo coprecipitation. The physiological significance of these findings, however, is supported by independent work carried out in yeast showing genetic interaction between the Pan1 protein (which contains two EH domains) and the Sjl1 protein [27], a yeast homolog of synaptojanin 1 which contains one NPF motif (B. Wendland and S. Emr, personal communication). The interaction between Eps15 and synaptojanin-170 may be very transient, or subject to regulation, and therefore difficult to detect in vivo.

Eps15 is present in nerve terminals (our unpublished observations). However, adult brain predominantly expresses synaptojanin-145 [4]. Thus, the direct binding of Eps15 to synaptojanin-170 does not appear to be critical for the specialized clathrin-mediated endocytosis of synaptic vesicles characteristic of synapses. Synaptojanin-170, which is relatively more enriched than synaptojanin-145 in developing brain as well as in non-neuronal cells [4,9], may instead be implicated in a house-keeping mechanism of endocytosis conserved from yeast to mammalian cells and possibly in additional functions. A similar situation may exist with proteins of the AP180 family, which are also thought to participate in the endocytic reaction [28–30]. The AP180 family member concentrated at synapses does not contain NPF motifs [28]. However, its house-keeping homologue CALM [31], as well as its two homologues in *S. cerevisae*, have multiple NPF motifs. Accordingly, genetic and biochemical interactions between Pan1 and AP180 homologues have also been observed in yeast (B. Wendland and S. Emr, personal communication).

Eps15 is associated with, and is phosphorylated by, the EGF receptor kinase upon EGF stimulation [11]. It is noteworthy that in transfected cells synaptojanin-145 has been shown to bind the activated EGF receptor, most likely via the adaptor protein Grb2 [32]. Thus, both isoforms of synaptojanin 1 may be involved in protein interaction cascades downstream of tyrosine receptor kinase signaling. Effects of the inositol 5-phosphatase activity of synaptojanin 1 on intracellular signaling may overlap with effects of this activity on endocytosis and actin function mediated by a modification of the phosphoinositide composition of the membrane [33]. In conclusion these findings represent yet another example of the important interconnections which are emerging between molecular mechanisms implicated in the endocytic reaction of clathrin-coated vesicles and signaling cascades activated by receptors which are internalized by clathrin-mediated endocytosis [34].

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