Dual Engagement Regulation of Protein Interactions with the AP-2 Adaptor α Appendage*

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Clathrin-mediated endocytosis depends upon the coordinated assembly of a large number of discrete clathrin coat components to couple cargo selection with rapid internalization from the cell surface. Accordingly, the heterotetrameric AP-2 adaptor complex binds not only to clathrin and select cargo molecules, but also to an extensive family of endocytic accessory factors and alternate sorting adaptors. Physical associations between accessory proteins and AP-2 occur primarily through DP(F/W) or FXDXF motifs, which engage an interaction surface positioned on the C-terminal platform subdomain of the independently folded α subunit appendage. Here, we find that the WXX(F/W)DX(D/E) interaction motif found in several endocytic proteins, including synaptotagmin 1, stomatin 2, AAK1, GAK, and NECTAP1, binds a second interaction site on the bilobal α appendage, located on the N-terminal β sandwich subdomain. Both α appendage binding sites can be engaged synchronously, and our data reveal that varied assemblies of interaction motifs with different affinities for two sites upon the α appendage can provide a mechanism for temporal ordering of endocytic accessory proteins during clathrin-mediated endocytosis.

Clathrin-coated vesicles are a major portal of entry into eukaryotic cells, carrying macromolecular nutrients, ligands, select transmembrane proteins, and even viruses into the cell interior from the plasma membrane (1, 2). Cargo selectivity of these short-lived transport intermediates is often thought to be governed by a central triad of proteins, the cargo receptors, clathrin, and the heterotetrameric AP-2 adaptor complex. Cargo receptors contain cytosolic internalization sequences, such as the YXXØ motif (where X is any amino acid and Ø represents a residue with a bulky hydrophobic side chain), found within proteins like the receptor for the endocytosed iron transport protein transferrin. Several distinct internalization sequences or tags are known, each specifying internalization by promoting preferential incorporation into assembling clathrin-coated vesicles (3). Clathrin functions as a trimer of heterodimers, composed of three 192-kDa heavy and three 20–25-kDa light chains, that polymerize to form the characteristic polyhedral clathrin lattice (4, 5). Assembled clathrin appears to act as a mechanical scaffold during the process of bud invagination. AP-2, the archetypical sorting adaptor, is composed of two large (~100 kDa) subunits (α and β2), a 50-kDa medium β2 subunit, and a 17-kDa small σ2 chain (2, 6). AP-2 binds physically to both clathrin, through the hinge and appendage domains of the β2 subunit (7), and to YXXØ-type internalization sequences, via the µ2 subunit, in a phosphorylation-regulated manner (6, 8–10). AP-2 is therefore a multifunctional protein that couples coat assembly with cargo selection.

Surprisingly, after small interfering RNA silencing of either the AP-2 α or µ2 subunit mRNA in HeLa cells to deplete cellular AP-2 adaptor levels, certain transmembrane receptors, like the epidermal growth factor and low density lipoprotein receptors, still internalize efficiently in a clathrin-dependent manner (11). This demonstrates that AP-2 is not absolutely essential for clathrin-mediated endocytosis in cultured mammalian cells. Yet, small interfering RNA knocking down of AP-2 decreases the abundance of clathrin-coated structures at the cell surface by >90% (11), and severe mutation of the AP-2 α subunit in Drosophila melanogaster (12) or targeted disruption of the µ2 subunit genes in mice1 is lethal. Thus, the AP-2 adaptor does play a pivotal role in clathrin coat dynamics at the plasma membrane.

In addition to binding YXXØ-type internalization sequences, AP-2 also binds, via the independently folded α and β2 subunit appendages that project off the heterotetrameric core, to at least 12 endocytic accessory proteins and alternate adaptors (2, 5, 13). These interactions depend upon short interaction motifs or ligands often tandemly arrayed in structurally disordered segments of the AP-2-binding proteins. Two discrete sequences, the DP(F/W) and FXDXF motifs, bind to a partially overlapping site on the α appendage (14). Several endocytic proteins contain both of these, as well as a recently identified third α appendage-binding sequence, the WXX(F/W)XR(D/E) motif (15–17). Although these interaction motifs seem responsible, in part, for placement of endocytic accessory proteins and alternate adaptors at bud sites on the plasma membrane, how the complex web of protein–protein interactions is regulated, how temporally ordered recruitment is achieved, and the physiologic benefit of one type of interaction motif over another are currently unknown. In this study, we show that the WXX(F/
WX19(D)E motif engages the crystallographically identified Trp-specific binding site on the β sandwich subdomain of the appendage that is distant from the major DP/FW/FXDFP-binding site on the platform subdomain of the αc appendage (14). The sandwich site increases the number of αc appendage binding modes, and we propose a model for hierarchical protein recruitment based on the representation of different interaction motifs with different affinities positioned within intrinsically disordered domains of endocytic αc appendage-binding proteins.

**EXPERIMENTAL PROCEDURES**

**DNA and Plasmids—**The GST-fused αc appendage and its mutants W840A, R905A, and R916A; GST-SJ170M1, GST-SJ170C2, SJ170C2/FD → A); and SJ170C2/W → A); and GST-stonin 2-(1–426) have been described previously (14, 16, 18). The GST-fused αc appendage mutants R707S, N712Y, G725N, K727A, R731A, F740D, and Q782A and the GST-stonin 2-(1–247), GST-stonin 2-(1–117), GST-stonin 2-(1–30), GST-stonin 2-(1–267), GST-stonin 2(W → A), GST-stonin 2(W → A), and GST-stonin 2(W1,2,3 → A) constructs were generated by site-directed mutagenesis using the QuickChange system (Stratagene) and the appropriate mutagenic primers. The full-length GFP-stonin 2 construct was generated by inserting the missing N-terminal residues into a GFP-stonin 2 vector (19) kindly provided by Dr. Juan Bonifacino. First, the internal Bcl site in the GFP-stonin 2-(204–905) plasmid was inactivated by QuikChange mutagenesis. Then, residues 1–426 of stonin 2 were amplified by PCR from human clone C14981 (Stratagene) with primers 5'-TTAGGCTTATATGCTTGACCTAGTGG-3' and 5'-AGGCGCGGCGTACGAGGCTGGGACCG-3'.

**Recombinant Proteins, Cell Extracts, and Antibodies—**GST and the GST fusion proteins were first each immobilized onto ~25-μl packed GSH-Sepharose by incubation at 4 °C for 2 h with continuous mixing. The Sepharose beads containing the required immobilized GST fusion proteins were washed and resuspended for assay buffer. Clarified rat brain cytosol, PC12 cell lysates, or purified thrombin-cleaved αc appendage (in the presence of 0.1 mg/ml carrier bovine serum albumin) was added, and the tubes were incubated at 4 °C for 60 min with continuous gentle mixing. For the competition assays, a WXXF(W/D/U/E) peptide (ISNWVQFEDDTP) or thrombin-cleaved proteins (in the presence of 25 mM N-Fhe-Pro-Arg-chloromethyl ketone) were added directly to the assay mixture. The GST-Sepharose beads were then recovered by centrifugation at 10,000 × g for 1 min at 4 °C, and an aliquot of each supernatant was removed and adjusted to 100 μl with SDS-sample buffer. After washing the GSH-Sepharose pellets four times each with ~1.5 ml of ice-cold phosphate-buffered saline by centrifugation, the supernatants were aspirated, and each pellet was resuspended in SDS sample buffer.

**The Experiment—**The SJ170 KGWVTFEE peptide was synthesized in the laboratory of Dr. Paul Allen (Washington University). Superdex S200-purified αc appendages were concentrated, and proteins and peptides were then prepared for ITC by overnight dialysis against buffer containing 50 mM phosphate (pH 7.5), 100 mM sodium chloride, and 1 mM tri(2-carboxyethyl)phosphine. All ITC experiments were carried out at 30 °C using a VP-ITC instrument (MicroCal) at Washington University. Typically, the instrument was balanced, and the SJ170 WXXF peptide, at a concentration of 1 mM, was titrated in 30 injections of 10 μl each. The exception was the αc appendage mutant R905A, which, because of low expression and solubility, was run with a protein concentration of 30 μM and a peptide concentration of 0.90 mM. Traces were corrected by subtracting blank measurements taken with buffer alone added to the ITC cell. Data were analyzed using Origin Version 5.0 (MicroCal). Binding constants were calculated by fitting the integrated data to a one-site binding model.

**Cells, Transfection, Immunofluorescence, and Freeze-eetch Electron Microscopy—**Normal rat kidney cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, and 2 mM L-glutamine. Undifferentiated PC12 cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum, 5% fetal calf serum, and 2 mM L-glutamine. HeLa S36 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. HeLa S36 cells were grown at 37 °C in a humidified 10% CO2 atmosphere. Cells were passaged onto 12-mm glass coverslips 1 day prior to transfection with LipofectAMINE 2000. One day after transfection, cells were fixed in 2% paraformaldehyde and prepared for immunofluorescence as described previously (20). For transferrin internalization, cells were serum-starved for 1 h, pulsed with 25 μg/ml biotin-labeled transferrin for 15 min at 37 °C, washed, and fixed.

**For freeze-eetch immunogold analysis, cells were cultured on small oriented pieces of carbon-coated glass coverslip and ruptured by sonication to generate “unroofed” cell cortices precisely as described (21). After fixation and rinsing with 30 mM Hepes-KOH (pH 7.2), 250 mM sucrose, and 2 mM EDTA supplemented with 1 mM phenylmethylsulfonyl fluoride and Complete protease inhibitor mixture (Roche Applied Science). The 105,000 × g supernatant is defined as cytosol and was stored in small aliquots at -80 °C. PC12 cell lysates were prepared after collecting the cells by trypsinization and washing with phosphate-buffered saline. Pelleted FC12 cells were solubilized in 25 mM Hepes-KOH (pH 7.2), 250 mM sucrose, and 2 mM EDTA supplemented with 1 mM phenylmethylsulfonyl fluoride and Complete protease inhibitor mixture and 1 mM phenylmethylsulfonyl fluoride. Following centrifugation at 20,000 × g for 15 min at 4 °C, aliquots of the lysate were stored frozen at -80 °C. Before use, thawed samples of either rat brain cytosol or PC12 cell lysates were adjusted to 25 mM Hepes-KOH (pH 7.2), 125 mM potassium acetate, 5 mM magnesium acetate, 2 mM EDTA, 2 mM EGTA, and 1 mM diethiothreitol (assay buffer) by addition of a 10× stock and then centrifuged at 245,000 × g (SIA-100,4 rotor) for 20 min at 4 °C to remove insoluble particulate material.

Polycystic serum against NECAP1 was generously provided by Dr. Peter McPherson, whereas the anti-μ subunit serum was a generous gift from Dr. Juan Bonifacino. Polyclonal antibodies against SNX9 (gorting negin-9) were generated in rabbits using residues 1–240 of SNX9 as the antigen. Affinity-purified anti-Numb antibodies were generously provided by Dr. Kojo Kaibuchi. Affinity-purified rabbit antibodies to epsin 1, Disabled-2 (Dab2), and synaptojanin 1 (recognizing both SJ145 and SJ170) have been described (14, 16, 20). Anti-AP-2 α subunit mAb 100/2 was a generous gift of Dr. Ernst Ungewickell, and the anti-CALM mAb was a generous gift from Dr. Jeong-Ah Kim. Monoclonal antibodies directed against AP180 and amphiphysin were from the Developmental Studies Hybridoma Bank, Department of Biological Science, University of Iowa, Iowa City, and from the National Institute on Aging, National Institutes of Health (Bethesda, MD).

**Supplementary References**

1. The abbreviations used are: GST, glutathione S-transferase; GFP, green fluorescent protein; mAb, monoclonal antibody; ITC, isothermal titration calorimetry.
2. Sequences are available upon request.
After washing with HKMgE buffer, the membranes were fixed in 2% glutaraldehyde in HKMgE buffer and then prepared for freeze-etch analysis (21).

**RESULTS**

A Second Functional αc Appendage Binding Site—The WXX(F/W)X(D/E) motif occurs in several endocytic proteins, including synaptojanin 1, a phosphoinositide polyphosphatase; the Ser/Thr kinases AAK1 and GAK/auxilin 2, which control YXXØ-type internalization sequence binding through adaptor subunit phosphorylation; and stonin 1/2 and NECAP1/2, the precise functions of which are currently unknown (15–17). Like the DP(F/W) and FXDXF motifs, the WXX(F/W)X(D/E) motif binds physically to the AP-2 α appendage (15–17). Yet, in affinity interaction assays in vitro, addition of a 250-fold molar excess of a stonin 2-derived ISNWVQFEDDTP peptide, which effectively prevented NECAP1 binding and significantly blunted SJ170 interactions with GST-αc appendage (Fig. 1A, compare lane f with lane d), has little effect on the association of epsin 1 or amphiphysin I with the immobilized appendage. This finding suggests that the WXX(F/W)X(D/E) motif might be accommodated by a site separate from the platform subdomain on the α appendage. Because the WXX(F/W)X(D/E) motif has an absolute requirement for Trp at the 0 position (16), we examined the contribution of residues located on the

![Diagram](http://www.jbc.org/...)

**Fig. 1. Separable protein interactions with the AP-2 αc appendage.** A, ~50 μg of GST (lanes a and b), GST-αc appendage (lanes c–f), GST-αc appendage mutant G725N (lanes g and h), or GST-αc appendage mutant Q782A (lanes i and j) immobilized on GSH-Sepharose were incubated with PC12 cell lysates in the absence or presence of 3 mM ISNWVQFEDDTP peptide. After centrifugation, aliquots corresponding to one-sixtieth each supernatant (S) and one-eighth 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 anti-synaptojanin 1 antibody AR1, anti-NECAP1 or anti-epsin 1 antibody, or anti-amphiphysin or anti-AP180 mAb. The positions of the molecular mass standards (in kilodaltons) are indicated on the left, and only the relevant portion of each blot is shown. The different forms of AP180 in the PC12 cell lysates could be due to alternatively spliced isoforms or different post-translational modifications. B, ~50 μg of GST (lanes a and b), GST-αc appendage (lanes c–f), or GST-αc appendage mutant Q782A (lanes g and h) immobilized on GSH-Sepharose were incubated with rat brain cytosol in the absence or presence of 3 mM ISNWVQFEDDTP peptide. After centrifugation, aliquots corresponding to one-sixtieth each supernatant and one-eighth of each washed pellet were resolved by SDS-PAGE and either stained with Coomassie Blue or transferred to nitrocellulose. Portions of the blots were probed with anti-NECAP1 or anti-epsin 1 antibody or anti-amphiphysin or anti-AP180 mAb. The identities of the Coomassie Blue-stained αc appendage-binding partners are indicated and validated by the immunoblots on the right.
Multisite Regulation of AP-2 α Appendage Interactions

sandwich subdomain of the αc appendage (Fig. 2, A–C) that we previously showed crystallographically create a Trp-specific binding site (14). Purified monomeric GST appendage bound to a minimal SJ170 (1478SNPKGVTWEE, GST-SJ170M1)-derived WXX(F/W)X(D/E) motif immobilized on GSH-Sepharose beads (Fig. 2D). The majority of the αc appendage sedimented with the GST-SJ170M1 fusion (lane d), whereas the appendage remained soluble (lane a) in the presence of GST alone (lane b). Alteration of select residues on one face of the N-terminal sandwich, composed of β strands A’, A, B, and E, perturbed the association of the αc appendage with the immobilized WXX(F/W)X(D/E) motif. The effect of a G725N substitution was most severe, whereas a Q782A mutation also blunted appendage binding significantly (Fig. 2D). Based on the structure of the

FIG. 2. A tryptophan-specific WXX(F/W)X(D/E)-binding site on the β sandwich subdomain of the αc appendage. A, shown is a schematic of the AP-2 adaptor based on the crystal structure of the heterotetrameric AP-2 core and illustrating the various protein and phospholipid interaction regions. PtdIns, phosphatidylinositol. B, shown is a surface representation of the AP-2 αc appendage illustrating the relative positions of the platform binding site and the sandwich subdomain distal DPW surface containing a Trp-accommodating site (14). The surface is colored according to sequence conservation (magenta is invariant, and yellow exhibits a conservation index of 7 or higher as scored by ALSCRIPT). Worm representations of the FXDFX and DPW peptides are shown in the platform and distal DPW sites as determined in our previous structural studies (14). The dashed line represents a hypothetical polypeptide chain connecting engagement of the two sites in concert. term. C, shown is a close-up view of the Trp-selective pocket on the sandwich subdomain. The positions of several side chains examined in this study are indicated, with carbon atom conservation colored as described for B. The position of the DPW peptide is displayed in worm form to indicate the Trp-binding site. D, ~400 µg of either GST (lanes a, b, e, f, i, j, m, and n) or GST-SJ170M1 (lanes c, d, g, h, k, l, o, and p) immobilized on GSH-Sepharose were incubated with thrombin-cleaved monomeric GST appendage (lanes a–d), αc appendage mutant G725N (lanes e–h), αc appendage mutant K727A (lanes i–l), or αc appendage mutant Q782A (lanes m–p) in the presence of carrier bovine serum albumin (BSA). After centrifugation, aliquots corresponding to one-fortieth of each supernatant (S) and one-eighth of each washed pellet (P) were resolved by SDS-PAGE and either stained with Coomassie Blue or transferred to nitrocellulose. The blot was probed with anti-AP-2 α subunit mAb 100/2. E, shown are ITC measurements of the binding of the SJ170 WXXF peptide to wild-type and mutant AP-2 α appendages. Traces are shown after subtraction of data from injection of the peptide into a buffer blank. The inset shows raw data from the SJ170 WXXF peptide (1 mM) injected into the wild-type α appendage (100 µM) in 10-µl aliquots. F, shown are the dissociation equilibrium constants (Kd) derived from the ITC experiments.

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Effect of sandwich domain point mutations on binding of the αc appendage to the SJ170 WXX(F/W)(D/E) motif and brain binding partners

| Protein                        | Relative binding to SJ170M1 | Relative binding to NECAP1 | Relative binding to Epsin 1 | Relative binding to AP180 | Relative binding to Amphipysin |
|--------------------------------|-----------------------------|----------------------------|-----------------------------|---------------------------|--------------------------------|
| GST                           | + + + +                      | ND                         | ND                          | ND                        | ND                             |
| GST-wild-type αc appendage     | + + + +                      | ND                         | ND                          | ND                        | ND                             |
| GST-αc appendage mutant R707S  | + + + +                      | ND                         | ND                          | ND                        | ND                             |
| GST-αc appendage mutant N712Y  | + + + +                      | ND                         | ND                          | ND                        | ND                             |
| GST-αc appendage mutant Q725A  | + + + +                      | ND                         | ND                          | ND                        | ND                             |
| GST-αc appendage mutant Q725N  | + + + +                      | ND                         | ND                          | ND                        | ND                             |
| GST-αc appendage mutant R727A  | + + + +                      | ND                         | ND                          | ND                        | ND                             |
| GST-αc appendage mutant R731A  | + + + +                      | ND                         | ND                          | ND                        | ND                             |
| GST-αc appendage mutant F740D  | + + + +                      | ND                         | ND                          | ND                        | ND                             |
| GST-αc appendage mutant Q782A  | + + + +                      | ND                         | ND                          | ND                        | ND                             |

*Semiquantitative indication of WXX(F/W)(D/E) sequence binding to the various AP-2 αc appendages relative to the wild-type protein (experiments performed as described for Fig. 2D).*

Not determined.

apo-αc appendage (Fig. 2B) (18, 23) and an epsin 1-derived DPW peptide co-crystallized at this site (Fig. 2A) (14), we suspect that Glu728 accepts a hydrogen bond from the indole nitrogen of the WXX(F/W)(D/E) Trp at the 0 position, accounting for the strict Trp selectivity (16). The G725N substitution likely fills much of the pocket required to accommodate the Trp residue (Fig. 2C). Altering several other side chains on the sandwich (Table 1) revealed that Phe740 is also required for productive interactions with the SJ170 WXX(F/W)(D/E) model protein, whereas a K727A substitution had a negligible effect (Fig. 2D). Several other side chains in the vicinity of the sandwich binding pocket did not affect appendage binding in this assay when mutated (Table 1).

The spatially distinct WXX(F/W)(D/E)-binding site on the sandwich subdomain was verified by ITC experiments. A synthetic KGWTVFEE peptide corresponding to the SJ170 WXX(F/W)(D/E) motif interacted with the wild-type αc appendage with a $K_d$ of 10.7 ± 0.3 μM (Fig. 2, E and F). Mutations of the platform subdomain that had an effect on DPF (R905A, $K_d = 9 ± 1 \mu M$) and FXDXF (R916A, $K_d = 13.8 ± 0.5 \mu M$) motif binding displayed minor differences in affinity for the WXX(F/W)(D/E) motif peptide. However, mutations of the Trp-selective binding site (G725N and Q782A) completely precluded detectable binding of the peptide. These results also reflect the strict requirement for the first Trp in the WXX(F/W)(D/E) motif (16), as substitution of the proximal Trp in the peptide with Ala completely abolished measurable peptide binding (Fig. 2F).

In pull-down assays with brain cytosol, the Q723A, G725N, F740D, and Q782A mutations each completely abolished the association of NECAP1 (which has a single C-terminal 272WVQF sequence (15)) with the immobilized αc appendage without perturbing epsin, AP180, or amphipysin binding (Fig. 1 and Table 1). In this assay, the α appendage mutation K727A permitted soluble NECAP1 binding less completely (Table 1). With PC12 cell extracts, the Q782A mutation also significantly blunted SJ170 binding (Fig. 1A); we attribute the remaining SJ170 binding to the intact FXDXF and DPF(F/W) (and WXX(F/W)(D/E); see below) motif binding to the unaltered platform subdomain site. The apparently normal binding of cytosolic SJ170 to GST-αc appendage mutant G725N may be related to the relatively weaker effect of this mutation on SJ170 binding (Fig. 2D). Thus, five of the seven residues that generate the sandwich subdomain Trp-selective region are variably important for WXX(F/W)(D/E) motif engagement.

A Phylogenetically Conserved α Subunit-Specific Binding Site—The sandwich subdomain interaction surface appears to have been conserved on the α appendage through evolution. Sequence alignments revealed that of the seven key residues that generate the Trp-binding site (Fig. 2B), three (Gln723, Gly725, and Gln728) are invariant from Schizosaccharomyces pombe to mammals and are also conserved in Arabidopsis thaliana (Fig. 3). The remaining residues (Gly714, Val715, Lys716, and Phe740) are all conservatively substituted (Fig. 3). In fact, surface-exposed side chain conservation is evident only on the A’/A/B/E sheet of the appendage sandwich, as the C/D/F/G strand-containing opposite side displays virtually no surface phylogenetic conservation (Fig. 3) (18). The strong sequence preservation argues strongly that the interaction surface on the sandwich subdomain plays a physiologically important role in AP-2 function.

The AP-2 β2 subunit appendage, which is structurally and functionally analogous to the α appendage (7), does not display a homologous interaction surface at the equivalent position on the sandwich subdomain. Indeed, the WXX(F/W)(D/E) sequence displayed a very high selectivity for the α appendage over the β2 appendage (SJ170 and NECAP1 binding) (Fig. 4) (15–17). The AP-1 γ subunit appendage and the structurally homologous GGA protein GAE (gamma-adaptin ear) domain (2, 24) bind to a D/F/W/GX0 motif (25, 26) superficially similar to the AP-2-binding WXX(F/W)(D/E) motif. In fact, the AP-1 γ appendage binds a 280WNSF sequence present in the hinge of GGA1 (27). Although the γ appendage/GAE domain is composed solely of a β sandwich domain and lacks the platform subdomain characteristic of the bilobal α and β subunit appendages, the binding site for the D/F/W/GX0 motif is located on the opposite side of the β sandwich from the WXX(F/W)(D/E) interface surface and also lacks the strict specificity for Trp at the 0 position (28–32). Together, these data argue that the WXX(F/W)(D/E)-binding site on the α appendage sandwich plays a unique role in AP-2 activity.

Multisite a Appendage Engagement—Human SJ170 has an alternatively spliced C-terminal extension containing a WXX(F/W)(D/E), a FXDXF, and two DPF motifs (see Fig. 5B) (14, 16, 33). In earlier work, we showed that the WXX(F/W)(D/E) and FXDXF sequences together promote optimal αc appendage engagement (16). Three lines of evidence suggest that multiple WXX(F/W)(D/E) motifs, as found in the endocytic protein stonin 2 (17, 18, 34), might enhance α appendage binding similarly. First, whereas addition of a WXX(F/W)(D/E) peptide completely abrogated binding of NECAP1 in PC12 cell extracts to immobilized GST-αc appendage, the peptide also weakly inhibited binding of AP180 (Fig. 1A, lane f), which does not contain a WXX(F/W)(D/E) motif. By contrast, the G725N or Q782A mutation prevented NECAP binding, but had no effect on AP180 association with the mutated αc appendage (Fig. 1A, lanes h and j). Similar experiments using brain cytosol also showed that the stonin 2 WXX(F/W)(D/E) peptide, while abolishing NECAP binding, diminished the association of AP180 and amphipysins I and II with the immo-
bilibilized αc appendage (Fig. 1B, compare lane f with lane d). One interpretation of these results is that the WXX(F/W)X(D/E) motif may also bind weakly to the major interaction surface on the platform subdomain of the αc appendage, although the ITC data with the αc appendage mutant Q782A indicated that the affinity of this interaction is poor.

Second, the binding of cytosolic SJ170 and NECAP1 to the GST-αc appendage fusion protein was perturbed not only by sandwich domain mutants (G725N, F740D, and Q782A) (Fig. 1 and Table I), but also by the platform domain mutants W840A and R916A and somewhat less by R905A (Fig. 4). Interestingly, the reduced recovery of NECAP1 in the supernatant fractions (compare lanes g, i, and k with lanes a and c) and the trace levels of NECAP1 in the GST-αc appendage pellet fractions (compare lanes h, j, and l with lane f) suggest that the platform mutations alter the off-rate of wild-type NECAP1 and that the bound pool of protein is lost during the washing steps. The positions of these modulatory side chains (Trp840, Arg905, and Arg916) relative to the WXX(F/W)X(D/E)-binding site on the sandwich subdomain (Fig. 2B), the large buried interfacial surface area between the
FIG. 5. Binding of the AP-2 adaptor to the stonin 2 XXX(P/W)X(D/E) repeats. A, shown is a schematic representation of the organization of human (Homo sapiens (Hs)) stonin 2 and the relative locations of the deletion constructs used in this study. SHD, stonin homology domain; MHD, μ subunit homology domain. B, ~100 μg of GST (lanes a and b), GST-SJ170C2 (lanes c and d), GST-stonin 2-1-426 (lanes e and f), GST-stonin 2-1-247 (lanes g and h), GST-stonin 2-1-117 (lanes i and j), or GST-stonin 2-1-30 (lanes k and l) immobilized on GSH-Sepharose were incubated with rat brain cytosol. After centrifugation, aliquots corresponding to one-fortieth of each supernatant (S) and one-eighth 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 anti-AP-2 α subunit mAb 100/2 or anti-μ2 subunit serum. Arrowheads indicate the positions of the large α and β subunits of the AP-2 complex, and asterisks indicate where protein transfer had been reduced by the large amount of GST fusion protein comigrating at that position. C, ~100 μg of GST (lanes a and b), GST-stonin 2-1-247 (lanes c and d), GST-stonin 2-1-247(W1,2,3 → A) (lanes e and f), GST-stonin 2-1-247(W2,3,4 → A) (lanes g and h), GST-stonin 2-1-247(W2,3,4 → A) (lanes i and j), GST-stonin 2-1-247(W2,3,4 → A) (lanes k and l), or GST-stonin 2-1-247(W2,3,4 → A) (lanes m and n) immobilized on GSH-Sepharose were incubated with rat brain cytosol. After centrifugation, aliquots corresponding to one-fortieth of each supernatant and one-eighth of each washed pellet were resolved by SDS-PAGE and either stained with Coomassie Blue or transferred to nitrocellulose. Portions of the blots were probed with anti-AP-2 α subunit mAb 100/2 or anti-μ2 subunit serum. Arrowheads indicate the positions of the large α and β subunits of the AP-2 complex.
FIG. 6. WX(F/W)(D/E) binding to the sandwich site enhances motif binding to the platform interaction surface. A, ~50 μg of immobilized GST (lanes a and b) or GST-αC appendage (lanes c–j) were incubated with rat brain cytosol alone (lanes a–d) or with cytosol supplemented with 20 μM stonin 2-(1–247) (lanes e and f), 20 μM stonin 2-(1–247)(W1,2,3→A) (lanes g and h), or 20 μM stonin 2-(1–117) (lanes i and j). After centrifugation, aliquots of approximately one-fortieth of each supernatant (S) and one-sixth of each washed pellet (P) were resolved.
sandwich and platform subdomains, and the rigidity of the two subdomains relative to one another (18) make it unlikely, in our view, that these platform mutations propagate a conformational effect on the sandwich subdomain.

Third, although truncation of the N-terminal region of stonin 2 containing three WXX(F/W)XX(D/E) motifs (Fig. 5A) to contain two motifs had little effect on AP-2 binding, a GST-stonin 2 fusion containing only the first WXX(F/W)XX(D/E) sequence bound AP-2 very weakly (Fig. 5B). Since GST-stonin 2 was present in large excess in these experiments, the data are consistent with the idea that tandemly arrayed WXX(F/W)XX(D/E) motifs cooperate to increase the apparent affinity for the AP-2 αC appendage. Similar results were obtained if individual WXX(F/W)XX(D/E) motifs were inactivated singly or in combination in the context of a GST-stonin 2-(1–247) fusion protein (Fig. 5C). Again, a single WXX(F/W)XX(D/E) sequence showed a marked reduction in AP-2 binding that, given the selectivity of the motif for the α appendage, appears inconsistent with a single binding site upon the appendage.

To extend these observations, we first established whether the N-terminal portion of stonin 2, which contains only WXX(F/W)XX(D/E) motifs, can inhibit protein partner binding to the αC appendage platform. Supplemen ting brain cytosol with 20 μM stonin 2-(1–247) largely prevented the association of AP180 and amphiphysins I and II with immobilized GST-αC appendage (Fig. 6A, compare lane f with lane d) without perturbing epsin 1 binding. This difference is due to avidity/chelate effects since epsin 1 has eight tandemly arrayed DPW motifs, whereas amphiphysin, for example, has only one DPF motif and one FXDXF motif. In these experiments, a portion of the added stonin 2 fragment associated with the sedimented appendage (open arrowhead), and as expected, NECAP1 binding was completely abolished. The capability of the stonin 2-(1–247) fragment to inhibit when bound at only substoichiometric levels suggests that multiple αC appendages can be engaged by a single stonin 2 molecule. An equivalent concentration of the stonin 2-(1–247)(W1,2,3 → A) mutant failed to bind to GST-αC appendage (closed arrowhead) and had no effect on the association of either NECAP1 or AP180 and amphiphysin with GST-αC appendage. Surprisingly, addition of a 5-fold molar excess of a smaller segment of stonin 2 (residues 1–117) containing only two WXX(F/W)XX(D/E) repeats inhibited AP180 binding very weakly (compare lane j with lane d) without perturbing amphiphysin binding. Nevertheless, stonin 2-(1–117) bound to the immobilized appendage (asterisk), abolishing the NECAP1 interaction. These results suggest that other residues adjacent to the WXX(F/W)XX(D/E) motifs can also contribute to binding to the platform once the polypeptide is bound to the sandwich.

To dissect the mode of inhibition further, we analyzed the effect of inactivating the sandwich site on the inhibitory action of the stonin 2-(1–247) and SJ170C2-(1454–1530) protein segments. When added to the cytosol, each segment bound to GST-αC appendage, preventing binding of AP180 and amphiphysin as well as NECAP1 (Fig. 6B, compare lanes f and h with lane d). Similar experiments with immobilized GST-αC appendage mutant Q782A revealed that an intact sandwich site is necessary for optimal inhibition by both the stonin 2-(1–247) and SJ170C2 proteins (compare lanes l and n with lanes d and j). Our interpretation of these results is that the WXX(F/W)XX(D/E) motif, by binding to the sandwich site, enhances the affinity of other interaction sequences located within these essentially unstructured protein regions for the platform site.

AP180 binding to GST-αC appendage mutant Q782A was inhibited by these protein fragments, but we believe this is because AP180 has the lowest apparent affinity for the αC appendage (18) and because freely soluble WXX(F/W)XX(D/E) motifs can perturb this interaction (Fig. 1).

For the SJ170C2 protein segment, which contains one FXDXF motif and one WXX(F/W)XX(D/E) motif, the concerted action of both motifs in αC appendage binding was clearly seen upon mutagenic inactivation of either motif separately (Fig. 6C). Importantly, in this case, altering the FXDXF motif to AXAXF (SJ170C2(FD → A)) reversed the competitive effect of the inhibitor on AP180 and amphiphysin binding, but had no effect on NECAP1 inhibition (compare lane j with lane h). By contrast, substituting the WXX(F/W)XX(D/E) motif with AXXX(F/W)XX(D/E) (SJ170C2(W → A)) still reversed the inhibitory effect of the fragment on AP180 and amphiphysin binding, but relieved the inhibition of NECAP1 binding (compare lane l with lanes h and j). Together, these data show that the capability of the SJ170C2 portion to inhibit αC appendage interactions requires both interaction motifs and both αC appendage binding sites.

Combinations of WXX(F/W)XX(D/E) and DPF/(F/W) motifs are also found tandemly arrayed in invertebrate endocytic components. In D. melanogaster Numb-associated kinase (accession number NP_477165), an Ark1/Prk1 family Ser/Thr protein kinase related to mammalian AAK1 and GAK/auxilin 2 (35), a 586WNPFEEE sequence is positioned between two DPF triplets in a central segment predicted to be unstructured. Related sequences are present in presumptive Anopheles gambiae (WNPFGP; accession number XP_321932) and Aps mellifera (WNPFEDV; accession number B1515476) orthologs with adjacent DPF triplets. The WNPF(X/D/E) sequence is homologous to a major AP-2 950NPFFDD interaction motif in human AAK1 (16) and conforms to the general consensus WXX/(F/W)(D/E) (16). In Caenorhabditis elegans protein that is a possible stonin 2 ortholog (APT-10; accession number NP_505566), a potential sandwich-binding sequence (WADFETS) lies between one proximal and two distal DPF repeats. In all these proteins, the individual motifs are separated by at least 15 amino acids, providing ~50 Å of flexible linker polypeptide. Although the activity of these sequences must be confirmed experimentally, the data are consistent with the phylogenetic conservation of
that expands the number of engagement modes to permit proteins with tandemly arrayed interaction motifs to regulate the occupancy of the platform.

Motif Arrays Govern Hierarchical Recruitment—Different apparent affinities of soluble αC appendage-binding partners in brain or PC12 cell extracts can be seen in titration experiments (Fig. 8A). In agreement with our competition studies, intact epsin 1 displayed the highest affinity for the α appendage, whereas AP180 and NECAP1 had relatively low apparent affinities (Fig. 8A). In general, there was a good correlation between the presence of multiple interaction motifs and/or the extent of motif repetition (Fig. 8B) and the observed apparent affinity for the immobilized αC appendage. Importantly, SJ170 had a high affinity for the appendage, and the difference in binding between SJ170 and the short, neuron-specific isoform of synaptojanin 1, SJ145, was clear. SJ145, which contains only a single WXX(F/W)X(D/E) motif, bound GST-αC appendage with an affinity roughly comparable with that of NECAP1. A striking finding is that several of the alternate adaptors, including epsin 1, Dab2, and HIP1, that expand the sorting repertoire of clathrin-coated vesicles that form at the plasma membrane had high apparent affinities for GST-αC appendage.

In no instance did we observe effective displacement of epsin 1 from the immobilized αC appendage, irrespective of the interaction motifs present in the competitor protein (Figs. 1 and 4). This indicates that, unlike dynamin and actin (37), epsin might not display dramatic temporal fluctuations during clathrin coat assembly. At steady state, there is a high degree of colocalization of endogenous epsin 1 with clathrin or AP-2 (38), demonstrating that epsin 1 populates the majority of clathrin-coated structures located at the cell surface. Immunogold analysis of the distribution of endogenous epsin 1 at the ventral surface of disrupted normal rat kidney or PC12 cells using freeze-etch electron microscopy showed that the protein was almost exclusively present within regions of assembled polygonal clathrin lattice (Fig. 9, B and C). Antibodies against the AP-2 α subunit and epsin 1 revealed an extensive presence of these endocytic proteins within regions of flat clathrin lattice. Significantly, epsin 1 labeling was not restricted only to flat lattices, but was also found in rounded structures and deeply invaginated profiles reflecting all stages of clathrin-coated vesicle assembly (Fig. 9C, arrows). A very recent report, using our anti-epsin antibodies, also showed that epsin 1 is found in flat clathrin lattices at the cell surface as well as in deeply invaginated clathrin-coated buds in HeLa cells (39). Together, these results suggest that epsin does not obligatorily exit the assembling clathrin bud prior to the fission event. Indeed, the only accessory factor identified in a recent proteomic analysis of purified brain clathrin-coated vesicles was epsin 1, and epsin is enriched in purified clathrin-coated vesicle preparations (40). These observations support the proposed action of epsin as an alternate adaptor (13, 41) and again underscore the differential dependencies conferred upon endocytic proteins by assemblies of different αC appendage interaction motifs.

**DISCUSSION**

Although the assembly of clathrin-coated vesicles at the plasma membrane requires only 1 or 2 min, the process is characterized by an intricate and complex series of protein-protein interactions. These interactions govern the assembly of a polyhedral clathrin lattice, the preferential retention of select cargo molecules, the invagination of the nascent transport intermediate, and finally, scission from the cell surface and uncoating. At least 20 accessory factors, in addition to the clathrin/AP-2/cargo triad, participate in these events (42). Several of these so-called accessory proteins (AP180, HIP1, epsin 1, and Dab2) appear to be bona fide alternate adaptors that cooperate
with AP-2 during lattice assembly while simultaneously expanding the sorting repertoire of the bud (13). Others, like amphiphysin, play a more structural role, whereas enzymatic proteins, such as AAK1, GAK, and synaptojanin 1, regulate cargo selection and clathrin coat disassembly (2, 35, 42). Despite these distinct functions, many of the accessory proteins contact the assembling coat in a similar fashion, by binding to the AP-2 appendage. A critical deficiency in our understanding of the molecular basis of clathrin-coated vesicle assembly is a lack of a detailed knowledge of the precise chronology and location of the myriad of protein-protein contacts necessary for the successful fabrication and release of a vesicle. We show here that associations with the AP-2 appendage utilize two spatially distinct binding surfaces and at least three discrete interaction sequence types. Furthermore, different assemblies of distinct interaction sequences produce proteins with different apparent affinities for AP-2 and generate a hierarchical set of interaction partners.

The measured affinities of the different α appendage-binding motifs are in accord with a hierarchical model for binding, with a $K_d$ of $10^{-12}$ M for the WXX(F/W)X(D/E) sequence in SJ170 making the sandwich site the highest affinity single interaction. In the alternatively spliced SJ170 C terminus, the WXX(F/W)X(D/E) sequence is the dominant interaction motif, as are the WNPF and WAAW sequences in AAK1 and GAK/auxilin 2, respectively (16). A single DPF motif has a $K_d$ of $10^{-12}$ M (23), and model proteins with one to three DPF triplets bind AP-2 extremely poorly (16, 38). The FXDXF motif likely has an intermediate affinity, as the stonin 2-(1–247) protein, with three WXX(F/W)X(D/E) motifs, effectively competes off amphiphysin and AP180, both with FXDXF sequences. The WNPF and WAAW sequences in AAK1 and GAK/auxilin 2 are atypical WXX(F/W)X(D/E) motifs that might enable these proteins to bind to both AP-2 and AP-1, through the sandwich domain of the γ subunit appendage, albeit via different interaction surfaces. The γ appendage binds a related WNSF sequence in GGA1 (27), and a bifunctional interaction motif could allow AAK1 to regulate cargo capture by phosphorylation of adaptor μ subunits (43) at different intracellular sites. Similarly, GAK is found in clathrin-coated vesicle preparations (40, 44, 45) and binds both the AP-1 γ subunit and AP-2 α subunit appendages and phosphorylates the μ subunits of these adaptors (45, 46). The lack of a discernible phenotype after AAK1 silencing by RNA interference in HeLa cells (36)
This accounts for the high apparent affinity of epsin 1 for the diffusion-limited exit by statistically favoring rapid rebinding. Yet, the multiplicity of binding motifs counteracts thresholds for binding to and residency on the platform. For endocytic proteins like epsin 1 and Eps15, tandemly repeated DP(F/W) sequences can promote simultaneous binding to multiple AP-2 molecules by both the C and D appendage. For endocytic proteins like epsin 1 and Eps15, tandemly repeated DP(F/W) sequences can promote simultaneous binding to multiple AP-2 molecules by both the C and D appendage. However, the $K_a$ values for these interactions are weak (>100 $\mu$m) (23) and, consequently, display interaction half-times of only a few seconds, which will allow other proteins access to the platform site. The majority of AP-2-binding partners have additional docking determinants (like modular phosphatidylinositol 4,5-bisphosphate-binding domains) or interaction sequences that can engage other clathrin coat components. The growing consensus is that it is the combinatorial effect of these associations that governs precise compartmental recruitment of structurally related adaptors like AP-1, AP-2, AP-3, epsin 1, and epsinR (2, 47, 48). For example, epsin 1 has a phosphatidylinositol 4,5-bisphosphate-binding N-terminal ENTH (epsin N-terminal homology) domain, eight DPW repeats, and two clathrin-binding sequences as well as NPF triplets that can bind to EH (Eps15 homology) domain-containing proteins like Eps15 and intersectin (2, 41). These additional contacts with the assembleable coat could also contribute to the steady presence of epsin 1 in clathrin-coated regions throughout the assembly and fission process. Phosphatidylinositol 4,5-bisphosphate engagement by the epsin ENTH domain involves the ordering of a new $\alpha$ helix that, once formed, inserts several aliphatic side chains into the bilayer (49). The penetration of these residues is thought to induce membrane curvature as clathrin lattice assembly progresses (49). We found extensive epsin 1 labeling of flat hexagonal clathrin arrays. These results indicate that the presence of epsin 1 does not obligatorily dictate membrane curvature.

A significant issue is whether, in individual cells, the entire cohort of binding partners is available to associate with the appendage or whether tissue-specific expression patterns limit the complexity of $\alpha$ appendage interactions. In brain, all but two of the 15 proteins shown schematically in Fig. 8B are expressed, and many have been directly localized to the presynaptic region, a site of intensive endocytic activity because regulated exocytosis of neurotransmitters is tightly coupled to compensatory clathrin-mediated endocytosis. Therefore, the hierarchical pattern of recruitment we see in our biochemical affinity association assays has direct physiological relevance. An important component that is not present in brain is SJ145, the long splice isoform of synaptotagmin 1. In neurons, the critical phosphoinositide polyphosphatase function is performed by SJ145, an alternate splice isoform that lacks the C-terminal extension that contains the DPF, FXDXF, and WXX(F/W)XD(E) motifs (50). A single WXX(F/W)XD(E) motif is present within SJ145, but the phosphatase is expressed at considerably higher concentrations in brain than in other tissues and is predominantly recruited to the bud site by endophilin or amphiphysin through proline-rich region-SH3 (Src homology 3) domain interactions (42). The abundance of SJ145 in nerve terminals may therefore facilitate function without a requirement for more complex adaptor interaction information. In fact, the interaction of SJ145 with endophilin and amphiphysin is regulated by phosphorylation (51, 52), and because of the higher concentration of synaptotagmin 1 at the synapse, the long splice isoform could conceivably be recruited prematurely to the developing bud and impede the rapid completion of the vesicle, which is dependent on phosphatidylinositol 4,5-bisphosphate.

Many of the AP-2-binding endocytic accessory proteins are

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**Fig. 9. Ultrastructural localization of epsin 1 in clathrin-coated structures at the cell surface.** Shown are fixed plasma membranes sheets prepared from either normal rat kidney (A and B) or PC12 (C) cells labeled with either anti-AP-2 $\alpha$ subunit (A) or anti-epsin 1 (B and C) antibody and secondary antibodies conjugated to 15-nm colloidal gold particles. Individual gold particles appear as white spheres, and representative freeze-etch images show the distribution of endogenous AP-2 and epsin 1. The extensive labeling for epsin in flat clathrin lattices as well as labeling of rounded structures that reflect the progressive invagination of the lattice to form clathrin-coated vesicles (arrows).
reversibly phosphorylated, and regulation of the physical interaction between the α appendage and binding partners by cycles of phosphorylation/dephosphorylation could add an additional layer of control by modulating the accessibility and/or affinity of α appendage interaction motifs (53). However, at the synapse, many of these proteins, collectively termed dephosphorylation (54), are coordinately dephosphorylated by calcineurin upon depolarization and Ca\(^{2+}\) influx.

In conclusion, our results identify a clear function for the α appendage sandwich subdomain in AP-2 interactions through its ability to bind the WXX(F/W)(X/D/E) sequence. The finding that WXX(F/W)(X/D/E) motif engagement allows other binding motifs to engage the adjacent major platform binding site suggests a potential manner to regulate α appendage occupancy. Finally, our data led us to predict that temporal ordering of alternate adaptors and accessory proteins necessary for proper clathrin-coated vesicle assembly is governed, in part, by particular sets of α appendage interaction motifs.

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Dual Engagement Regulation of Protein Interactions with the AP-2 Adaptor α Appendage
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