The proline-rich domain of synaptojanin 1, a synaptic protein with phosphatidylinositol phosphatase activity, binds to amphiphysin and to a family of recently discovered proteins known as the SH3p4/8/13, the SH3-GL, or the endophilin family. These interactions are mediated by SH3 domains and are believed to play a regulatory role in synaptic vesicle recycling. We have precisely mapped the target peptides on human synaptojanin that are recognized by the SH3 domains of endophilins and amphiphysin and proven that they are distinct. By a combination of different approaches, selection of phage displayed peptide libraries, substitution analyses of peptides synthesized on cellulose membranes, and a peptide scan spanning a 252-residue long synaptojanin fragment, we have concluded that amphiphysin binds to two sites, PIRPSR and PTIPPR, whereas endophilin has a distinct preferred binding site, PKRPPPR. The comparison of the results obtained by phage display and substitution analysis permitted the identification of proline and arginine at positions 4 and 6 in the PIRPSR and PTIPPR target sequence as the major determinants of the recognition specificity mediated by the SH3 domain of amphiphysin 1. More complex is the structural rationalization of the preferred endophilin ligands where SH3 binding cannot be easily interpreted in the framework of the “classical” type I or type II SH3 binding models. Our results suggest that the binding repertoire of SH3 domains may be more complex than originally predicted.

SH3 domains bind to proline-rich peptides that fold into a polyproline type 2 helix. Many SH3-binding proteins contain relatively long proline-rich domains (PRD) with multiple potential SH3 interaction sites (1–4). Given the relatively low specificity of peptide recognition mediated by SH3 domains, it is not clear whether all these interactions, which are identified in vitro, are of functional significance. A second question that arises is whether SH3 domains bind rather unspecifically to many sites along the PRD or rather form specific complexes by binding to unique and distinct sites.

Dynamin, synaptojanin, and synapsin, three proteins that are concentrated in the pre-synaptic components of nerve terminals, bear proline-rich regions that bind to diverse SH3-containing proteins. Synapsin I is the main synaptic ligand of the SH3 domain of the adapter protein Grb2 in vitro (2). Recently it has been reported that the same proline-rich D region of synapsin I interacts with c-Src and stimulates its tyrosine kinase activity (5). The physiological significance of these interactions is not clear yet. In contrast, strong evidence supports the notion that disruption of the interaction between amphiphysin and the PRD of dynamin impairs synaptic vesicle endocytosis (6). Dynamin is a GTPase that forms a collar at the neck of forming endocytic vesicles and participates in the fission process that results in the formation of free vesicles (7). Several other SH3-containing proteins have been shown to bind to dynamin in vitro (1, 8–10).

Synaptojanin is a third protein, concentrated in the pre-synaptic compartment, that contains a carboxyl-terminal PRD. This protein was initially discovered as it binds to the SH3 domains of Grb2 (2) and was subsequently characterized as an inositol 5-phosphatase that dephosphorylates inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetrakisphosphate, and phosphtidylinositol 4,5-bisphosphate at the 5 position of the inositol ring (11). A direct involvement of synaptojanin in vesicle endocytosis has not been demonstrated. However, its localization, and the recognition that phosphate metabolism is implicated in a variety of membrane trafficking events (12), has steered considerable interest in its potential role in endocytosis. Confirming this notion, the disruption of three synaptojanin orthologous yeast genes, single and in pairs, resulted in mutant strains with abnormal vacuolar and plasma membrane morphology as well as increased sensitivity to osmotic stress and defects in endocytosis (13, 14). Finally the carboxy terminus of synaptojanin binds to the SH3 domains of amphiphysins (isoforms 1 and 2), an eutheridemic protein with an established role in endocytosis (6, 15–17). Recently, another SH3-containing protein of 40 kDa was found to bind to synaptojanin in overlay assays or in the yeast 2-hybrid system (3, 18). This 40-kDa protein is a member of a family of three very homologous proteins that were originally identified in a mouse expression library (19) and independently cloned by a degenerate oligonucleotide amplification approach from human brain cDNA (20). The members of this family were named SH3p4, SH3p8, and SH3p16 in mouse and GL2, GL1, and GL3 in man. Recently, Micheva et al. (22) have proposed to rename SH3p4/
GL2 into endophilin, based on its affinity for several endocytic proteins. Here, for sake of clarity, we will refer to all three members of this family as endophilins while maintaining the SH3-GL numbering. Thus endophilins 1, 2, and 3 correspond to SH3-GL1/SH3p3, SH3-GL2/SH3p4, and SH3-GL3/SH3p13, respectively.

The three members of the endophilin family bind to synaptojanin isoform 1 but not isoform 2 (21). Endophilin 2 has been more extensively characterized because of its prominent localization in the central nervous system (18, 20).

The suggestion that the SH3-mediated binding of amphiphysin and endophilin 2 to synaptojanin 1 is of physiological significance is reinforced by the observation that the three proteins form two distinct complexes that can be immunoprecipitated from brain extracts (22). Amphiphysin is found in a complex with synaptojanin 1 and dynamin, whereas endophilin 2 can be immunoprecipitated with synaptojanin 1.

In this work we describe the recognition specificity of the SH3 domains of amphiphysin 1 and endophilins and the mapping of their binding sites on the synaptojanin 1 PRD.

**EXPERIMENTAL PROCEDURES**

**Phage Display Methodology**—Library construction and panning were performed as described (23, 24). Briefly, 2–20 μg of GST-SH3 fusion protein bound to glutathione-Sepharose 4B gel (Amersham Pharmacia Biotech) were incubated with 10¹⁰ infectious particles from a nonapeptide library. After washing 10 times with PBS, 0.5% Tween 20, the bound phage was eluted with 100 mM glycine HCl, pH 2.2. After three selection cycles, the binding of isolated clones was confirmed by ELISA. Microtiter wells were coated with 10⁴ particles of a clonal phage stock and incubated with 0.2 μg of GST-SH3 fusion protein. The wells were then washed 10 times with PBS, 0.1% Tween 20, and bound protein was detected with anti-GST goat primary antibody (Amersham Pharmacia Biotech) and a secondary anti-goat monoclonal alkaline phosphatase-conjugated antibody (Sigma). Clones with strong SH3 binding activity were selected for further analysis. The sequence of the peptides displayed by positive clones were determined by manual and automated (ABI PRISM 310 Perkin-Elmer) sequencing of phage single-stranded DNA using universal M13–40 primer.

**Production of GST Fusion Proteins**—An endophilin 1 clone from a human fetal brain cDNA library (20) was used as template in PCR to generate endophilin 1-SH3 coding fragment (residues 302–368) with the forward primer 5'-AGGATCATCGCCGCAGCAGGTGAGCGTCGCC-3' (GL1-F12) and the reverse primer 5'-AGGATCATCGCCGCAGCAGGAGAC-3' (GL1-R16). Similarly an endophilin 2 clone was used as template to generate an endophilin 2-SH3 coding fragment (residues 286–352) with the forward primer 5'-AGGATCATCGCCGCAGCAGGTGAGCGTCGCC-3' (GL2-F8) and with the reverse primer 5'-AGGATCATCGCCGCAGCAGGTGAGCGTCGCC-3' (GL2-R4). Finally, the endophilin 3 SH3 coding fragment was cloned in frame with GST using BamHI-EcoRI sites of pGEX-2T (Amersham Pharmacia Biotech). The GST fusion proteins were expressed and purified as suggested by the producer.

**Peptides Synthesis**—Peptides bound to continuous cellulose membrane supports were prepared by automated spot synthesis (Abimed, Langenfeld, Germany) using Whatman 50 cellulose membrane (Whatman, Maidstone, UK) as described previously in detail (26–29). All peptides were amino-terminally acetylated using acetyladhyde and diisopropylethylamine.

**RESULTS**

**Binding Specificity of the SH3 Domains of Amphiphysin and Endophilins**—In order to determine the recognition specificity of the human amphiphysin and endophilins SH3 domains, we used these domains to select peptide ligands from a peptide repertoire displayed by fusion to the major capsid protein of filamentous 1 phage. The SH3 domains of amphiphysin 1 and endophilins 1, 2, and 3 were produced by cloning their coding sequence into a GST fusion expression vector, and the affinity purified domains were used to pan a nonapeptide library (23). After three selection cycles, 20 single clones, derived from each of the four panning experiments, were tested by phage ELISA, and the amino acid sequence of the peptides displayed by the positive ones (approximately 50%) were derived from the DNA sequence of the gene VIII insert. In Fig. 1, we have aligned the peptide sequences, obtained from each selection experiment, to maximize peptide homology. Endophilins 1 and 2 selected a limited number of peptides that were found repeatedly and whose amino acid sequence can be represented by the consensus P+RPPXpr, where the residues in capital letters are always found at the corresponding position in each selected peptide, + represents either Lys or Arg, and X any amino acid. The SH3 domain of endophilin 3 is more tolerant in the second position of the consensus, where other residues aside from Arg and Lys can be accepted. The P+RPPXpr motif is always preceded either by a positively charged residue or by the phenylalanine that in the PVIII phage coat protein immediately precedes the inserted peptide.

The SH3 domain of amphiphysin, in contrast, selects peptides that conform to the consensus RXPR. Since the amino acid sequences of these peptides can be aligned without shifting...
their frame, it is possible that flanking residues in the pVIII coding sequence may be important in the binding process.

To confirm their recognition specificity, we tested the four domains, plus the SH3 of the MYO3 yeast protein as a control, by phage ELISA against a panel of phage clones whose sequences were considered representative of the consensus in Fig. 1. As illustrated in Fig. 2, phages displaying peptides containing the PKRPP or PRRPP motifs were recognized by the three SH3 of the endophilins but not from the ones of amphiphysin 1 or the control MYO3p. In contrast, peptides containing a single positively charged residue, PRPR or PQRPP, only reacted with endophilin 3. Finally, peptides conforming to the consensus derived from the amphiphysin 1 panning experiment predominantly bound to the amphiphysin SH3.

In order to confirm the results obtained by phage display, we performed a competition experiment in which overlay binding of endophilin 2 SH3 was carried out in the presence of 100 μM of the biotinylated peptide GSGSAPTYPPPLPPS. In these conditions, the dynamin and synaptogin signals (3) are reduced by approximately 70% and completely disappear when the peptide that precedes the peptide in the phage context is in italics. Numbers refer to the number of times that the specific peptide was found to be displayed by independently isolated clones. Boxed below each sequence list, we have represented the consensus sequences where we have reported in capital letters the residues that are conserved in all peptides and in lowercase letters the residues that are found in more than 50% of the peptide sequences. + represents either Arg or Lys, x indicates any amino acid, and # any hydrophobic residue.

**Fig. 1. Peptides selected by phage display.** Hybrid GST-SH3 proteins were linked to glutathione-Sepharose and utilized to affinity purify phage displaying peptide ligands. After three panning cycles approximately 20 clones were tested by phage ELISA and the amino acid sequence of the peptides displayed by the positive clones deduced from the DNA sequence of the corresponding gene VIII. Peptide sequences are in the one-letter amino acid code. The F that precedes the peptide in the phage context is in italics. Numbers refer to the number of times that the specific peptide was found to be displayed by independently isolated clones. Boxed below each sequence list, we have represented the consensus sequences where we have reported in capital letters the residues that are conserved in all peptides and in lowercase letters the residues that are found in more than 50% of the peptide sequences. + represents either Arg or Lys, x indicates any amino acid, and # any hydrophobic residue.

Peptide competition. Approximately 15 μg of rat brain extract were electrophoresed on a 10% polyacrylamide gel and, after transfer to a nitrocellulose membrane, overlaid either with anti-dynamin and anti-synapsin antibodies (lane 1) or with a GST-endophilin 2-SH3 hybrid protein (lanes 2–8). The interaction of the endophilin 2-SH3 with the target proteins in the extract was competed with (lane 3) 100 μM peptide GSGSAPTYPPPLPPS (lane 4), 50 μM of the same peptide complexed with streptavidin (lane 5), 50 μM of a control peptide GSGSPTYPPPLPP complexed with streptavidin (lane 7), 2.5 × 10⁶ phage/ml displaying the peptide PRRPPPPRPM or (lane 8) 5 × 10⁶ of control helper phage.

**Fig. 2. Phage ELISA.** Approximately 10⁸ phage particles, displaying the peptide indicated above each diagram, were adsorbed to each vial of a microtiter plate and incubated with 0.2 μg of the relevant GST-SH3 fusion proteins. The retained domain was revealed by an anti-GST antibody and a secondary alkaline phosphatase-conjugated antibody. The SH3 domain of the yeast protein MYO3p was used as a control. The peptide RYPPSYSFP was selected by the MYO3p-SH3 from a nonapeptide phage displayed library (G. Cestra, unpublished results).

**Fig. 3. Peptide competition.** Approximately 15 μg of rat brain extract were electrophoresed on a 10% polyacrylamide gel and, after transfer to a nitrocellulose membrane, overlaid either with anti-dynamin and anti-synapsin antibodies (lane 1) or with a GST-endophilin 2-SH3 hybrid protein (lanes 2–8). The interaction of the endophilin 2-SH3 with the target proteins in the extract was competed with (lane 3) 100 μM peptide GSGSAPTYPPPLPPS (lane 4), 50 μM of the same peptide complexed with streptavidin (lane 5), 50 μM of a control peptide GSGSPTYPPPLPP complexed with streptavidin (lane 7), 2.5 × 10⁶ phage/ml displaying the peptide PRRPPPPRPM or (lane 8) 5 × 10⁶ of control helper phage.
corresponds to a peptide that, as a result of mutagenesis, contains an RPXR motif (LEPKRPPPRP).

Surprisingly, and somewhat in contrast with the phage ELISA experiment, the endophilin 2 SH3 domain binds with higher affinity to peptide Syn1064–1074 than to peptide Syn103–1113 (Fig. 4B). The substitution analysis reveals that peptide 2 does not bind to the endophilin SH3 because of the negatively charged residue that precedes the PKRP motif. Whenever the Glu at position 2 is changed into a residue with either a positive or a hydrophobic side chain, binding is restored. This is in accord with the results of the phage display experiment that indicated that positive residues and Phe are preferred at that position (see Fig. 1). When a 5-fold higher membrane-bound peptide concentration is used in a similar experiment, a stronger signal is obtained, and residues that are important for recognition specificity are revealed (Fig. 4C). Cys and negatively charged residues are hardly admitted at any position. Consistent with the phage display results, the residues in the PKRPPXPR motif do not tolerate the vast majority of substitutions.

The endophilin SH3 binds to peptide Syn1064–1074 only marginally less efficiently than the amphiphysin SH3. Binding, however, is less specific and displays a different sensitivity to amino acid substitutions. Substitution of the first Arg of the RPXR motif severely affects binding, whereas the second Arg tolerates hydrophobic side chains. The remaining peptide residues are rather tolerant as long as Cys, Asp, Glu, or Tyr are avoided.

**Peptide Scanning of the Synaptojanin Pro-rich Carboxy-terminal Region**—According to the substitution analysis, the peptide LEPKRPFPFRP is a suboptimal ligand for the endophilin SH3 domain. In order to identify alternative sequences in synaptojanin that may be involved in endophilin and amphiphysin SH3 binding, we synthesized 126 overlapping undecapeptides spanning the entire carboxy-terminal region of human synaptojanin (Fig. 5).

In agreement with the phage display experiment, the amphiphysin SH3 reacts with cellulose-bound peptides containing the sequence LPPRSSR (region A1) that exactly matches the RPXR consensus. Two more regions, containing the PTPPR (region A2) and PPQPR (region A3) sequences, showed significant binding in agreement with the substitution experiment that indicated that the first arginine in the motif could be substituted with isoleucine or proline. More complex is the binding pattern obtained with the endophilin SH3. We identified three main regions as putative ligands of this domain. Regions E1 and E3 overlap sequences that were already mapped as amphiphysin targets (A1 and A3), whereas region E2 probably including two binding motifs, encompasses the endophilin binding consensus PKRP, and extends approximately 15 amino acids beyond. The likely biological significance of these SH3 target sites is supported by the observation that their sequences and binding properties are conserved in the rat synaptojanin (not shown).

**Binding of SH3 Domains to Synaptojanin Fragments**—To confirm the mapping of SH3 targets obtained with the Pep-Scan experiment, we expressed three different fragments of the synaptojanin carboxy terminus, as fusion to GST. Fragment 1 (F1) contains targets A1 (E1) and E2, fragment 2 (F2) E2’ and A2, and fragment 3 (F3) A3 (E3) (Fig. 6A). Fig. 6B reports the results of an overlay experiment where the three fragments were transferred to nitrocellulose filters and probed with 32P-labeled chimeric GST-SH3 proteins. Both the amphiphysin and endophilin SH3 bind to fragment F1. F2 binds to amphiphysin and to a lesser degree to endophilin. Interestingly the endophilin SH3 binds to the degradation products of the GST-F2 protein as efficiently as to the full-length protein, whereas the amphiphysin SH3 only binds to the non-degraded protein. This suggests that the first domain binds to the NH2-terminal side of the F2 fragment, whereas the latter binds to a peptide target that is close to the COOH-terminal side. Finally fragment F3 contains a target site for amphiphysin only.

These results are in agreement with the Pep-Scan experiment that suggested that synaptojanin contains multiple binding sites for the amphiphysin and endophilin SH3s. At the same time, they contribute to rank the affinities of the putative peptide targets in a larger protein context: A1 ~ A2 > A3 and E1 + E2 > E2’ > E3.

To map precisely the SH3 target peptides, predicted by the phage display and Pep-Scan experiments, in this larger protein context, we expressed fusion proteins containing fragments of synaptojanin mutated in the strongest putative binding sites, A1 (E1), E2, and A2 (Fig. 6C).

The endophilin SH3 binds to the synaptojanin fragment car-
Fig. 6. Mutant analysis of the SH3 target sites in the synaptojanin PRD. A is a schematic representation of the carboxyl-terminal region of synaptojanin and of the fragments, F1, F2, and F3, that were produced by fusion to GST. The sequence of the mutants in the A1, E2, and A2 putative target sites are also indicated. B, 3 μg of GST hybrid proteins fused to the indicated synaptojanin fragments were electrophoresed on a 10% polyacrylamide gel, blotted onto a nitrocellulose filter, and probed with in vitro phosphorylated (approximately 2 × 10^6 cpm) GST-endophilin 2-SH3 or GST-amphiphysin_SH3. After washing the membrane were scanned with a STORM PhosphorImager, and the radioactivity in each band was quantitated. C, the experiment was done in the same conditions as in B. The synaptojanin fragments electrophoresed in the gel contained the mutations indicated above each lane. D, 1 μg of GST-endophilin 2-SH3 (or GST-amphiphysin 1-SH3), cross-linked to tosyl-activated magnetic beads, were incubated with different concentrations of ^32P-labeled GST-F1 (wild type or carrying mutations in the A1 or E2 SH3 target sites). The bound and free label was measured after separation of the magnetic beads. Amp, amphiphysin; End, endophilin; wt, wild type.

Fig. 7. Putative SH3 targets in the dynamin PRD. Top, alignment of the carboxyl-terminal proline-rich domain of five dynamins from Drosohila, rat, and humans. Four regions in the PRD sequence of human and rat dynamins were identified as putative amphiphysin (Amph) and endophilin 2 (End 2) SH3 binding regions on the basis of the phage display and Pep-Spot experiments. The corresponding peptides were synthesized on nitrocellulose membrane and incubated with GST-SH3 hybrid proteins cross-linked to horseradish peroxidase. The bound hybrid proteins were revealed by incubation with a chemiluminescent substrate.

By registering a mutation in the A1(E1) site as efficiently as the wild type fragment, whereas most of the affinity is lost when the two positively charged residues in the PKRPPP motif are changed into PTSPPP (a2m). In contrast, amphiphysin recognition of the F1 fragment is almost abolished when the LPIRPSR motif in the F2 fragment is dependent on the PTIPPR peptide (or similar ones) in all the dynamin sequences shown. The amphiphysin 1 SH3 binds predominantly to the PSRPNR sequence in the proline-rich domain (PRD, amino acids 733-738) (17, 31), whereas at least one endophilin target should be located in the amino-terminal region of PRD (32). By exploiting the information extracted from the phage display and pep-spot analysis, we have synthesized the dynamin peptides characterized by a proline-rich region and positively charged residues on a cellulose membrane (Fig. 7).

The amphiphysin 1 SH3 binds predominantly to the PSRPNR peptide (or similar ones) in all the dynamin sequences that we have considered. A second minor site corresponding to the sequence PPVPSRPGASP also binds to the amphiphysin-SH3 in this assay. The endophilin 2-SH3 recognizes two binding sites with comparable apparent affinity. The first one coincides with the amphiphysin-binding site, whereas the second one is located closer to the amino terminus of the PRD and corresponds to peptide SPTPQRRAVP.

**DISCUSSION**

Binding of SH3 domains to proline-rich regions of pre-synaptic proteins plays a prominent role in vesicle recycling (1-3, 5-8, 16-18). At least three proteins that are concentrated in the presynaptic region, synapsin, dynamin, and synaptojanin, contain a carboxyl-terminal proline-rich region with several PXPF motifs that are often considered signatures for SH3-binding sites (33). Because of the abundance of putative SH3-binding sites and the relative low specificity of SH3 binding to polyproline peptides, it is not clear whether the binding of SH3-containing proteins to the polyproline domains is the result of a fairly unspecific recognition of multiple sites or rather each SH3 domain has a preferred target site. Grabs et al. (31) have recently reported that the SH3 domain of amphiphysin 1 binds to dynamin at a single site (PSRPNR) with relatively high affinity (190 nM).

To answer this question we have characterized the binding specificity of the SH3 domains of human amphiphysin and...
The preferred ligands for endophilin 2 and amphiphysin SH3 domains cannot be easily adapted to this binding model. The pXRpXR amphiphysin consensus (where lowercase P indicates that the Pro is not absolutely required at that position) could be portrayed as a novel, type II, peptide ligand with an unusual positive charge at position P0 and a tolerance for hydrophobic residues at P+2 (Fig. 8B) (the numbering of the ligand residues is as in Ref. 37). The extra positive residue, with respect to the classical type II ligand consensus, is consistent with the high density of negative charges in the hydrophilic pocket of the amphiphysin SH3-binding site, as deduced from the three-dimensional structure modeled on the amphiphysin SH3 structure (17).

More complex is the interpretation for the endophilin-preferred ligand (PKRPPXPXR), where it is difficult to identify, without ambiguity, the core PXXP SH3-binding motif. We favor a type I binding orientation with the second Pro of the PXXP stretch corresponding to P0. We are currently carrying out site-directed mutagenesis experiments to support our model. It has to be emphasized, however, that SH3 binding to type I ligands with positive residues at position P−2 and P−3 and an arginine at P+3 would represent a novel binding mode that cannot be easily derived from the current model. The detailed structural analysis of the interaction between the SH3 domain of endophilin and their target peptides should reveal novel structural features that should add to the description and prediction of SH3-ligand interaction.

Detailed conclusions drawn from comparison of the intensities of each spot in the substitution experiment should await confirmatory results from quantitative experiments. It is clear, however, that both the SPOT and phage display methods, although unrelated, give comparable results, underscoring the importance of the PKRPPXPXR and the p#XRpXR motifs for endophilin 2 and amphiphysin SH3 recognition specificity, respectively.

By looking at the intensities of the spots in the grid of the substitution experiment and by selecting at each position the spot with the best signal, one may tentatively design a peptide that would recognize an SH3 domain with high affinity and specificity. Assuming that the contributions of each side chain to the binding energy, at the different positions, are additive, a good binder for endophilin 2 SH3 should be #PRPPPPXR. This approach opens the way to the design of peptides that, by binding with high affinity and specificity to the SH3 domains of any member of the endophilin family or of amphiphysin, would...
permit to probe the function of these proteins by in vitro or in vivo inhibition experiments. Furthermore, the precise identification of the endophilin and amphiphysin binding sites on synaptotagmin should permit the engineering of cells or organisms in which the interaction between synaptotagmin and either of these two molecules is impaired.

The detailed characterization of the peptide recognition specificity of a given SH3 domain should in principle assist in the discovery of proteins that are natural targets and in the identification of their binding sites along the primary sequence. We have tested the reliability of this approach by predicting the amphiphysin- and endophilin-binding sites on the PRD of synaptojanin isofrom 1.

Scanning of the human synaptojanin sequence with the consensus motifs identified by phage display and substitution analysis of membrane-bound synthetic peptides permits the identification of four candidate amphiphysin targets: PIRPSR, PTIPPR, PQPQPR, and PAPPQR. Accordingly, when 126 peptides spanning the entire PRD were synthesized and tested for their ability to bind to the SH3 domain of amphiphysin, only the peptides containing the predicted motifs were positive in the assay. Furthermore, when binding was tested, by an overlay assay, in a larger protein context, we could confirm these results and prove, by site-directed mutagenesis, that synaptojanin possesses (at least) two target sites that bind to the amphiphysin SH3 with comparable apparent affinity. Alteration of the putative target sequences by site-directed mutagenesis reduced or even abolished binding thus supporting the conclusion that the SH3 domain of amphiphysin recognizes these targets in the proline-rich region. All mammalian dynamin sequences contain a PXXRX motif that, as already demonstrated in the case of dynamin I, is the major amphiphysin-binding site (Fig. 7).

On the contrary there is a single synaptojanin sequence PKRPQPQPQPQPPPRP that exactly matches the endophilin consensus. However, this is not predicted to be a good ligand since it is preceded by glutamate that is not acceptable at that position. In fact, in the Pep-Scan experiment, the endophilin SH3 was found to bind better to peptides derived from other regions of the synaptotagmin PRD. In agreement with the substitution experiment (Fig. 4B), one of these corresponds to the amphiphysin target sites PIRPSR and two more, PAPPQR and PQQPPR, can be reconciled with the amphiphysin consensus.

Direct mapping of the endophilin target sites on synaptotagmin fragments by site-directed mutagenesis is consistent with PKRPQPQPQPQPQPQP as being the preferred endophilin-binding site. The apparent contradiction between the results of the peptide experiments and of the mapping of target sites on more extended protein fragments underscores the importance of protein context in determining the binding affinity of a given peptide target. It is not clear at present whether this is obtained by additional contacts of the SH3 domain with distal protein residues, as demonstrated in the Ne/Hex-SH3 interaction (42, 43), or rather by a conformational change (stabilization) of the polypolyeptide target induced by the structural context.

In summary the amphiphysin SH3 domain has a high specificity for the extended consensus outlined in Fig. 8, whereas the endophilin SH3 displays a lesser specificity and binds to peptides that can be grouped into at least three classes characterized by the consensus (i) PR/KRPRXXX, (ii) PXPRXP (similar to the amphiphysin one), and finally (iii) a third class exemplified by the dynamin 1 peptide SPTQPQRARAP.

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The SH3 Domains of Endophilin and Amphiphysin Bind to the Proline-rich Region of Synaptojanin 1 at Distinct Sites That Display an Unconventional Binding Specificity

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