AP-2 Recruitment to Synaptotagmin Stimulated by Tyrosine-Based Endocytic Motifs

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Clathrin-mediated endocytosis is initiated by the recruitment of the clathrin adaptor protein AP-2 to the plasma membrane where the membrane protein synaptotagmin is thought to act as a docking site. AP-2 also interacts with endocytic motifs present in other cargo proteins. Peptides with a tyrosine-based endocytic motif stimulated binding of AP-2 to synaptotagmin and enhanced AP-2 recruitment to the plasma membrane of neuronal and non-neuronal cells. This suggests a mechanism by which nucleation of clathrin-coated pits is stimulated by the loading of cargo proteins.

Clathrin-coated vesicles participate in several intracellular transport steps including endocytosis and the export of proteins from the trans Golgi (*1*). A specialized form of clathrin-mediated endocytosis occurs at the synapse, where clathrin-coated vesicles recycle synaptic vesicle components (*2*). Clathrin-mediated endocytosis involves the internalization of plasma membrane components into vesicles that are surrounded by a coat H complex of clathrin and the adaptor complex AP-2 (a heterotetramer composed of α,β2, μ2, and σ2 subunits) (*1*). AP-2 interaction with both lipids as well as with tyrosine- (*3*) or dileucine-based (*4*) endocytic motifs present in cargo proteins may contribute to coat recruitment (*5, 6*). A distinct proteinaceous docking site for AP-2 at the plasma membrane has also been postulated (*7, 8*). Synaptotagmin, a transmembrane protein that binds to AP-2 through its C2B domain, was proposed to represent this site (*9, 10*).

If synaptotagmin is a docking site for AP-2, its interaction with AP-2 should not be competed by the binding of AP-2 to cargo proteins bearing endocytic motifs. Glutathione *S*-transferase (GST) fusion proteins composed of either the C2A or C2B domains of synaptotagmin I were incubated with rat brain cytosol (*11*) (Fig. 1A, left) or a crude clathrin coat fraction isolated from calf brain (*12*) (Fig. 1A, right) in the presence or absence of a peptide containing the tyrosine-based endocytic motif YXXØ (X represents any amino acid, and Ø amino acids with bulky and hydrophobic side chains) (*3*). AP-2 bound to the C2B domain only (*9*) (Fig. 1A). The presence of a 14–amino acid oligomeric peptide containing the YXXØ-based endocytic motif [YQRL (Tyr Gln Arg Leu)] of TGN38 (a recycling protein of the trans Golgi network) (*3, 13*) did not inhibit the interaction of AP-2 with GST-C2B: instead, the peptide stimulated it (Fig. 1A, right). This effect was dependent on the concentration of

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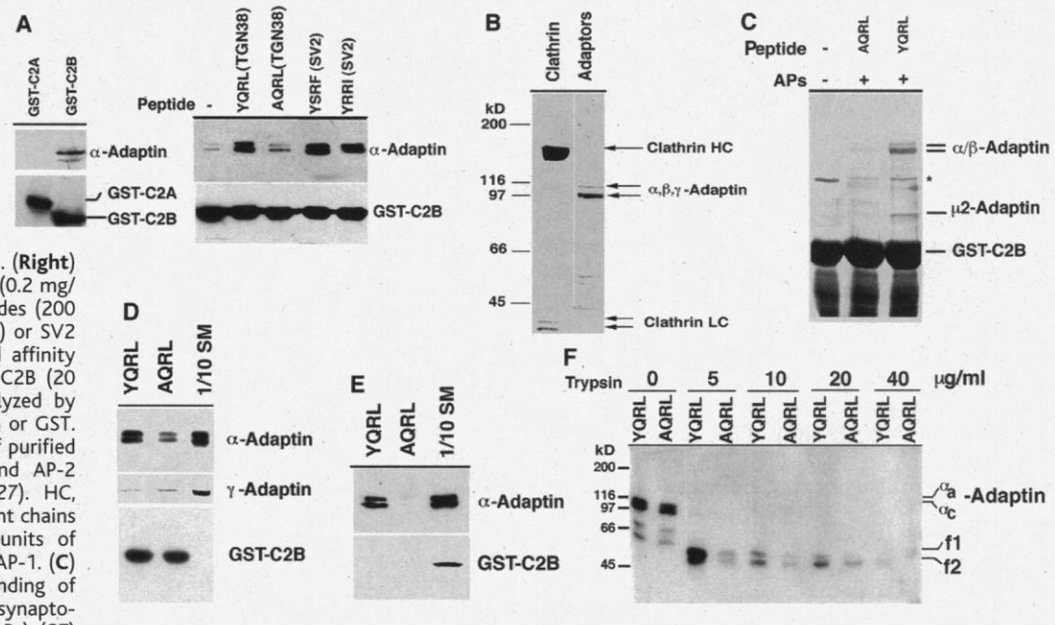

Fig. 1. Effect of YXXØ peptides on the interaction of AP-2 with synaptotagmin I. (**A**) YXXØ peptides stimulate AP-2 binding to the C2B domain of synaptotagmin I. (**Left**) Affinity purification of AP-2 from rat brain cytosol (2 mg/ml) with GST-C2A or GST-C2B domain fusion proteins (20 μg) bound to glutathione Sepharose beads (*26*). (**Right**) A crude clathrin coat fraction (0.2 mg/ml) was incubated with peptides (200 μM) derived from TGN38 (*13*) or SV2 (*15*) for 30 min at 37°C and affinity purified on immobilized GST-C2B (20 μg) (*26*). Samples were analyzed by immunoblotting for α-adaptin or GST. (**B**) Coomassie blue staining of purified clathrin (15 μg) or AP-1 and AP-2 adaptor proteins (10 μg) (*27*). HC, heavy chain of clathrin; LC, light chains of clathrin; α,β-adaptin, subunits of AP-2; γ-adaptin, a subunit of AP-1. (**C**) YXXØ peptides stimulate binding of purified adaptorproteins to synaptotagmin I. Adaptor proteins (APs) (*27*) (10 μg) were incubated with YQRL or AQRL peptides (200 μM) (A, Ala) and affinity purified on immobilized GST-C2B (20 μg) as in (A). Samples were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining . The asterisk indicates DnaK, a bacterial heat-shock protein that copurifies with overexpressed GST fusion proteins. (**D**) YXXØ peptides do not stimulate binding of synaptotagmin I to AP-1. Immobilized GST-C2B (20 μg) was incubated with the crude clathrin coat fraction (0.2 mg/ml) as described in (A), and associated proteins were detected by immunoblotting for GST or for α- and γ-adaptin (1/10 SM= 1/10 of the total amount of starting material added to the reaction). (**E**) Immobilized YXXØ peptides bind to AP-2, but not to GST-C2B. YQRL or AQRL peptides were immobilized on CNBr-Sepharose and incubated with crude clathrin coat or GST-C2B (each at 0.1 mg/ml). Bound proteins were detected by immunoblotting for α-adaptin or GST. (**F**) YXXØ peptides inhibit proteolysis of AP-2 by trypsin. Crude clathrin coat fraction (0.2 mg/ml) was incubated with the YQRL or AQRL peptides as in (A). Trypsin was added for 30 min at room temperature. Proteins were detected by immunoblotting with antibodies (AC1-M11) to α-adaptin. α_a and α_c are α-adaptin isoforms, and f1 and f2 are proteolytic fragments of α-adaptin.

peptide present and is consistent with previous studies on the affinity of YXX∅-based endocytic motifs for AP-2 (*4*). A peptide in which the tyrosine was replaced by alanine had no effect. The peptide directly affected the AP-2–synaptotagmin interaction because the stimulation also occurred when purified adaptor proteins containing AP-2 were used (Fig. 1, B and C). The effect was specific for AP-2, because the small amount of bound γ-adaptin, a subunit of the clathrin adaptor complex AP-1, was not affected by the peptide (Fig. 1D). Furthermore, the effect was mediated by binding of the YQRL peptide to AP-2, because the immobilized YQRL peptide bound AP-2 but not GST-C2B (Fig. 1E). It is possible that the binding of YXX∅-based endocytic motifs to AP-2 induces a structural change in AP-2 that increases its affinity for synaptotagmin. Low concentrations of trypsin remove the hinge and ear domains of AP-2, generating AP-2 "cores" that contain a 50-kD fragment of α-adaptin (*1*). In the presence of the YQRL peptide, the α-adaptin core fragment of AP-2 was more resistant to low concentrations of trypsin, consistent with the occurrence of a peptide-induced structural change in the AP-2 complex (Fig. 1F) (*14*).

These findings suggest a mechanism by which the interaction of AP-2 with synaptotagmin is enhanced by the presence of additional cargo proteins to be loaded into the nascent endocytic vesicle. Because these co-

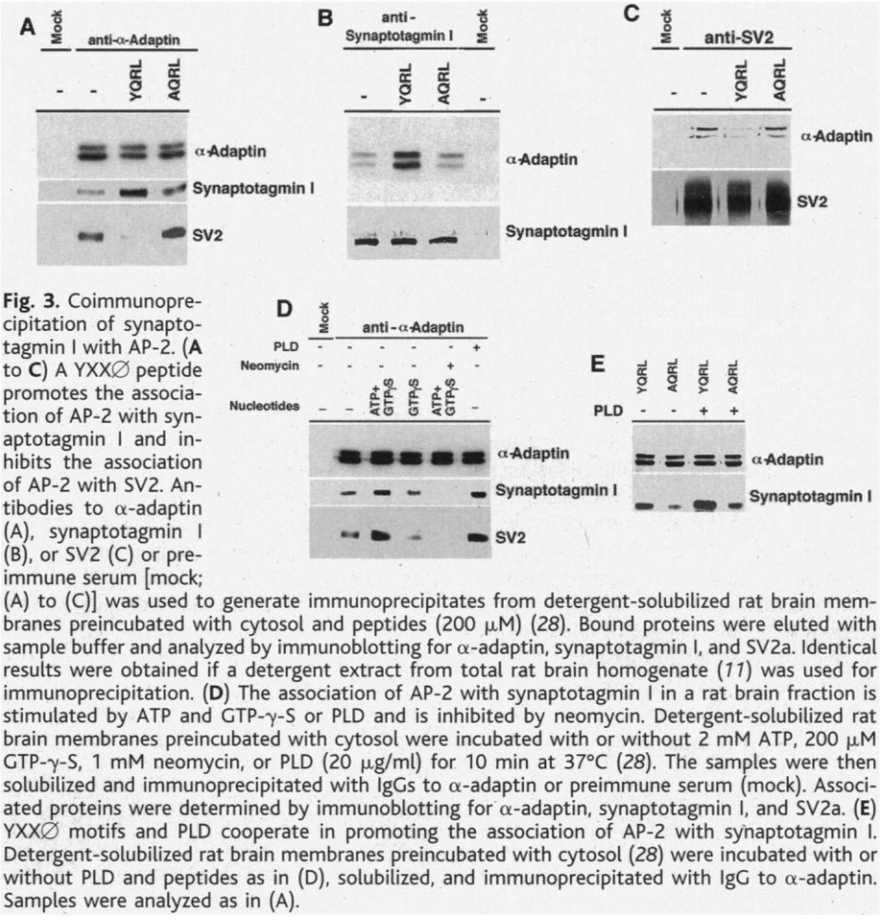

Fig. 3. Coimmunoprecipitation of synaptotagmin I with AP-2. (**A** to **C**) A YXX∅ peptide promotes the association of AP-2 with synaptotagmin I and inhibits the association of AP-2 with SV2. Antibodies to α-adaptin (A), synaptotagmin I (B), or SV2 (C) or preimmune serum [mock; (A) to (C)] was used to generate immunoprecipitates from detergent-solubilized rat brain membranes preincubated with cytosol and peptides (200 μM) (*28*). Bound proteins were eluted with sample buffer and analyzed by immunoblotting for α-adaptin, synaptotagmin I, and SV2a. Identical results were obtained if a detergent extract from total rat brain homogenate (*11*) was used for immunoprecipitation. (**D**) The association of AP-2 with synaptotagmin I in a rat brain fraction is stimulated by ATP and GTP-γ-S or PLD and is inhibited by neomycin. Detergent-solubilized rat brain membranes preincubated with cytosol were incubated with or without 2 mM ATP, 200 μM GTP-γ-S, 1 mM neomycin, or PLD (20 μg/ml) for 10 min at 37°C (*28*). The samples were then solubilized and immunoprecipitated with IgGs to α-adaptin or preimmune serum (mock). Associated proteins were determined by immunoblotting for α-adaptin, synaptotagmin I, and SV2a. (**E**) YXX∅ motifs and PLD cooperate in promoting the association of AP-2 with synaptotagmin I. Detergent-solubilized rat brain membranes preincubated with cytosol (*28*) were incubated with or without PLD and peptides as in (D), solubilized, and immunoprecipitated with IgG to α-adaptin. Samples were analyzed as in (A).

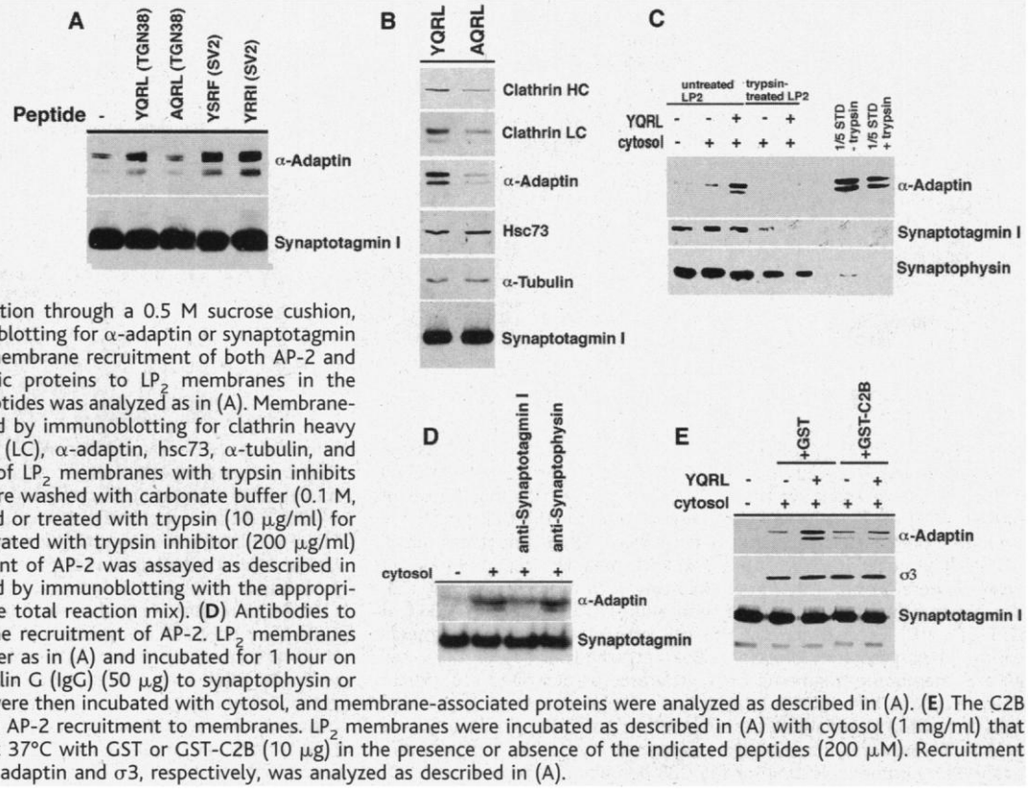

Fig. 2. Effect of YXX∅ peptides on the recruitment of AP-2 to synaptic membranes. (**A**) YXX∅ peptides stimulate membrane recruitment of AP-2. LP_2 synaptosomal membranes (50 μg) (*11*) were washed with carbonate buffer (0.1 M, pH 11.5) and incubated with rat brain cytosol (3 mg/ml) for 15 min at 37°C in the presence of the indicated peptides (200 μM) in cytosolic buffer (*21*). LP_2 membranes were then isolated by centrifugation through a 0.5 M sucrose cushion, washed, and analyzed by immunoblotting for α-adaptin or synaptotagmin I. (**B**) YXX∅ peptides stimulate membrane recruitment of both AP-2 and clathrin. Recruitment of cytosolic proteins to LP_2 membranes in the presence of the YQRL or AQRL peptides was analyzed as in (A). Membrane-associated proteins were analyzed by immunoblotting for clathrin heavy chain (HC), clathrin light chains (LC), α-adaptin, hsc73, α-tubulin, and synaptotagmin I. (**C**) Treatment of LP_2 membranes with trypsin inhibits AP-2 recruitment. Membranes were washed with carbonate buffer (0.1 M, pH 11.5) and either left untreated or treated with trypsin (10 μg/ml) for 1 hour on ice. Trypsin was inactivated with trypsin inhibitor (200 μg/ml) and PMSF (1 mM), and recruitment of AP-2 was assayed as described in (A). Bound proteins were analyzed by immunoblotting with the appropriate antibody (1/5 STD, 1/5 of the total reaction mix). (**D**) Antibodies to synaptotagmin I inhibit membrane recruitment of AP-2. LP_2 membranes were washed with carbonate buffer as in (A) and incubated for 1 hour on ice with or without immunoglobulin G (IgG) (50 μg) to synaptophysin or to synaptotagmin I. Membranes were then incubated with cytosol, and membrane-associated proteins were analyzed as described in (A). (**E**) The C2B domain of synaptotagmin inhibits AP-2 recruitment to membranes. LP_2 membranes were incubated as described in (A) with cytosol (1 mg/ml) that had been incubated for 30 min at 37°C with GST or GST-C2B (10 μg) in the presence or absence of the indicated peptides (200 μM). Recruitment of the AP-2 and AP-3 subunits α-adaptin and σ3, respectively, was analyzed as described in (A).

operative interactions may occur in the nerve terminal, we examined synaptic vesicle proteins for the presence of YXXØ-based endocytic motifs. SV2a, an abundant transmembrane protein of synaptic vesicles, contains at least two such motifs in its cytoplasmic domains (YSRF and YRRI) (*15*). The 14–amino acid oligomeric peptides containing these two YXXØ-based endocytic motifs (*15*) also stimulated AP-2 binding to the C2B domain of synaptotagmin (Fig. 1A).

To assess the physiological significance of these findings, we examined the recruitment of AP-2 onto native synaptic membranes. LP_2 synaptosomal membranes [a fraction of small synaptic membranes (*11*)] were first washed with carbonate buffer to remove endogenous cytosolic endocytic proteins and then incubated with desalted rat brain cytosol (*11*) in the presence or absence of the various YXXØ–containing peptides. Addition of peptides stimulated the membrane recruitment of AP-2 (Fig. 2A) and clathrin (Fig. 2B), but not of other cytosolic proteins such as tubulin or hsc73 (*16*). No stimulation of AP-2 binding occurred with trypsin-treated LP_2 membranes (Fig. 2C), indicating that a protease-sensitive component is required for the peptide-induced stimulation of AP-2 binding to LP_2 membranes. Antibodies to the cytoplasmic domain of synaptotagmin I, but not antibodies to synaptophysin, another abundant synaptic vesicle membrane protein, also inhibited AP-2 binding to membranes (Fig. 2D). Moreover, GST-C2B inhibited the stimulatory effect of the YQRL peptide on AP-2 binding to membranes (Fig. 2E). In contrast, membrane association of σ3, a component of the AP-3 adaptor complex, was not affected by the YQRL peptide, or by GST-C2B (Fig. 2E).

Immunoprecipitation experiments performed on detergent-solubilized rat brain membranes in the presence of brain cytosol showed that AP-2 coimmunoprecipitated both synaptotagmin I and SV2a (Fig. 3A). In the presence of the YQRL peptide, more synaptotagmin I was recovered in the immunoprecipitate. In contrast, less SV2a was coimmunoprecipitated, suggesting that SV2a associates with AP-2 through YXXØ-based endocytic motifs (Fig. 3A). Likewise, the YQRL peptide stimulated the recovery of AP-2 in anti–synaptotagmin I immunoprecipitations (Fig. 3B), but decreased the recovery of AP-2 in anti-SV2a immunoprecipitations (Fig. 3C). The mutant AQRL peptide had no effect in any of these experiments (*17*). Thus, synaptotagmin and SV2 appear to use different mechanisms for binding to AP-2. SV2 may associate with AP-2 through the YXXØ–motif binding site of its μ2 subunit (*3*), which is critically important for the internalization of some plasma membrane receptors (*18*). Synaptotagmin may bind to AP-2 at another site through a cluster of lysine residues in its C2B domain which bears no resemblance to YXXØ–motifs (*19*).

The binding of coat proteins to membranes may involve their interactions with both membrane proteins and lipids (*20–22*). Because phospholipase D (PLD) activity enhances membrane recruitment of AP-2 (*6*), PLD may also affect the association of AP-2 with synaptotagmin. AP-2 was immunoprecipitated from detergent-solubilized rat brain membranes in the presence of brain cytosol that had been treated with either PLD, various nucleotides, or neomycin. PLD as well as ATP plus guanosine 5′-*O*-(3′-thiotriphosphate) (GTP-γ-S), which can activate PLD through ARF family proteins (*6*), stimulated the interaction of AP-2 with synaptotagmin I. Neomycin, an inhibitor of PLD, inhibited this stimulation. GTP-γ-S alone had no effect. Similar results were observed for the interaction of SV2 with the AP-2 complex (Fig. 3D). Both PLD and the YQRL peptide stimulated the formation of AP-2–synaptotagmin I complexes. However, if PLD and the YQRL peptide were added together, the AP-2 interaction with synaptotagmin I was further enhanced (Fig. 3E). Thus, PLD and YXXØ-based endocytic motifs act synergistically to promote the association of AP-2 with synaptotagmin.

Because synaptotagmin isoforms are widely expressed in all tissues (*9*), we examined the effect of YXXØ-containing peptides on AP-2 recruitment to non-neuronal plasma

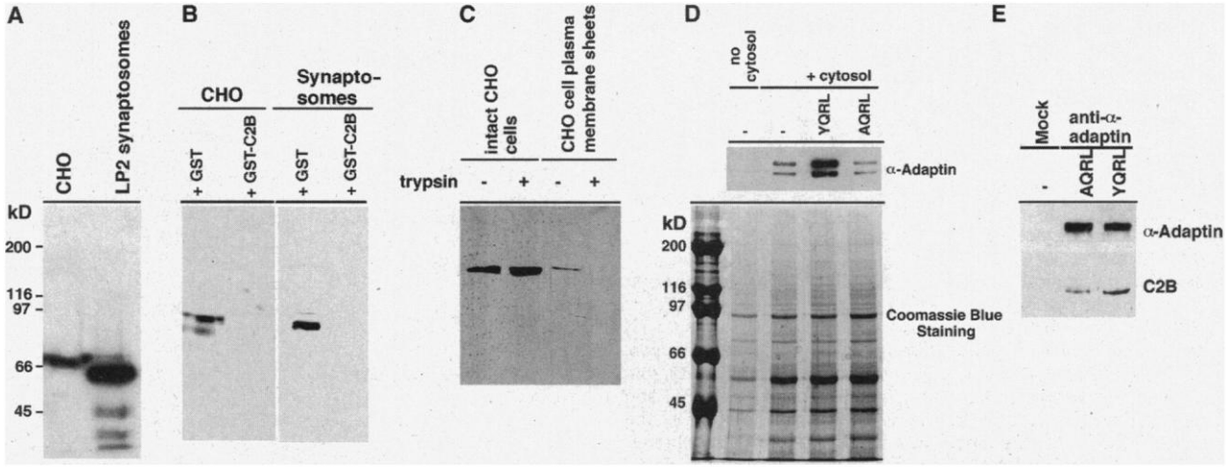

Fig. 4. Effect of YXXØ peptides on AP-2 recruitment to CHO cell plasma membranes. (**A**) CHO cells express an isoform of synaptotagmin. A CHO cell homogenate and a rat brain LP_2 synaptosomal fraction [50 μg of protein (*11*)] were analyzed by immunoblotting for the C2B domain of synaptotagmin I. (**B**) Specificity of the antiserum to synaptotagmin. A CHO cell homogenate or a rat brain synaptosomal LP_2 fraction (40 μg of protein) were analyzed by immunoblotting with antibodies to the C2B domain that had been incubated with either 10 μg of purified GST or GST-C2B. (**C**) Bead-attached CHO cell plasma membrane fragments expose their cytoplasmic surface. Bead-attached inside-out CHO cell plasma membrane fragments were generated as described (*23*). Intact CHO cells (4×10^5) or bead-attached plasma membrane sheets (4×10^5 cells of starting material) were treated with trypsin (20 μg/ml) for 1 hour at 4°C. Trypsin was inactivated as described in Fig. 2C. Samples were analyzed by immunoblotting for the C2B domain of synaptotagmin. (**D**) Effect of a YXXØ peptide on AP-2 binding to bead-attached plasma membrane sheets. Membrane-coated beads (*23*) were incubated for 15 min at 37°C with rat brain cytosol (4 mg/ml) in the presence or absence of the indicated peptides (200 μM), washed, and analyzed either by SDS-PAGE and immunoblotting for α-adaptin (upper panel) or by staining with Coomassie blue (lower panel). (**E**) A YXXØ peptide promotes the association of AP-2 with synaptotagmin in CHO cell lysates. CHO cells (10×10^6) were washed with phosphate-buffered saline, then with cytosolic buffer, and resuspended in 1/20 volume cytosolic buffer (*21*) in the presence or absence of 200 μM YQRL or AQRL peptides. Samples were incubated for 5 min at room temperature, then solubilized by 1% triton X-100 for 10 min at room temperature. Extracts were cleared by centrifugation and immunoprecipitated with monoclonal antibodies to α-adaptin or preimmune serum (mock). Bound proteins were analyzed by immunoblotting for the C2B domain of synaptotagmin or for α-adaptin.

membranes. The presence of a putative synaptotagmin isoform (70 kD) in CHO cells was detected with a polyclonal antiserum to the C2B domain of synaptotagmin (Fig. 4A). GST-C2B blocked this immunoreactivity (Fig. 4B). CHO cells were bound to poly-D-lysine–coated beads, ruptured by sonication, and washed to generate bead-attached plasma membrane sheets with their cytosolic face exposed to the medium (*23*) (Fig. 4C). Plasma membrane–coated beads were then incubated with rat brain cytosol in the presence or absence of the YQRL or AQRL peptides. Recruitment of AP-2 was stimulated by the YQRL peptide, but not by its AQRL mutant (Fig. 4D) (*24*). The YQRL peptide also increased the amount of the putative synaptotagmin isoform recovered in anti–AP-2 immunoprecipitates from CHO cell extracts (Fig. 4E). These data show that in non-neuronal cells, YXX∅-based endocytic motifs cooperate with a docking site, which most likely includes synaptotagmin, in recruiting AP-2 to the membrane.

Binding of YXX∅-based endocytic motifs to AP-2 may increase AP-2's affinity for synaptotagmin and thereby facilitate coated pit formation. A similar mechanism for coupling coat nucleation and cargo protein selection has been reported for the assembly of COPII-coated buds at the endoplasmic reticulum (*25*). Thus, the common picture that emerges from these studies is that cargo proteins participate in the nucleation of coat assembly and facilitate their own sorting into transport vesicles.

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16. Similar results were obtained if adenosine triphosphate (ATP), which may regulate clathrin-coated pit assembly, was present in the cytosol.
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27. AP-2 and AP-1 adaptor proteins were prepared by separation of the clathrin coat proteins (3 mg/ml) on a Sepharose CL4B gel filtration column in 0.5 M tris-HCl pH 7.4. The adaptor (AP-2 plus AP-1) and clathrin peaks were pooled and concentrated to 0.5 mg/ml and 1 mg/ml, respectively, and either used directly or frozen in liqid nitrogen and stored at–70°C.
28. LP_2 membranes (1 mg; at 2 mg/ml) were prepared from a total rat brain homogenate, mixed with 2 mg of dialyzed rat brain cytosol (20 mg/ml) (*11*), solubilized in 1% triton X-100 containing cytosolic buffer (*21*), and cleared by centrifugation. To examine the effect of nucleotides or PLD, we first incubated the LP_2-cytosol mix for 10 min at 37°C with these reagents before solubilization in 1% triton X-100 containing cytosolic buffer (*21*).

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Identification of a Mating Type–Like Locus in the Asexual Pathogenic Yeast *Candida albicans*

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Candida albicans, the most prevalent fungal pathogen in humans, is thought to lack a sexual cycle. A set of *C. albicans* genes has been identified that corresponds to the master sexual cycle regulators a1, α1, and α2 of the *Saccharomyces cerevisiae* mating-type (*MAT*) locus. The *C. albicans* genes are arranged in a way that suggests that these genes are part of a mating type–like locus that is similar to the mating-type loci of other fungi. In addition to the transcriptional regulators a1, α1, and α2, the *C. albicans* mating type–like locus contains several genes not seen in other fungal *MAT* loci, including those encoding proteins similar to poly(A) polymerases, oxysterol binding proteins, and phosphatidylinositol kinases.

The yeast *C. albicans* is the most common human fungal pathogen causing most cases of oral and vaginal thrush as well as severe mucosal and systemic infections in immunocompromised individuals (*1*). A principle difficulty in studying *C. albicans*, compared with other yeasts, is that *C. albicans* has no known sexual cycle and is therefore not amenable to conventional genetic analysis. It is a diploid organism for which no haploid state has been observed, nor has any process resembling meiosis or spore formation been detected. Sexual reproduction in fungi is typically controlled by genes that reside in a genetic locus called a mating-type, or *MAT*, locus. In the budding yeast *Saccharomyces cerevisiae*, the genes residing at the *MAT* locus can be either the **a** type or the α type. These genes, which code for transcriptional regulators, specify the three cell types involved in the *S. cerevisiae* sexual cycle (*2*, *3*).

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