A Novel AP-2 Adaptor Interaction Motif Initially Identified in the Long-spli...
structures (26), showing that hydrolysis is not a prerequisite for internalization.

Synaptotagin 1 is composed of two tandemly arrayed catalytic phospha- tase domains, the SacI and inositol 5’-phosphatase homology domains, followed by a carboxy-terminal pro- line-rich domain (23, 27). The central inositol 5’-phosphatase domain dephosphorylates PtdIns(4,5)P2 to phosphatidylinosi- tol 4-phosphate. The proximal SacI homology domain is also a phosphatidylinositol phosphatase (28) that preferentially hy- drolizes phosphatidylinositol 3-phosphate or phosphatidyli- nositol 4-phosphate. Thus, synaptotagin 1 is a phosphoinositide polyphosphatase that can regenerate phosphatidylinositol by sequential dephosphorylation reactions. In humans, the gene for synaptotagin 1 is located on chromosome 21 (29). There is some evidence for increased expression in Down’s syndrome (30, 31) and possible connection to 21q22-linked bipolar disorder (32).

The relatively abundant 145-kDa synaptotagin 1 isoform (SJ145) is thought to be recruited to the clathrin lattice on the presynaptic plasma membrane in the brain by the interaction of the carboxy-terminal proline-rich sequences with the Src homology 3 (SH3) domains of endocytic proteins like endophilin or amphiphysin (33, 34). In this study, we show that the non- neuronal 170-kDa long splice isoform of synaptojanin 1, termed SJ170, harbors a novel AP-2 adaptor-binding element absent from SJ145. This interaction determinant engages the α-subunit appendage and likely promotes efficient targeting of the polyphosphatase to the assembling clathrin lattice in non-neu- ronal cells. A functionally analogous WXX(W/F) sequence is also found in the endocytic protein kinases AAK1 and GAK/auxilin.

EXPERIMENTAL PROCEDURES

DNA Constructs—Plasmids encoding the murine AP-2 α, appendage (α-, subunit residues 701–938), the W840A, R905A, and R916A α, appendage point mutants, the rat β2 appendage (residues 714–951), the rat epsin 1 DPW domain (residues 229–407), the autosomal recessive hypercholesterolemia (ARH) protein carboxy-terminal segment (resi- dues 180–308), and the YSKV internalization sequence of the cation- dependent mannose 6-phosphate receptor each fused to the carboxy- terminal end of glutathione S-transferase (GST) have been described (35–38). A plasmid encoding a GST-Grb2 fusion protein was kindly provided by Greg Longmore (Washington University School of Medi- cine, St. Louis). The preparation of the GST-SJ170C1 (residues 1440–1530) and GST-SJ170C2 (residues 1454–1530) plasmids from human EST n51b08.s1 was detailed previously (37). The entire alternatively spliced segment of human SJ170 (residues 1305–1575) was amplified using KIAA0910 (Kazusa Institute, Chiba, Japan) as the template and inserted similarly into pGEX-4T-1. The SJ170M1 fusion protein (resi- dues 1478–1489) was prepared by ligation of complementary oligonu- cleotides, after annealing and digestion, into EcoRI/XhoI cleaved pGEX-4T-1. The AAK1 plasmids GST-AAK1C1 (residues 547–862), GST- AAk1C2 (residues 671–862), and GST-AAK1C3 (residues 741–862) were prepared in pGEX-4T-1 by PCR utilizing KIAA1048 (Kazusa Insti- tute, Chiba, Japan) as the template and the template and ligated into pGEX-4T-1. All mutations were introduced into the appropriate vectors using the QuikChange system (Stratagene) with the required muta- genic primer pairs (the sequences of which are all available upon request). All the clones and mutations were verified by automated dyeoxygenucleotide sequencing.

Antibodies—Anti-synaptotagin antisera was generated in rabbits by immunization with a synthetic peptide (ARKDNIGRNQSPQAG) derived from the carboxy-terminal region of rat synaptotagin 1 that is common to both SJ145 and SJ170. Affinity purified antibodies, desig- nated AE/1, were prepared from the serum on covalently immobilized peptide by standard procedures. The monoclonal antibodies (mAbs) directed against amphiphysin (which recognizes both amphiphysin I and -II) and AP180 were purchased from BD Transduction Laborato- ries. Anti-clathrin mAb TD.1 and Cl57.3, the AP-1 β1/AP-2 β2-subunit- reactive mAb 101/3, the AP-2 α-subunit mAb 100/2, the anti-AP-1 γ subunit antibody AE/1, the anti-AP-1 μ subunit antibody BY1, and the affinity purified rabbit anti-epsi-aminobutyric acid receptor have been described (35, 36). Affinity purified anti-SJ170-specific antibodies (designated IωA) were generously supplied by Peter McPherson (McGill University), whereas the rabbit anti-AP-2 μ subunit serum was kindly pro- vided by Juan Bonifacio (NICHD, National Institutes of Health). The cholinergic anti-εopi515 antiserum was generously supplied by Ernst Ungewickell.

Protein and Tissue Extract Preparation—GST and the various GST fusion proteins were produced in Escherichia coli BL21 cells. The stand- ard induction protocol entails shifting log-phase cultures (A600 = 0.6) from 37 °C to room temperature and then adding isopropyl-1-thio-β- galactopyranoside to a final concentration of 100 μM. At room temperature with constant shaking, the bacteria were recovered by centrifugation at 15,000 × gmax at 4 °C for 15 min and were stored at 80 °C until used. Bacteria were lysed on ice in 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.2% (w/v) Triton X-100, 10 μM β-mercaptoethanol with sonication or in B-PER reagent (Fermentas). Insoluble material was removed by centrifugation at 53,700 × gmax at 4 °C for 15 min and then the GST fusions were collected on GSH-Sepharose. After extensive washing in phosphate-buffered saline, GST fusions were eluted with 10 mM Tris-HCl, pH 8.0, 10 mM GSH, 5 mM dithiothreitol on ice and dialyzed into phosphate-buffered saline, 1 mM dithiothreitol before use in binding assays. Several of the purified fusion proteins were cleaved from the GST with thrombin. Amersham Biosciences GST-Sepharose was immobil- ized upon GSH-Sepharose. Digestion was as recommended by the manufacturer, followed by addition of the irreversible thrombin inhibi- tor n-Phe-Pro-Arg chloromethyl ketone (Calbiochem) to a final concentra- tion of 25 μM.

Cytosol was prepared from frozen rat brain (PelFreez) by sequential differential centrifugation after homogenization in 25 mM Hepes-KOH, pH 7.2, 250 mM sucrose, 2 mM EDTA, and 2 mM EGTA supplemented with 1 mM phenylmethylsulfonyl fluoride and Complete (Roche Diag- nostics) protease inhibitor mixture. The 105,000 × gmax supernatant is defined as cytosol and was stored in small aliquots at −80 °C. Undiff- erentiated PC12 cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and stored frozen at −80 °C. Cytosol of these cells was prepared as described (36, 39). Typically, 50–400 μg of GST and the GST fusion proteins were first each immobilized upon 25 μl of packed GSH-Sepharose by incubation at 4 °C for 2 h with continuous mixing. The Sepharose beads containing the required immobilized proteins were then washed and resuspended to 50 μl in assay buffer. Clarified rat brain cytosol, PC12 cell lysates, or purified, thrombin-cleaved α or β appendage (in the presence of 0.1 mg/ml carrier bovine serum albumin) were added and the tubes were incubated at 4 °C for 60 min with continuous gentle mixing. For the competition assays, thrombin- cleaved α and β appendages were added directly into the assay mixture to a final concentration of 20 μM in the presence of 25 μM n-Phe-Pro-Arg chloromethyl ketone, an irreversible thrombin inhibitor. The GST-Sepha- rose beads were then recovered by centrifugation at 10,000 × gmax at 4 °C for 1 min and an aliquot of each supernatant was removed and adjusted to 100 μl with SDS sample buffer. After washing the GSH-Sepharose beads with −1.5 ml cold phosphate- buffered saline by centrifugation, the supernatants were aspirated and each pellet resuspended in SDS sample buffer.

Electrophoresis and Immunoblotting—Samples were resolved on polyacrylamide gels prepared with an altered acrylamide: bis-acryla- mide (30:0.4) stock solution. The decreased cross-linking generally allows better separation of the relatively large endocytic proteins, most noticeably AP180 and epsin 1. After SDS-PAGE, pro- teins were either stained with Coomassie Blue or transferred to nitro- cellulose in ice-cold 15.6 mM Tris, 120 mM glycine. Blots were usually blocked overnight in 5% nonfat milk in 10 mM Tris-HCl, pH 7.8, 150 μM...
Novel AP-2 Binding Sequence in Endocytic Proteins

**RESULTS**

Selective Binding of SJ170 to the AP-2 a Appendage—SJ170 is expressed at much lower levels in peripheral tissues than is SJ145 in the central nervous system. Using antibodies against an epitope common to both isoforms, SJ145 is easily detectable in rat brain extracts, whereas the abundance of SJ170 is below the level of detection in extracts of adult brain, liver, lung, testis, or heart (40). The SJ170 isoform only becomes detectable after enrichment with an SH3 domain-binding partner like Grb2 or amphiphysin (40). Consequently, it has been proposed that the alternatively spliced carboxyl-terminal extension in SJ170 facilitates efficient recruitment to endocytic buds forming at the cell surface (41). Indeed, in extracts from undifferentiated PC12 cells, which contain both SJ145 and SJ170, only the SJ170 isoform associates with immobilized GST-αc appendage in pull-down type assays (Fig. 1, lane d). Importantly, SJ170 does not sediment with immobilized GST alone (lane b), whereas the more abundant SJ145 isoform remains in the soluble fraction under both conditions (lanes a and c). Similar pull-down experiments utilizing rat brain cytosol, with a much higher SJ145 concentration, still shows no interaction with the αc appendage (data not shown). The enrichment of SJ170 in the GST-αc appendage pellet fraction is similar to other known endocytic accessory proteins, including amphiphysin, AP180, and epsin (lane d). By contrast, both SJ145 and SJ170 bind to the SH3 domain of Grb2 (lane f), as reported (40). Therefore, the failure to detect SJ145 in the GST-αc appendage pellet fraction rules out the possibility that SJ170 is being recruited by the SH3 domain of appendage-bound amphiphysin. These results underscore the importance of the unique SJ170 extension in precise intracellular targeting.

**A Third AP-2 Binding Sequence within the SJ170 Extension**—The alternatively spliced segment unique to SJ170 contains two known types of AP-2 interaction motif, two DPF triplets (1323DPF and 1557DPF) and a 1463F motif. We have shown previously that a carboxyl-terminal portion of SJ170 devoid of both DPF motifs (GST-SJ170C2, Fig. 2) binds to AP-2 with good apparent affinity (37). Yet mutation of the DXXFXDP motif to AXAXFXDP or AXAXFXW only reduces AP-2 association marginally (37). Therefore, to determine whether an additional AP-2 adaptor-binding element is present within the SJ170C2 residues (1454–1530), we sequentially truncated the carboxyl end in the context of a GST fusion protein. Constructs containing only residues 1454–1497 (GST-SJ170C5) or 1454–1489 (GST-SJ170C5.5) bind to AP-2 efficiently (Fig. 2). However, further removal of 7 amino acids (GST-SJ170C6, residues 1454–1482) abolishes the stable interaction with the AP-2 adaptor complex near completely. This identifies residues 1482–1489 as a region that contributes to AP-2 adaptor interactions.

This localized tract of SJ170 (residues 1482–1489) could either harbor an independent AP-2 binding motif or contribute structurally to the optimal presentation of the proximal FXDXXF sequence. To distinguish between these two possibilities, we fused a restricted region of the carboxyl terminus of SJ170 to GST and assessed adaptor engagement in vitro using rat brain cytosol. Importantly, appending only 12 amino acids of the SJ170 sequence, 1478SNPKGWVTFEEE, to GST (GST-SJ170M1) facilitates a substantial AP-2 interaction (Fig. 3A, lane d compared with lane b). The extent of adaptor binding to the GST-SJ170M1 (lane d) is lower than that seen with a larger fusion that also contains the FXDXXF sequence (lane j) but is clearly above that seen with the FXDXXF sequence alone (lane i). No AP-2 associates with GST alone under the same assay conditions (lane b). This clearly validates that the mapped region houses a novel AP-2 interaction motif that is both autonomous and transplantable. For comparison, when similarly fused to GST, a YXXØ-type endocytic internalization sequence,
A Dominant Tryptophan/Phenylalanine-based Motif—Within the region necessary for AP-2 binding, the primary sequence of SJ170 contains one Trp and one Phe residue (Fig. 2). Because phenylalanine side chains contribute an important hydrophobic binding component to several characterized AP-2 appendage interaction motifs (37), we separately mutated to Ala the two Phe residues close to the putative binding region (F1486A and F1492A). The proximal Phe is essential for productive AP-2 binding; only residual AP-2 interaction remains upon substitution with Ala (Fig. 2), which we attribute to the intact proximal intact FXDXF motif in this model protein. Phe\textsuperscript{1486} however, is not required for AP-2 engagement, in agreement with the capacity of the GST-SJ170C5.5-(1454–1489) fusion protein to associate with AP-2. Like the required Phe\textsuperscript{1486} substitution of the Trp for Ala (W1483A) abolishes the adaptor binding capacity of the GST-SJ170M1 protein (Fig. 3A, lane f). We therefore tentatively term this interaction sequence the WXXF motif. Experiments with longer SJ170 carboxy-terminal tail fusions rule out the possibility that the observed AP-2 binding is artifically because of expression of small isolated segments out of context of the native protein. The entire alternatively spliced region found in SJ170, when immobilized as a GST fusion, affinity isolates AP-2 (41) (Fig. 3B, lane d). A single W1483A substitution in the protein almost totally abolishes the interaction with AP-2 (lane f compared with lane d). This reveals that in this type of assay, the WXXF motif is a dominant endocytic interaction sequence within SJ170; we attribute the residual binding in the W1483A mutant to the intact\textsuperscript{1556}DPF and\textsuperscript{1463}FXDXF motifs. A similar effect is seen with the smaller SJ170C2 (residues 1454–1530) fusion (Fig. 2). We also confirm that the entire SJ170 carboxy-terminal segment binds weakly to clathrin as well (Fig. 3, lanes d and f) (41), but the W1483A substitution does not perturb this interaction detectably.

In other experiments with the GST-SJ17C5 fusion we find that the proximal Trp side chain cannot be replaced with Tyr, Phe, His, or Leu and still function in AP-2 interactions as does the wild-type fusion protein (data not shown). The distal aromatic, Phe\textsuperscript{1486}, can be altered to a Trp without a discernible effect on adaptor binding, however (data not shown). The results of these experiments clearly establish the existence of a novel WXXF AP-2 interaction determinant within SJ170 and argue strongly against the possibility that this association is an atypical form of YXX\textsuperscript{X} interaction with the AP-2 \text{\mu}2 subunit.

**Binary SJ170-AP-2 \text{\alpha}_{c\text{C}} Appendage Interactions**—Previously, we showed that the SJ170C2 protein, when cleaved from GST, could compete with soluble endocytic accessory proteins for binding to GST-\text{\alpha}_{c\text{C}}, appendage (37) (Fig. 4, lane h compared with lane d). When added into brain cytosol to a concentration of 20 \text{\mu}M, the inhibition of AP180 and amphiphysin I and II binding approaches that seen with the 20 \text{\mu}M epsin 1 DPW domain (residues 229–407) (lane h compared with lane f). However, unlike the DPW domain of epsin 1, which contains 8 tandemly arrayed DPW triplets (45), the SJ170C2 is unable to prevent binding of soluble brain epsin 1 to GST-\text{\alpha}_{c\text{C}} (lanes g and h compared with lanes e and f). Because the WXXF motif appears to be a dominant AP-2 interaction motif in pull-down type assays (Fig. 3), we tested the ability of SJ170C2 mutated at either the 1463\text{FXDXF} or 1463\text{FXDXF} site to inhibit binding to GST-\text{\alpha}_{c\text{C}}. Unexpectedly, mutation of either sequence dramatically reduced the inhibitory capacity of the protein (Fig. 4, lanes j and l compared with lane h). With the exception of limited inhibition of AP180 association, the remainder of the endocytic interaction partners bind similarly to that observed in the absence of an added SJ170C2 competitor (lanes j and l compared with lane d). Analysis of the different SJ170C2 pro-
teins by SDS-PAGE (not shown) shows that they were all added to equivalent concentrations, a 5-fold molar excess over the immobilized \( \alpha_c \) appendage. Also, secondary structure prediction algorithms indicate that this region of SJ170 is likely to be disordered, as are the AP-2- and clathrin-binding regions of both epsin 1 and AP180 (17, 37), making misfolding improbable in our view. Our interpretation of the data is that both the WXXF and the FDXDF motifs are required for effective competition at the platform interaction surface of the \( \alpha_c \) appendage in the configuration of this type of assay.

Appendage Specificity of the WXXF Motif—If the WXXF and FDXDF sequences can each associate physically with the \( \alpha_c \) appendage, why is there such a marked difference in the AP-2 binding capacity of these sequences in pull-down assays (Figs. 2 and 3)? One explanation could be that unlike the FDXDF motif, which cannot bind to the structurally and functionally analogous and epsin, AP180, and epsin 1 present in a PC12 cell lysate (Fig. 5B, lane f), the GST-\( \beta_2 \) appendage binds to epsin and AP180 but not to SJ170 (lane d). These experiments suggest that the WXXF motif does not interact effectively with \( \beta \) appendages, and this finding is validated by in vitro binding assays using purified proteins (Fig. 5C). Thrombin-released, monomeric \( \alpha_c \) appendage binds robustly to both GST-SJ170C5 (lane d) and the minimal GST-SJ170M1 fusion protein (lane f) but not to GST alone (lane b). In contrast, neither of these SJ170 fusion proteins associates with the monomeric \( \beta_2 \) appendage (lanes j and l compared with lane h). Instead, the \( \beta_2 \) appendage remains in the supernatant fraction (lanes g, i, and k, solid arrowheads), as does the \( \alpha_c \) appendage in the presence of GST (lane a, open arrowhead). We conclude that the WXXF motif harbored by SJ170 is highly selective for the \( \alpha \) appendage domain of AP-2.

Next, we examined the relative affinities of the WXXF and FDXDF motifs for AP-2 we compared the GST-SJ170C2 fusion protein (Fig. 2) to the GST-SJ170C2 (Phe-Asp \( \rightarrow \) Ala) and GST-SJ170C2 (Trp \( \rightarrow \) Ala) mutants. From the binding profile of cytosolic AP-2 to increasing amounts of the wild type SJ170C2 (Fig. 6) we estimate that half-maximal adaptor binding occurs at \(-20 \mu g \) (Fig. 5B). Inactivation of the FDXDF motif in the context of the SJ170C2 segment causes a modest (-5-fold) shift in the amount required for half-maximal binding (to \(-100 \mu g \)). By contrast, altering the WVTDF sequence to AVTF has at least a 25-fold effect, increasing the amount needed for 50% binding to \( >500 \mu g \). These values substantiate the dominant role that the WXXF sequence plays in binding the AP-2 \( \alpha \) appendage in pull-down assays and are in general agreement with the competition data presented in Fig. 4.

Finally, to resolve whether the WXXF motif engages the same interaction surface upon the \( \alpha_c \) appendage utilized by epsin, eps15, amphiphysin, and AP180, we compared the binding of soluble SJ170 to immobilized wild type GST-\( \alpha_c \) or GST-\( \alpha_c \)(W840A), GST-\( \alpha_c \)(R905A), or GST-\( \alpha_c \)(R916A) mutant appendages. The Trp\(^{465}\) side chain contributes a major portion of the platform interaction surface (35, 47) and a W840A substitution cases severe and general disruption of partner interactions (Fig. 5B, lane h compared with lane b and f) (37, 47) including inhibition of SJ170 binding (lane h). These data sug-
The WXXF sequence is highly selective for the AP-2 α appendage. A. approximately 100 µg of GST (lanes a and b), GST-DPW (residues 229–407 of epsin 1; lanes c and d), SJ170C5 (lanes e and f), or GST-ARHC1 (residues 180–308 of ARH; lanes g and h) immobilized on GSH-Sepharose was incubated with rat brain cytosol. After centrifugation, aliquots corresponding to 1/75 of each supernatant (S) and 1/6 of each washed pellet (P) were resolved by SDS-PAGE and either stained with Coomassie Blue or transferred to nitrocellulose. Portions of the blots were probed with the anti-AP-2 α-subunit mAb 100/2, anti-AP-2 β-subunit antiserum, the anti-AP-1 γ-subunit antibody AE/1, or anti-AP-1 µ1-subunit serum RY/1.

B. approximately 50 µg of GST (lanes a and b), GST-β2 appendage (lanes c and d), GST-αc appendage (lanes e and f), or the GST-αc appendage point mutants W840A (lanes g and h), R905A (lanes i and j), or R916A (lanes k and l) immobilized on GSH-Sepharose were incubated with PC12 cell lysate. After centrifugation, aliquots corresponding to 1/60 of each supernatant (S) and 1/8 of each washed pellet (P) were resolved by SDS-PAGE and either stained with Coomassie Blue (left panel) or transferred to nitrocellulose (right panels). Portions of the blots were probed with the anti-synaptojanin 1 antibody AR/1, an anti-AP180 mAb, or anti-epsin 1 antibodies. The asterisk indicates a likely degradation product of epsin 1.

C. approximately 400 µg of GST (lanes a, b, g, and h), GST-SJ170C5 (lanes c, d, i, and j), or GST-SJ170M1 (lanes e, f, k, and l) immobilized on GSH-Sepharose was incubated with either thrombin-cleaved, monomeric αc appendage (lanes a–f) or β2 appendage (lane g–l) in the presence of thrombin (lanes a–f). After centrifugation, aliquots corresponding to 1/60 of each supernatant (S) and 1/8 of each washed pellet (P) were resolved by SDS-PAGE and either stained with Coomassie Blue (left panel) or transferred to nitrocellulose (right panels). Portions of the blots were probed with the anti-AP180 mAb or anti-epsin 1 antibodies. BSA is used as a control.
of carrier bovine serum albumin. After centrifugation, aliquots corresponding to 1/75 of each supernatant (S) and 1/16 of each washed pellet were resolved by SDS-PAGE and stained with Coomassie Blue. The amount of AP-2 recovered in each lane was quantified by scanning densitometry using Quantity One (Bio-Rad) software. After background subtraction, data are plotted as percent of AP-2 binding relative to 400 µg of GST-SJ170C2WT (100%). Results from a single representative experiment are shown.

Fig. 6. Different apparent affinities of the WXXF and FXDXF sequences for AP-2. Increasing amounts (6–400 µg) of GST-SJ170C2WT (residues 1454–1530, closed circles), GST-SJ170C2 (Phe-Asp → Ala) (open triangles), or GST-SJ170C2 (W1483A) (filled squares) immobilized on GSH-Septarose were incubated with clarified rat brain cytosol. After centrifugation, aliquots of 1/10 of each washed pellet were resolved by SDS-PAGE and stained with Coomassie Blue. The amount of AP-2 recovered in each lane was quantified by scanning densitometry using Quantity One (Bio-Rad) software. After background subtraction, data are plotted as percent of AP-2 binding to the AP-2 complex (43, 44), has the sequence 695WNPF. This motif participates directly in AP-2 binding although residues outside the WXX(FW) motif probably contribute toward optimal engagement. Interestingly, each of these proteins contains 2 DPF triplets located within regions of the polypeptide likely to be unstructured (Figs. 2 and 7, A and C). The combination of several discrete binding motifs and conformational plasticity could allow simultaneous engagement of multiple adaptor molecules within an assembled clathrin lattice.

We also note that three copies of the WXX(FW) motif are tandemly arrayed within the first 250 residues of the brain isoform of the endocytic protein stonin 2/stonin B (Table I), a polypeptide tract predicted to be essentially disordered (not shown) and with no significant homology to other known proteins. An orthologue of the Drosophila stonin B protein, stonin 2 also contains NPF triplets (52, 53) and, like GAK (49, 50), has been suggested to participate in clathrin uncoating events (53). Available EST sequences indicate that the WXX(FW) sequences in stonin 2 are phylogenetically conserved from zebrfish to mammals. When fused in-frame with GST, the amino-terminal 426 amino acids of human stonin 2 bind to AP-2 in a pull-down assay (Fig. 8, lane d). The extent of binding is similar to that observed with the GST-SJ170C2 fusion (lane b). Although the association between stonin 2 and AP-2 is thought to be indirect, being mediated by eps15 (52), we still observe AP-2 binding following near-quantitative removal of eps15 from cytosol by preincubation with GST-αc appendage. The pellet obtained after preincubating the cytosol with GST-αc appendage shows the recovery of eps15 together with the sedimented beads (lane h compared with lane g). Yet, the AP-2 remaining in the depleted cytosol still binds to GST-stonin 2 to a similar extent (lane f) as the AP-2 in the mock-depleted cytosol (lane d). When overexpressed in HeLa cells, full-length AP-2 (data not shown). Taken together, the AAK1 results are in accord with the SJ170 data presented above showing that the WXXF sequence has a higher apparent affinity for AP-2.

The AAK1 695STWNPFDD sequence is invariant in the presumptive rodent AAK1 orthologues (rat accession number XM_232172 and partial mouse clone AAH43125) and a nearly identical sequence (SGWNPFGE) is also found within the carboxyl-terminal segment of mouse (NM_080708) and human (Q8NSY1) BMP2-inducible protein kinase (BIKe) (48). The kinase domains of AAK1 and BIK are 77% identical and the rodent and human BIK sequences each also contain two DPF triplets. Interestingly, another related Ser/Thr kinase, GAK/auxilin 2, contains the sequence 1037WAAW (Fig. 7C). The anchor aromatic side chains are conserved between the rodent (WDTW) and human (WAAW) GAK/auxilin 2 orthologues, and we have shown for the SJ170 WXXF that WXXW functions comparably. We therefore determined whether the GAK WAAW participates in AP-2 interactions. In a manner analogous to the AAK1 fusion, a GAK fusion (GST-GAKC1, residues 945–1311; Fig. 7D, lane d) binds AP-2 from brain cytosol in pull-down assays, as reported by others (49). This interaction is almost completely abrogated by a single W1036A mutation within the WAAW sequence (Fig. 7D, lane f); again we attribute the residual binding to the intact DPF sequences. GAK is involved in clathrin coat disassembly following fission and also binds physically to clathrin (49, 50) (possibly via clathrin binding DLL sequences (51), Fig. 7C) but the W1036A substitution has no effect on this interaction (lane f compared with lane d). We conclude that in SJ170, AAK1, and GAK, the WXX(WF) motif participates directly in AP-2 binding although residues outside the WXX(FW) motif probably contribute toward optimal engagement. Interestingly, each of these proteins contains 2 DPF triplets located within regions of the polypeptide likely to be unstructured (Figs. 2 and 7, A and C). The combination of several discrete binding motifs and conformational plasticity could allow simultaneous engagement of multiple adaptor molecules within an assembled clathrin lattice.

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Novel AP-2 Binding Sequence in Endocytic Proteins

A

Hs AAK1

1 kinase
poly Gln

AAK1C1 (547-862)
AAK1C2 (57-186)
AAK1C2 (W695A/F898A/WF-A)
AAK1C3 (741-862)

αβ subunit
αC subunit

B

Hs GAK/auxilin 2

1 kinase
tensin homology

GAK1C1 (945-1311)
GAK1C1 (W1037A)

αβ subunit
GST-αβ subunit
αC subunit

C

D

FIG. 7. Functional WXX(FW) motifs in other endocytic proteins. A and C, schematic illustration of the overall domain organization of the AAK1 (A) and GAK/auxilin 2 (C) proteins. The positions of the amino-terminal Ser/Thr kinase, tensin/PTEN homology, and DnaJ homology (J) domains are indicated. The location of the different interaction motifs (WXX(FW)), purple; DPF, blue; DLL, green) is demarcated by the vertical bars, whereas the various GST-AAK1 and GST-GAK constructs used are shown below in precise relation to the protein schematic. The position of the mutated Trp and Phe residues tested is indicated by a red asterisk. B, approximately 200 μg of GST (lanes a and b), GST-AAK1C1 (lanes c and d), GST-AAK1C2 (lanes e and f), GST-AAK1C2 (W695A) (lanes g and h), GST-AAK1C2 (F898A) (lanes i and j), GST-AAK1C2 (WF-A) (lanes k and l), or GST-AAK1C3 (lanes m and n) immobilized on GSH-Sepharose was incubated with rat brain cytosol (which was prepared by preincubation with either 150 μg of immobilized GST or GST-αC appendage; the resulting pellets (lanes g and h) demonstrate capture of AP-2 binding partners, including eps15). After centrifugation, aliquots corresponding to 1/60 of each supernatant (S) and 1/8 (lanes b, d, and f) or 1/12 (lanes g and h) of each washed pellet (P) were resolved by SDS-PAGE and either stained with Coomassie Blue or transferred to nitrocellulose. The position of the bound AP-2 α- and β-subunits are indicated with open arrowheads, whereas the intact GST-AK1C2 fusion protein is indicated with filled arrowheads. Portions of the gels were probed with the anti-AP-2 α-subunit mAb 100/2, anti-AP-2 β2-subunit, or anti-eps15 antiserum. * cross-reactivity of the anti-eps 15 antiserum with the intact GST-AK1C2 fusion protein.

FIG. 8. The WXX(FW)X(DE) domain of stonin 2 binds AP-2 independently of eps15. Approximately 100 μg of either GST-SJ170C2 (lanes a and b) or GST-stonin 2 (residues 1–426) (lanes c–f) immobilized on GSH-Sepharose was incubated with either mock (GST)-depleted (lanes a and d) or GST-αC appendage-depleted (lanes e and f) rat brain cytosol (which was prepared by preincubation with either 150 μg of immobilized GST or GST-αC appendage; the resulting pellets (lanes g and h) demonstrate capture of AP-2 binding partners, including eps15). After centrifugation, aliquots corresponding to 1/60 of each supernatant (S) and 1/8 (lanes b, d, and f) or 1/12 (lanes g and h) of each washed pellet (P) were resolved by SDS-PAGE and either stained with Coomassie Blue or transferred to nitrocellulose. The position of the bound AP-2 α- and β-subunits are indicated with open arrowheads, whereas the intact GST-stonin 2 fusion protein is indicated with filled arrowheads. Portions of the gels were probed with the anti-AP-2 α-subunit mAb 100/2, anti-AP-2 β2-subunit, or anti-eps15 antiserum. * cross-reactivity of the anti-eps 15 antiserum with the intact GST-stonin 2 fusion protein.

Table I

| 0+4 | 0+4 |
|-----|-----|
| 1482KGWVTTEEE | Hs SJ170 |
| 693SNFDD | Hs AAK1 |
| 1035GGGQAAMTE | Hs GAK |
| 1054GSDTFW | Rn GAK |
| 1805SERVRENE | Hs stonin 2 (1) |
| 1502SNHVQED | Hs stonin 2 (2) |
| 2288SVTTFDD | Hs stonin 2 (3) |
| 2702SNHVQEE | Hs NECAP 1 |

a) The 0 position residue, corresponding to the first anchor aromatic side chain, and the +4 position aromatic are indicated. Conserved residues used in defining a tentative consensus sequence are also underlined.

b) The human (Hs) or rat (Rn) sequence accession numbers are: SJ170, NM_003895; AAK1, NM_014911; GAK, NM_005255 and NM_031030; brain stonin 2, NM_033194; brain NECAP 1, NP_006524; epsin R, AY289196; epsin R, BC004467; rabaptin-5, NM_004703; and γ-synergin, NM_080550.

c) The human neuronal stonin 2 sequence displays three tandemly arrayed WXX(FW)X(DE) sequences designated (1), (2) and (3).

d) Asterisk indicates the extreme carboxyl-terminal residue of the protein.

TABLE I

| Trp/Phe-based appendage interaction sequences |
|---------------------------------------------|
| 0+4 | 0+4 |
| 1482KGWVTTEEE | Hs SJ170 |
| 693SNFDD | Hs AAK1 |
| 1035GGGQAAMTE | Hs GAK |
| 1054GSDTFW | Rn GAK |
| 1805SERVRENE | Hs stonin 2 (1) |
| 1502SNHVQED | Hs stonin 2 (2) |
| 2288SVTTFDD | Hs stonin 2 (3) |
| 2702SNHVQEE | Hs NECAP 1 |

a) The 0 position residue, corresponding to the first anchor aromatic side chain, and the +4 position aromatic are indicated. Conserved residues used in defining a tentative consensus sequence are also underlined.

b) The human (Hs) or rat (Rn) sequence accession numbers are: SJ170, NM_003895; AAK1, NM_014911; GAK, NM_005255 and NM_031030; brain stonin 2, NM_033194; brain NECAP 1, NP_006524; epsin R, AY289196; epsin R, BC004467; rabaptin-5, NM_004703; and γ-synergin, NM_080550.

c) The human neuronal stonin 2 sequence displays three tandemly arrayed WXX(FW)X(DE) sequences designated (1), (2) and (3).

d) Asterisk indicates the extreme carboxyl-terminal residue of the protein.
stonin 2 prevents internalization of transferrin, low density lipoprotein, and epidermal growth factor (52). The overexpressed GFP-stonin 2 alters the intracellular localization of AP-2 causing the adaptor complex to cluster in large aggregates that prevent proper placement at the cell surface (52). Importantly, there are no other recognizable α-appendage binding sequences within stonin 2. Altogether, these experiments show that the WXX(F/W) motif is functional in several endocytic proteins, and the compiled sequences (Table I) indicate that WXX(F/W)(X)(DE) is a predictive consensus for this third type of AP-2 interaction motif.

**DISCUSSION**

We have defined a third sequence type that engages the AP-2 adaptor, WXX(F/W)(X)(DE), which is characterized by key anchor aromatic side chains, as are the DP(F/W) and FDXF motifs. Very recently, a WDH sequence was also shown to bind directly to the AP-2 α-appendage (54) although the interaction surface was not delineated. The WTVFEE sequence is evolutionarily conserved between rat and human SJ170 but is not found in the related polyphosphatase synaptojanin 2 (55). Whereas synaptojanin 2 is clearly implicated in endocytic uptake (56, 57), it appears to act at a different step in clathrin-coated vesicle formation, earlier than SJ170 (57). In addition, synaptojanin 2, unlike synaptojanin 1, is targeted to membranes via the small GTPase Rac1 (55, 56), and this might account for the difference in the representation of AP-2 interaction determinants in these two enzymes.

Our data clearly show the high selectivity of the SJ170 WTVF sequence for the AP-2 α-appendage. Whereas it appears that the WXX(F/W)(X)(DE) motif binds to the platform subdomain of the αc-appendage, the results of the competition studies, the motif deletion analysis in the GST-SJ170C2 model protein, and the capacity of some of the αc-appendage mutants to still bind intact SJ170 indicate that the WXXF binding site does not overlap the DP(F/W)/FDXF interaction site completely. At present, we cannot rule out that the effects of the αc-appendage platform mutants we tested are because of conformational perturbation of an adjacent WXX(F/W)(X)(DE) binding surface. During revision of this paper, an analogous WQVF sequence was identified in two clathrin-associated proteins, designated NECAP 1 and 2, that mediates binding to AP-2 (58) (Table I). The NECAP WQVF sequence does not compete with either DP(F/W) or FDXF motifs for αc-appendage binding (58). In this regard, it is interesting to note that several groups have shown recently that the AP-1 γ-subunit appendage, as well as the structurally/functionally related GGA γ-adaptin ear (GAE) domain, bind a DFXXØ sequence (where Ø represents a bulky hydrophobic side chain) (59–65) (Table I). This superficially related AP-1/GGA interaction motif binds to the sandwich domain of the γ appendage. The overall 8-stranded β-sandwich fold of the γ appendage is analogous to the amino-terminal sandwich subdomain of the α appendage that supports the platform subdomain (35, 47). In the GGA1 GAE-DFGGF (p58; see Table I interaction), the proximal Phe packs into a cavity created partly by GGA1 appendage residues Pro656, Arg807, and Arg1099 (61). Analogous residues are required for the GGA3 GAE-DFGGPL (rabaptin-5) (64) and DFXXØ-γ appendage associations (59, 60, 62). Yet none of the side chains necessary for the DFXXØ interaction are conserved at the equivalent position of the αc-appendage sandwich subdomain (59) making it unlikely in our view to be the binding site for the WXX(F/W) motif. Furthermore, close inspection of the WXX(F/W)(X)(DE) sequences delineated here shows obvious side chain differences from the DFSXXØ-type sequence (Table I). It is clear that there is no strong preference for acidic residues before the proximal Trp (0 position) in the WXX(F/W) but, instead, conservation of an acidic residue at the +5 position relative to the anchor Trp (0). In NECAP 1 and 2, the WXXF sequence is positioned at the extreme carboxyl terminus (Table I) and is similarly positioned in the Xenopus NECAP orthologue (AAH54244). Here, the terminal carboxyl group may replace a distal acidic side chain, as is seen in the truncated LLDDL-type clathrin box sequences in the S. cerevisiae epsin orthologues Ent1p and Ent2p (66). The favored Gly at the +1 position of the DFXXØ sequence is not present in any of the identified AP-2 α-appendage binding sequences. Indeed, we do not detect any interaction between a minimal SJ170 AP-2-binding fusion (GST-SJ170C5) and AP-1 (Fig. 5A).

Systematic analysis of the contextual rules and side chain preferences for the WXX(F/W)(X)(DE)/αc-appendage interaction should begin to explain the molecular basis for selectivity compared with the DFXXØ γ-bind motif. Intriguingly, the minimal AP-2 binding region in numb, a known endocytic protein (67, 68), has been mapped to the sequence 611−612VPD-PFEAQWALENKQRTNPSPT (67). Given our failure to detect robust binding of AP-2 to model proteins containing only one or two DPF triplets, it will also be interesting to determine whether a 620−629WALEN sequence present in both numb and numb-like contributes at all to AP-2 appendage binding. Ultimately, however, unambiguous appreciation of the precise orientation of a SJ170, AAK1, GAK, and/or stonin 2 WXX (F/W)(X)(DE) sequence bound to the α appendage must await structural studies.

One important question that is currently unresolved is why multiple discrete adaptor interaction motifs are necessary biologically? From our current studies, it is clear that the DP(F/W), FDXF, and WXX(F/W)(X)(DE) sequences each differ in apparent affinity for the αc-appendage. One interesting possibility for the presence of multiple interaction motifs within the carboxyl-terminal extension of SJ170 is that they might function cooperatively to promote release of other accessory factors late in the budding process. Secondary structure predictions suggest that the SJ170 extension is probably unstructured so the carboxyl-terminal extension of SJ170 is that they might function cooperatively to promote release of other accessory factors late in the budding process. Secondary structure predictions suggest that the SJ170 extension is probably unstructured so the embedded DPF, FDXF, and WXX(F/W)(X)(DE) sequences could bind to multiple appendages within the clathrin lattice (in a random coil conformation, the AP-2 interaction harboring region can extend over 60 nm). Concomitant enzymatic dephosphorylation of PtdIns(4,5)P2 would terminate ENTH/ANTH or PTB domain associations with the lipid bilayer. Binding to a distinct αc-appendage surface via the WXX(F/W)(X)(DE) motif would allow SJ170 (and AAK1, GAK, stonin 2, and NECAP) to engage AP-2, whereas the principal platform site is occupied by other endocytic adaptors or accessory proteins. Once docked, SJ170 could potentially compete other endocytic accessory proteins from the bud site, a model that would explain why accessory proteins like epsin and amphipathin are not enriched within purified clathrin-coated vesicles, and begin to provide a clue to the temporal regulation of accessory protein presence at the clathrin-coated bud.

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**REFERENCES**

1. Stauffer, T. P., Ahn, S., and Meyer, T. (1998) *Curr. Biol.* 8, 343–346
2. Varnai, P., and Balla, T. (1998) *J. Cell Biol.* 143, 511–510
3. Jost, M., Simpson, F., Kvarn, J. M., Lemmon, M. A., and Schmid, S. L. (1998) *Curr. Biol.* 8, 1399–1402
4. Gaidarov, I., Chen, Q., Falck, J. R., Reddy, K. K., and Keen, J. H. (1996) *J. Biol. Chem.* 271, 20922–20929
5. Gaidarov, I., and Keen, J. H. (1999) *J. Cell Biol.* 146, 755–764
6. Collins, B. M., McCoy, A. J., Kent, H. M., Evans, P. R., and Owen, D. J. (2002) *Cell* 109, 525–535
7. Norris, F. A., Ungewickell, E., and Majerus, P. W. (1995) *J. Biol. Chem.* 270, 214–217
Novel AP-2 Binding Sequence in Endocytic Proteins

9. Ford, M. G., Pearse, B. M., Higgins, M. K., Vallis, Y., Owen, D. J., Gibson, A., Hopkins, C. R., Evans, P. R., and McMahon, H. T. (2001) *Science* **291**, 1051–1055

10. Itoh, T., Kishiba, S., Kigawa, T., Kikuchi, A., Yokoyama, S., and Takenawa, T. (2004) *Science* **291**, 1047–1051

11. Mishra, S. K., Agostinelli, N. R., Brett, T. J., Mizukami, I., Ross, T. S., and Traub, L. M. (2001) *J. Biol. Chem.* **276**, 46230–46236

12. Ford, M. G., Keyel, P. A., Hawryluk, M. J., Agostinelli, N. R., Watkins, C. C., and Traub, L. M. (2002) *EMBO J.* **21**, 4915–4926

13. Ford, M. G., Milis, I. G., Peter, B. J., Vallis, Y., Praefcke, G. J., Evans, P. R., and McMahon, H. T. (2002) *Nature* **419**, 361–366

14. Guiducci, C., Krupnick, J. F., Falck, J. R., Benovic, J. L., and Keen, J. H. (1999) *EMBO J.* **18**, 871–881

15. Arnesen, L. S., Kunz, J., Anderson, R. A., and Traub, L. M. (1999) *J. Biol. Chem.* **274**, 17794–17805

16. Wenk, M. R., Pellegrini, L., Klenchin, V. A., Di Paolo, G., Chang, S., Daniell, L., Kalthoff, C., Alves, J., Urbanke, C., Knorr, R., and Ungewickell, E. J. (2002) *J. Biol. Chem.* **277**, 8289–8296

17. Padron, D., Wang, Y. J., Yamamoto, M., Yin, H., and Roth, M. G. (2003) *J. Biol. Chem.* **268**, 693–701

18. Shupliakov, O., Low, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., de Camilli, P., and Brodin, L. (1997) *J. Biol. Chem.* **272**, 9625–9629

19. McPherson, P. S., Garcia, E. P., Slepeny, V. I., David, C., Zhang, X., Grabs, D., Sossin, W. S., Bauerfeind, R., Nemoto, Y., and De Camilli, P. (1996) *Nature* **379**, 353–357

20. Kim, W. T., Chang, S., Daniell, L., Cremona, O., Di Paolo, G., and De Camilli, P. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 17143–17148

21. Harris, T. W., Hartwig, E., Horvitz, H. R., and Jorgensen, E. M. (2000) *J. Cell Biol.* **150**, 589–600

22. Stefan, C. J., Audhya, T., and Emr, S. D. (2002) *Mol. Biol. Cell* **13**, 542–557

23. Hughes, W. E., Cooke, F. T., and Parker, P. J. (2000) *Biochem. J.* **350**, 337–352

24. Guo, S., Stoll, D., Lemovar, S. M., and York, J. D. (1999) *J. Biol. Chem.* **274**, 12990–12995

25. Cremona, O., Nimmakayalu, M., Haffner, C., Bray-Ward, P., Ward, D. C., and De Camilli, P. (2000) *Cytochemistry* **13**, 88–90

26. Arai, Y., Ijima, T., Takenawa, T., Becker, L. E., and Takahashi, S. (2002) *Brain Res. Bull.* **54**, 67–72

27. Cheon, M. S., Kim, S. H., Ovod, V., Kopitar Jerala, N., Morgan, J. I., Hatefi, Y., Ijima, T., Takenawa, T., and Luheec, G. (2003) *Amino Acids* **24**, 127–134

28. Saito, T., Guan, D., Poplos, D. F., Lau, S., Klein, M., Fann, C. S., and Lachman, H. M. (2001) *Mol. Psychiatry* **6**, 387–395

29. Brodon, L., Low, P., and Shupliakov, O. (2000) *Curr. Opin. Neurobiol.* **10**, 312–320

30. Slepeny, V. I., and De Camilli, P. (2000) *Nat. Rev. Neurosci.* **1**, 161–172

31. Traub, L. M., Down, M. A., Wirstch, J. L., and Fremont, D. H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8907–8912

32. Drake, M. T., Down, M. A., and Traub, L. M. (2000) *J. Biol. Chem.* **275**, 6479–6489

33. Macauley, J., Nissey, T., and Traub, L. M. (2000) *Science* **291**, 797–809

34. Mishra, S. K., Watkins, S. C., and Traub, L. M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 16099–16104