Bimodal Interaction of Coatomer with the p24 Family of Putative Cargo Receptors

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Cytoplasmic domains of members of the p24 family of putative cargo receptors were shown to bind to coatomer, the coat protein of COPI-coated transport vesicles. Domains that contained dilysine endoplasmic reticulum retrieval signals bound the α-, β′-, and ε-COP subunits of coatomer, whereas other p24 domains bound the β-, γ-, and ζ-COP subunits and required a phenylalanine-containing motif. Transit of a CD8-p24 chimera from the endoplasmic reticulum through the Golgi complex was slowed when the phenylalanine motif was mutated, suggesting that this motif may function as an anterograde transport signal. The either-or bimodal binding of coatomer to p24 tails suggests models for how coatomer can potentially package retrograde-directed and anterograde-directed cargo into distinct COPI-coated vesicles.

Vesicles coated with COPI mediate anterograde transport at various stages from the endoplasmic reticulum (ER) through the Golgi complex (*1–3*). Coatomer (*4*), the coat subunit of these vesicles, is also implicated in the retrograde retrieval of escaped ER proteins from the Golgi apparatus by interacting with the COOH-terminal, cytoplasmically oriented dilysine ER retrieval signals (*5–7*).

Members of the p24 family of transmembrane proteins (*8–10*) have been proposed to operate as cargo receptors, selecting proteins for inclusion in budding COPI- and COPII-coated vesicles. A mammalian p24 protein, chop24a, was identified as an abundant component of Golgi-derived COPI-coated vesicles and is present (considered together with another family member, p23) in stochiometric quantities in vesicles relative to coatomer (9). A yeast member, Emp24p, is present in COPII-coated vesicles, a second class of vesicles also required for ER-to-Golgi transport (*3, 11*). The luminal domains of p24 proteins are predicted to contain a membrane-proximal coiled-coil region, and an NH_2-terminal region that is poorly conserved except for a pair of cysteine residues that may form a conserved disulfide bond (9). The topography of p24 proteins, the presence of several species in coated vesicles, and the fact that disruption of the *EMP24* gene differentially affects the kinetics of transport of diverse cargo proteins are consistent with the proposed role of p24 proteins as cargo receptors (9, *10*).

The p24 family currently comprises 16 homologous proteins (Fig. 1). All p24 proteins possess a phenylalanine residue in their cytoplasmic domain close to the transmembrane segment. The amino acid preceding this phenylalanine is exclusively another phenylalanine in all known mammalian p24 proteins, but can be a large hydrophobic or aromatic residue in other species (for example, yeast). Moreover, in all known p24 proteins with one exception (yeast Emp24p), the absolutely conserved phenylalanine is followed by two or three basic residues near the COOH-terminus. One subset of p24 proteins contains the sequence KKXX-COOH or KXKXX-COOH (*12*), known to constitute an ER retrieval signal (*13, 14*). A second subset of p24 proteins contains one or more basic residues COOH-terminal to the conserved phenylalanine, but not the sequence KKXX-COOH or KXKXX-COOH.

Cargo receptors are expected to interact with one or more subunits of the coats of coated vesicles to ensure their efficient packaging along with the cargo they bind (*1*). We analyzed the binding of coatomer to a series of cytoplasmic domains of p24 proteins representing each of the two subsets of tail sequences. The cytoplasmic domains were fused to the COOH-terminus of glutathione-S-transferase (GST) and expressed as wild-type proteins or as mutants in which the conserved phenylalanine residue was replaced by alanine (F→A mutants). The fusion proteins were bound to glutathione-agarose beads and incubated with cell extracts. Binding was detected by immunoblot analysis with antibodies specific to each coatomer subunit.

As might have been expected from studies of dilysine motifs of non-p24 proteins lacking the conserved phenylalanine (5), the dilysine-containing cytoplasmic domains of hp24d and yp24c bound coatomer (Fig. 2A) and preferentially interacted with α-, β′-, and ε-COP, which we term the B (basic) subcomplex (Fig. 2B). Similar binding occurred with the wild-type and F→A mutant proteins. These same three COP proteins were selectively bound to a similar extent to dilysine motifs of the ER proteins WBP1, a component of the yeast *N*-oligosaccharyl transferase (*15*), and E3/19K (E19) (*16*), an adenoviral protein (Fig. 3) (5).

Three p24 tails that lack the dilysine motif—from chop24a, Emp24p, and

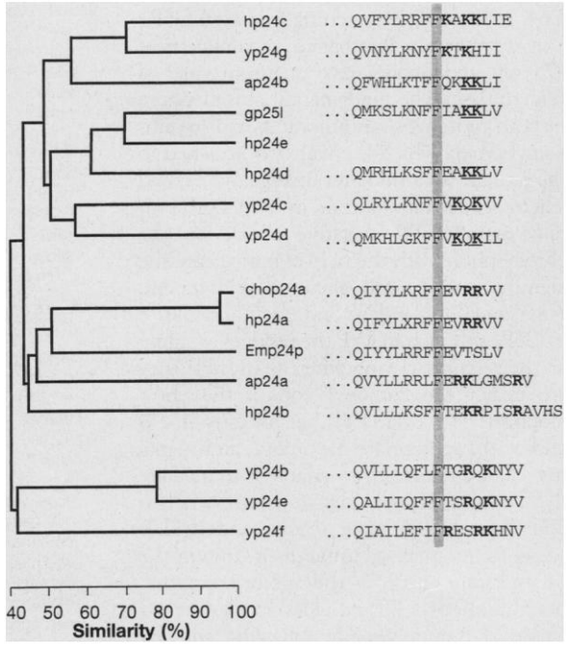

Fig. 1. The p24 family. A dendrogram was generated with the GCG program pileup (*26*) and the entire available sequences of 14 previously described p24 members (*9*) and of the entries yp24f and yp24g. The prefix h refers to human proteins, y to yeast, a to *Arabidopsis thaliana*, and cho to CHO cells; gp25l is from dog (*8*) and Emp24p is from yeast (*10*). The horizontal branch lengths are proportional to the similarity between the sequences. The amino acid sequences of the COOH-terminal cytoplasmic domains are indicated in single-letter code (*12*). The first residue, glutamine, is predicted to be located at the border of the cytoplasmic domain and transmembrane span. The absolutely conserved phenylalanine residue is shaded. Basic residues distal to the conserved phenylalanine are in bold; basic residues in positions −3 and −4 or −3 and −5 matching the dilysine ER retrieval motif are bold and underlined (*27*).

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yp24f—also bound coatomer (Fig. 2A). However, these tails preferentially interacted with another set of coatomer subunits, β-, γ-, and ζ-COP. We term this group the F (phenylalanine) subcomplex (Fig. 2B). This interaction was specific because (i) it was greatly reduced when the conserved phenylalanine was replaced with alanine; (ii) it was selective for the F subcomplex; and (iii) other p24 tails (hp24b and yp24g) bound much less coatomer. Because there is no reason to believe that all p24 proteins would be transported in COPI-coated vesicles, it is not surprising that some tails do not bind coatomer. Emp24p is known to enter COPII-coated vesicles departing the ER (*10*); our observation that Emp24p binds coatomer suggests that Emp24p may also enter COPI-coated vesicles and thus be a dual coat receptor.

To confirm that the interaction of COP proteins with the p24 tails did not require additional proteins, we performed similar experiments with purified coatomer and obtained similar results. The possible interaction of δ-COP with the panel of fusion proteins could not be reliably analyzed because this subunit was recovered in highly variable amounts, despite the inclusion of protease inhibitors.

Our observation of phenylalanine-independent binding of p24 tails containing KKXX-COOH or KXKXX-COOH is consistent with the fact that the coatomer B subcomplex can be released from coatomer by exposure to high salt concentrations and alone binds KKXX-COOH in proteins lacking the conserved phenylalanines (*17*). Apparently, the salt conditions used previously (*5*), and now by us, also result in the removal of some or all coatomer subunits not directly attached to the cytoplasmic domains of p24 proteins. Thus, we suggest that one or more of the F subcomplex subunits (β-, γ-, and ζ-COP) binds to a subset of p24 cytoplasmic domains in a manner that requires the conserved phenylalanine located at position −7 to −9 proximal to the COOH-terminus. Residues adjacent to the phenylalanine may also be important for coatomer interaction because hp24b and yp24g showed little coatomer binding (Fig. 2).

If it is physiologically relevant, the extent of F subcomplex binding would be expected to be similar to that of B subcomplex binding. We therefore compared coatomer binding to the cytoplasmic domains of WBP1 (*15*), E19 (*16*), and a set of p24 proteins. Binding to WBP1 and E19 was dependent on the dilysine motif; it was abolished when the lysines were replaced with serines (Fig. 3) (*5*). The amounts of coatomer subunits that interacted with hp24d, Emp24p, and chop24a were similar to those that bound to WBP1 and E19 (Fig. 3).

Cargo receptors, probably including p24 proteins and possibly the VIP36 family of lectins (*18*), must move bidirectionally as an essential aspect of their function. Such proteins can thus be expected to contain a mixture of anterograde and retrograde transport signals in their cytoplasmic tails. The dilysine motifs that direct binding to the B subcomplex operate as retrograde transport signals in vivo (*5*, *13*, *14*). However, the possibility suggested by our binding data that p24 tails that lack dilysine motifs may also constitute a transport signal requiring the conserved phenylalanine has not been analyzed. To test the physiological relevance of the phenylalanine-containing motif of F subcomplex–binding p24 tails, we analyzed CD8-p24 chimeras in transiently transfected mammalian cells. The CD8 protein has been used previously to define cytoplasmic- and transmembrane span–targeting sequences (*13*, *14*, *19*). The transmembrane segment and cytoplasmic domain of chop24a were joined to the luminal domain of human CD8; either the wild-type tail (24a FF) or a mutant in which the two phenylalanines of the chop24a cytoplasmic tail were replaced by alanine (24a AA) was used. We mutated both phenylalanine residues because they are conserved in all known mammalian p24 proteins, and because established vesicle sorting motifs typically contain two conserved residues (*1*). Immunofluorescence analysis showed that the 24a FF protein was localized to the cell surface in unpermeabilized cells (Fig. 4C). In permeabilized cells, 24a FF was also detected in a juxtanuclear area, presumably the Golgi complex (Fig. 4A). The mutant 24a AA was present at the cell surface in unpermeabilized cells (Fig. 4D); however, in contrast to 24a FF, marked labeling of

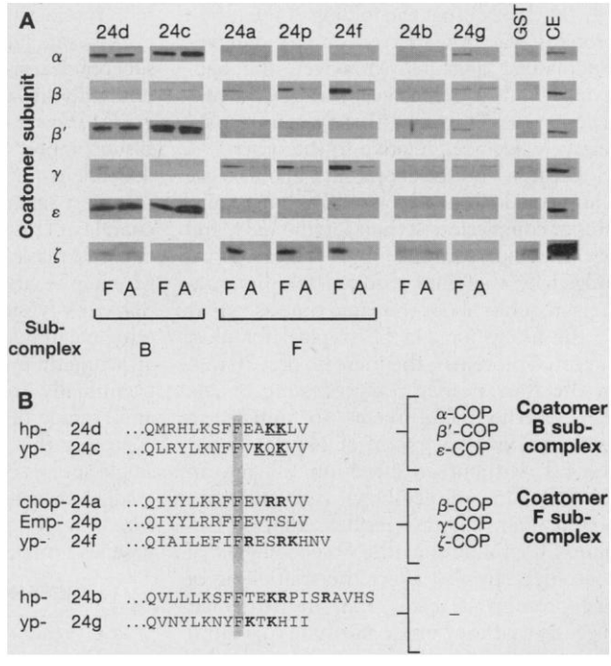

Fig. 2. Coatomer binding to GST-p24 fusion proteins. (**A**) Fusion proteins were expressed in *Escherichia coli* and bound to glutathione-agarose beads (*24*). The beads were incubated with CHO cell extract, and bound coatomer subunits were detected by immunoblot analysis (*25*). p24 members were expressed as wild-type (F) or Phe→Ala mutant (A) proteins. 24d refers to the hp24d protein, 24c is yp24c, 24a is chop24a, 24p is Emp24p, 24f is yp24f, 24b is hp24b, and 24g is yp24g. Lane labeled GST refers to binding to GST protein as a control; CE refers to immunoblot analysis with 1 μl of CHO cell extract for comparison. (**B**) Summary of cytoplasmic domains (*12*) and coatomer interactions. The absolutely conserved phenylalanine residue of p24 proteins is shaded. Basic residues distal to the conserved phenylalanine are in bold; basic residues in positions −3 and −4 or −3 and −5 matching the dilysine ER retrieval motif are bold and underlined (*27*).

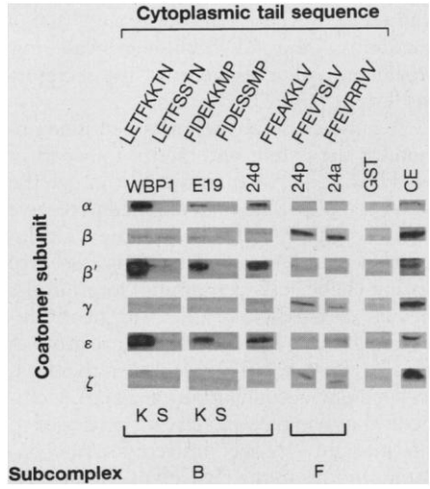

Fig. 3. Coatomer binding to WBP1 and E19. Fusion proteins were expressed in *E. coli* and bound to glutathione-agarose beads, which were then incubated with CHO cell extract. Bound coatomer subunits were detected by immunoblot analysis. WBP1 and E19 were expressed as wild-type (K) or mutant (S) (lysine residues at positions −3 and −4 replaced with serines) proteins (*28*). 24d refers to the hp24d protein, 24p is Emp24p, and 24a is chop24a. Lanes labeled GST and CE are as described for Fig. 2A. See (*12*) for amino acid abbreviations.

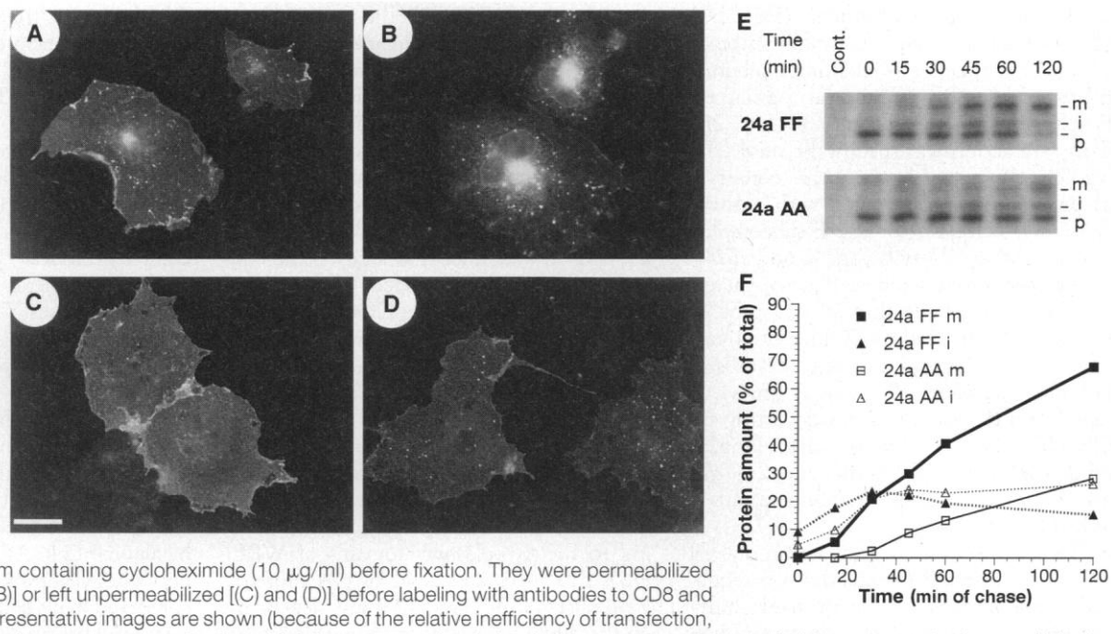

Fig. 4. Localization and pulse-chase analysis of CD8-chop24a. (**A** through **D**) Intracellular localization of CD8 chimeras by immunofluorescence microscopy. The transmembrane segment and cytoplasmic domain of chop24a were transferred to the luminal domain of CD8. The chimeras, expressed as wild-type forms (24a FF) [(A) and (C)] or as mutants in which the two phenylalanines of the chop24a tail were replaced by alanine (24a AA) [(B) and (D)], were analyzed in transiently transfected COS-7 cells 42 hours after transfection (*29*). The cells were incubated for 3 hours in medium containing cycloheximide (10 μg/ml) before fixation. They were permeabilized with Triton X-100 [(A) and (B)] or left unpermeabilized [(C) and (D)] before labeling with antibodies to CD8 and secondary antibodies. Representative images are shown (because of the relative inefficiency of transfection, it was difficult to show more than two transfected cells per field in a common focal plane). The punctate staining probably represents background labeling also present on nonpermeabilized cells. Bar, 10 μm. (**E**) Pulse-chase analysis of COS-7 cells transfected with CD8 chimeras. Forty-two hours after transfection with the 24a FF or 24a AA constructs, COS-7 cells were labeled for 20 min with [^{35}S]methionine and [^{35}S]cysteine and then incubated for 0, 15, 30, 45, 60, or 120 min in medium containing unlabeled methionine and cysteine. The cells were lysed with Triton X-100, and the CD8 chimeras were isolated by immunoprecipitation (*30*) and subjected to SDS–polyacrylamide gel electrophoresis on a 12% gel. The CD8-chop24a precursor (p), intermediate (i), and mature (m) O-glycosylated forms (*20*) are indicated. Control (Con.), immunoprecipitation from nontransfected cells. The intermediate form comigrated with the 29-kD molecular size standard. (**F**) Quantitation of the pulse-chase analysis. The amounts of the mature and intermediate O-glycosylated forms (*20*) of 24a FF and 24a AA in (E) were determined by densitometric scanning of autoradiograms (exposed for 48 or 72 hours with similar results; data from the 48-hour exposure are shown).

juxtanuclear structures, presumably the Golgi complex, and labeling of the nuclear envelope and ER were apparent in permeabilized cells (Fig. 4B), suggesting that a fraction of the CD8 chimera was now present in earlier segments of the secretory pathway.

A pulse-chase analysis was performed to monitor the extent and rate of transport of 24a FF and 24a AA from the ER through the Golgi complex. The time required to receive O-linked glycans (attached to the CD8 luminal domain) that had been processed to mature (sialic acid–containing) forms in the medial- or trans-Golgi (*20*) was greatly increased for the 24a AA chimera relative to 24a FF (Fig. 4, E and F). Moreover, there was an apparent accumulation of 24a AA chimeras that had received glycans processed to intermediate (*N*-acetylgalactosamine–containing) forms in the cis-Golgi (*20*), suggesting that intra-Golgi transport of 24a AA was slowed relative to that of 24a FF. The overall time required for half of the chimeras to receive intermediate and mature glycan forms, considered together, was increased from ~35 min for 24a FF to ~105 min for 24a AA, and the appearance of the intermediate form was delayed for 24a AA, suggesting that the rate of ER export of 24a AA was reduced relative to that of 24a FF. However, the extent of dimerization, as judged by the appearance of disulfide-linked CD8 dimers on nonreducing gels (*20, 21*), was similar for 24a FF and 24a AA, with between 40 and 50% dimers at 30 min of chase in both instances, confirming the expectation (*13, 14, 19, 21, 22*) that the folding of the fusion proteins was not affected by the different cytoplasmic domains. Moreover, the half-lives of both chimeras (~5 hours) were similar, suggesting that neither protein was selectively degraded relative to the other.

Because viral glycoprotein oligomers are transported to the Golgi within a few minutes of completion of their folding (*23*), and because the folding of both chimeras (as judged by the time required for dimerization) requires about the same time as export to the Golgi for 24a FF (typical for most exported proteins), the increase of ~70 min in the time needed for processing of 24a AA by the Golgi seems to indicate a marked slowing of export of 24a AA out of the ER, without an effect on the rate of folding. This result shows that the conserved phenylalanine residues that are required for binding to the F subcomplex of coatomer can also affect the trafficking of p24 proteins in cells, and, in particular, suggests that they may contribute to a motif that signals forward transport from the ER to the Golgi and within the Golgi.

In summary, coatomer binds a variety of p24 proteins, not only those with dilysine retrograde transport signals but also others that lack this motif, implying that proteins of this family may be both cargo and coat protein receptors. Coatomer is at least bivalent, interacting differentially with different cytoplasmic tail sequences through different subcomplexes. Specifically, coatomer can bind dilysine-containing backward (retrograde) transport signal peptides through its B subcomplex and can bind phenylalanine-dependent potential forward (anterograde) transport signal peptides through its F subcomplex. This scenario raises the possibility of conformational switching mechanisms by which p24 and other proteins such as v-SNAREs [vesicle-specific soluble NSF (*N*-ethylmaleimide–sensitive fusion protein) attachment protein receptors], by binding to mutually exclusive sites present in either anterograde or retrograde conformations of coatomer (but not in both), could direct a single species of coatomer to collect anterograde- versus retrograde-directed membrane cargo and targeting proteins into separate vesicles formed by distinct conformers.

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12. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; and X, any amino acid.
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24. The cDNAs for the expression of fusion proteins were constructed by annealing sense and antisense oligonucleotides encoding the amino acid sequences shown in Fig. 2B. The cytoplasmic tail sequences were preceded by a linker sequence encoding the amino acids GGSGG (*12*) and were subcloned into the pGEX-2T polylinker (Pharmacia). The sequences were verified by DNA sequencing. For protein expression, *E. coli* DH5α cultures in log phase were shifted from 37° to 25°C and induced with 1 mM isopropyl-β-D thiogalactopyranoside. After 6 hours, the bacteria were harvested, washed in lysis buffer [phosphate-buffered saline (PBS) containing 0.05% Tween-20, 2 mM EDTA, 0.1% β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF)], and passed twice through a French press (10,000 psi) at 4°C in lysis buffer. The lysates were centrifuged for 30 min at 4°C and 334,000*g* to separate insoluble material, and the resulting supernatants were frozen in liquid nitrogen. Thawed supernatants were recentrifuged and then incubated with 60 μl of glutathione-agarose (1:1 dilution of slurry) for 1 hour in PBS containing 0.05% Tween-20 and 1 mM PMSF. The quantities of lysate were adjusted to obtain equal amounts of each fusion protein bound (quantified by densitometric scanning of Coomassie blue R-250–stained gels). All procedures were performed at 4°C except when indicated. The beads were washed with 1 ml of buffer A [0.5% Triton X-100, 50 mM tris-HCl (pH 7.4), 300 mM NaCl, 1 mM PMSF, 1 mM dithiothreitol] and incubated for 10 min at room temperature with 1 ml of buffer A containing 2 mM adenosine triphosphate and 10 mM $MgCl_2$. The beads were further washed with 1 ml of buffer A containing 1.3 M NaCl followed by 1 ml of buffer A.
25. CHO extracts were prepared from ~3 ml of centrifuged cells with 40 ml of buffer A containing leupeptin and antipain each at 20 μg/ml. The extracts were rotated for 30 min at 4°C. Insoluble material was removed by centrifugation for 45 min at 180,000*g* and 4°C, and the resulting supernatants were frozen in liquid nitrogen. Thawed CHO extract was recentrifuged and 800 μl in buffer A were incubated for 3 hours at 4°C (with rotation) with the beads containing bound GST fusion proteins. The beads were washed three times with 1 ml of buffer A and once with 50 mM Hepes (pH 7.2). After aspiration with a Hamilton syringe, bound proteins were eluted by boiling in reducing SDS sample buffer. SDS–polyacrylamide gel electrophoresis and immunoblot analysis by enhanced chemiluminescence (ECL, Amersham) were performed as described [S. Hara-Kuge *et al.*, *J. Cell Biol.* **124**, 883 (1994)]. Antibodies were used at the following dilutions: anti–α-COP (883, affinity-purified), 1:1500; anti–β-COP (M3A5, ascites), 1:800; anti–β′-COP (C1PL, affinity-purified), 1:3000; anti–γ-COP (serum), 0.4 μg/ml; anti–ε-COP (affinity-purified), 50 ng/ml; and anti–ζ-COP (affinity-purified), 20 ng/ml.
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27. GenBank-EMBL accession numbers: hp24a (T27390, F06012, T34121, R14234, T27339, T11000, T48545); hp24b (T48838, R25915, T17481); hp24c (T32238); hp24d (T98284); hp24e (F07445); chop24a (U26264); gp25l (X53592); ap24a (Z34726); ap24b (T46519); Emp24p (X67317); yp24b (L22015); yp24c (U00059); yp24d (L22015); yp24e (X87331, T36996); yp24f (Z48432); and yp24g (Z49810).
28. The constructs encoding wild-type GST-WBP1 (GST-KKLETFKKTN) (*12*), mutant GST-WBP1 (GST-KKLET-FSSTN), wild-type E19 (GST-KYKSRRSFIDEKKMP), and mutant E19 (GST-KYKSRRSFIDESSMP) in the pGEX-3X vector were described previously (*5*). All procedures were performed as described (*24, 25*).
29. The CD8 chimeras were constructed by the polymerase chain reaction such that the 165 amino acids of the human CD8 extracellular domain were preserved. Codon 166 was changed to glycine to introduce a unique Apa I restriction site and was followed by a conserved proline, a stop codon, and an Eco RI site. Oligonucleotides encoding the COOH-terminal 34 amino acids of chop24a [RVVLWSFFEALVL-VAMTLGQIYYLKR(F/A)(F/A)EVRRVV] (*12*), preceded by an Apa I site and followed by a stop codon and an Eco RI site, were subcloned into the CD8 construct and inserted into the pECE vector [L. Ellis *et al.*, *Cell* **45**, 721 (1986)]. Sequences were verified by DNA sequencing. Transfection of COS-7 cells was performed with Lipofectin and Lipofectamine (Gibco BRL) for immunofluorescence and pulse-chase analysis, respectively. The OKT8 monoclonal antibody to CD8 (Ortho) and fluorescein-conjugated goat antibodies to mouse immunoglobulin (Molecular Probes) were used at a dilution of 1/30 and 1/100, respectively, for immunofluorescence.
30. Pulse-chase analysis and immunoprecipitation with the OKT8 monoclonal antibody were performed essentially as described [M. R. Jackson, T. Nilsson, P. A. Peterson, *J. Cell Biol.* **121**, 317 (1993)] but with protein G–agarose (Boehringer).
31. We thank T. Söllner, C. Blobel, and M. Craighead for discussions; C. Harter, F. Wieland, and T. Kreis for antibodies; F. Letourneur and P. Cosson for the GST-WBP1 and GST-E19 constructs; S. Ponnambalam and T. Nilsson for the CD8 cDNA construct; and M. Spiess for the pECE vector. Supported by NIH and the Mathers Charitable Foundation (J.E.R.); the Human Frontier Science Program Organization and the Swiss National Science Foundation (K.F.); and the Deutsche Forschungsgemeinschaft (M.V.).

7 March 1996; accepted 18 July 1996

A Neostriatal Habit Learning System in Humans

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Amnesic patients and nondemented patients with Parkinson's disease were given a probabilistic classification task in which they learned which of two outcomes would occur on each trial, given the particular combination of cues that appeared. Amnesic patients exhibited normal learning of the task but had severely impaired declarative memory for the training episode. In contrast, patients with Parkinson's disease failed to learn the probabilistic classification task, despite having intact memory for the training episode. This double dissociation shows that the limbic-diencephalic regions damaged in amnesia and the neostriatum damaged in Parkinson's disease support separate and parallel learning systems. In humans, the neostriatum (caudate nucleus and putamen) is essential for the gradual, incremental learning of associations that is characteristic of habit learning. The neostriatum is important not just for motor behavior and motor learning but also for acquiring nonmotor dispositions and tendencies that depend on new associations.

Students of brain and behavior have long recognized that double dissociations (*1*) provide the strongest evidence for separating the functions of brain systems. Recent work with experimental animals has dissociated hippocampal and dorsal striatal learning systems (*2*). Rats with lesions of the hippocampus or anatomically related structures were impaired on tasks thought to require spatial, relational memory, but they were intact at tasks of habit learning that require the gradual, incremental learning of associations. Lesions of the dorsal striatum produced the opposite pattern of results.

Evidence for separate memory systems has also been obtained in humans (*3*). For example, amnesic patients are profoundly impaired on conventional tests of declarative (explicit) memory that assess recall and recognition, but they are intact at a variety of nondeclarative (implicit) memory tasks that assess skill learning, simple forms of

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