Disabled-2 exhibits the properties of a cargo-selective endocytic clathrin adaptor

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Clathrin-coated pits at the cell surface select material for transportation into the cell interior. A major mode of cargo selection at the bud site is via the μ2 subunit of the AP-2 adaptor complex, which recognizes tyrosine-based internalization signals. Other internalization motifs and signals, including phosphorylation and ubiquitylation, also tag certain proteins for incorporation into a coated vesicle, but the mechanism of selection is unclear. Disabled-2 (Dab2) recognizes the FXNPXY internalization motif in LDL-receptor family members via an N-terminal phosphotyrosine-binding (PTB) domain. Here, we show that in addition to binding AP-2, Dab2 also binds directly to phosphoinositides and to clathrin, assembling triskelia into regular polyhedral coats. The FXNPXY motif and phosphoinositides contact different regions of the PTB domain, but can stably anchor Dab2 to the membrane surface, while the distal AP-2 and clathrin-binding determinants regulate clathrin lattice assembly. We propose that Dab2 is a typical member of a growing family of cargo-specific adaptor proteins, including β-arrestin, AP180, epsin, HIP1 and numb, which regulate clathrin-coat assembly at the plasma membrane by synchronizing cargo selection and lattice polymerization events.
Keywords: cargo selection/clathrin/Disabled-2/ phosphoinositide/receptor-mediated endocytosis

Introduction

Clathrin-coated buds only assemble at the cell surface to convey select macromolecules into the cell interior. To gain entry into the clathrin coat, many transmembrane proteins utilize short endocytic sorting determinants located within the cytosolic domain. The best understood internalization signal employs the tyrosine-based YXXØ sequence, where Ø represents a residue with a bulky hydrophobic side chain (Bonifacino and Dell'Angelica, 1999). This particular class of internalization signals binds directly to the μ2 subunit of the AP-2 adaptor heterotetramer (Ohno *et al*., 1995). Structurally, the interaction occurs via an extended YXXØ sequence engaging the μ2

β-sandwich in a β-sheet augmentation, with specificity provided by two hydrophobic pockets that accommodate the key tyrosine and bulky hydrophobic side chains (Owen and Evans, 1998). As AP-2 also binds to clathrin directly, these findings explain nicely how the adaptors can efficiently coordinate clathrin lattice assembly with cargo selection.

It is clear that not all cargo molecules cluster into clathrin-coated buds by direct engagement of the adaptor μ2 subunit. Heptahelical G protein-coupled receptors utilize an alternate system centered around β-arrestin (Miller and Lefkowitz, 2001). Normally soluble, β-arrestin translocates onto activated, and thus phosphorylated, heptahelical receptors while synchronously interacting with both clathrin and the β2 subunit of AP-2 (Goodman *et al*., 1996; Laporte *et al*., 1999, 2000). The multivalent attachments involving β-arrestin recruit activated receptors into assembling clathrin-coated regions for down-regulation. Thus, β-arrestin meshes the receptor with the standard cellular endocytic machinery (Santini *et al*., 2002; Scott *et al*., 2002). A role akin to that of β-arrestin for alternate classes of cargo molecules is probably fulfilled by other proteins. Mutagenic inactivation of the μ2 subunit potently blocks transferrin receptor uptake (which uses a YTRF internalization motif), but fails to affect epidermal growth factor (EGF) receptor endocytosis (Nesterov *et al*., 1999). This indicates that the sorting of transferrin and EGF receptors into clathrin buds is mechanistically distinct. In fact, when one is over-expressed, transferrin, EGF and low density lipoprotein (LDL) receptors do not appear to compete with each other for incorporation into clathrin-coated vesicles, despite all containing tyrosine-based internalization signals (Warren *et al*., 1998). Dedicated connector proteins, functionally analogous to β-arrestin, were postulated to account for the independent selection of these proteins into clathrin coats at the cell surface (Warren *et al*., 1998).

Evidence for one additional connector-type protein comes from genetic disruption of UNC-11, the *Caenorhabditis elegans* ortholog of AP180 (Nonet *et al*., 1999). Remarkably, in *unc-11* mutant worms, synaptobrevin/ VAMP fails to be appropriately recycled together with the synaptic vesicle membrane following exocytosis. Instead, the protein diffuses widely over the presynaptic plasma membrane (Nonet *et al*., 1999). The size distribution of neuronal clathrin-coated vesicles is also aberrant in these worms, suggesting that UNC-11 could integrate cargo selection temporally with clathrin-coat assembly. The N-terminal ENTH domain of AP180 binds to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). With the adjacent AP-2- and clathrin-binding segment of the protein, the ENTH domain facilitates the assembly of highly ordered, invaginated clathrin-coated buds in the presence of soluble AP-2 and clathrin (Ford *et al*., 2001).
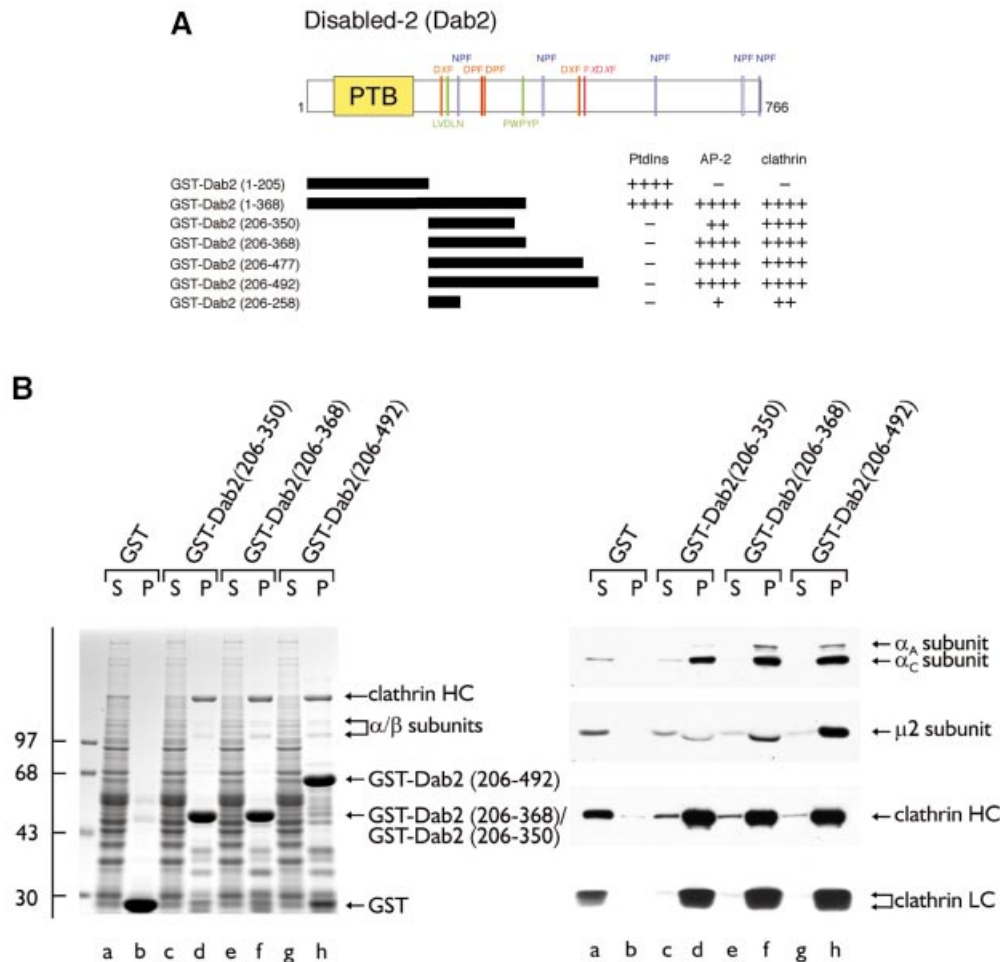
Fig. 1. Dab2 binds to both the AP-2 adaptor complex and clathrin. (**A**) Schematic illustration of the overall domain organization of Dab2 and the various GST–Dab2 constructs used. Phosphoinositide-, AP-2- and clathrin-binding properties of each fusion protein are indicated qualitatively. (**B**) Approximately 50 µg of either GST (lanes a and b), GST–Dab2(206–350) (lanes c and d), GST–Dab2(206–368) (lanes e and f) or GST–Dab2(206–492) (lanes g and h) immobilized on GSH–Sepharose were incubated with rat brain cytosol. After centrifugation, aliquots corresponding to 1/60 of each supernatant (S) and 1/5 of each washed pellet (P) were resolved by SDS–PAGE and either stained with Coomassie Blue or transferred to nitrocellulose. Portions of the blots were probed with the anti-AP-2 α-subunit mAb 100/2, anti-AP-2 µ2-subunit antiserum, the anti-clathrin heavy chain (HC) mAb TD.1 or the anti-clathrin light chain (LC) mAb Cl 57.3. The position of the molecular mass standards (in kDa) is indicated on the left and only the relevant portion of each blot is shown.

We have recently shown that two endocytic accessory proteins, epsin and huntingtin-interacting protein 1 (HIP1), each of which has similar overall domain organization to AP180, can also promote optimal clathrin-coat assembly upon liposome templates (Mishra *et al.*, 2001). These proteins too could be cargo-selective endocytic connectors.

The NP*XY* internalization motif within the LDL receptor might utilize an alternate connector-type protein because transferrin receptor overexpression does not interfere with LDL receptor internalization (Warren *et al.*, 1998). One candidate connector is Disabled-2 (Dab2). Dab2 contains an N-terminal phosphotyrosine-binding (PTB) domain with a marked preference for non-phosphorylated NP*XY* motifs, as is found in the LDL receptor and amyloid precursor protein (Morris and Cooper, 2001). At steady state, the intracellular distribution of Dab2 co-localizes well with AP-2 and clathrin. Dab2 binds to AP-2 directly via a central portion that, like epsin, AP180 and HIP1, engages the independently folded appendage domain of the AP-2 α-subunit (Morris and

Cooper, 2001). We show here that Dab2 also binds robustly to clathrin, independently of the AP-2 association, and, like other PTB domains, the PTB domain of Dab2 associates directly with phosphoinositides. With synthetic liposome templates, Dab2 drives clathrin-coat assembly in the presence of purified AP-2 and clathrin and, consequently, displays all the properties of a cargo-specific adaptor protein.

Results

Dab2 binds the AP-2 α-subunit appendage directly using the same interaction surface engaged by epsin, eps15, amphiphysin, HIP1 and AP180 (Morris and Cooper, 2001). Tandem DPF triplets, positioned at [293]DPF-RDDPF, are potential interaction motifs for the α-appendage, yet amino acids 206–350 of murine Dab2 fused to glutathione *S*-transferase (GST) bind to soluble AP-2 relatively weakly (Morris and Cooper, 2001) (Figure 1B, lanes d). A GST fusion protein of Dab2 residues 206–492 appears to bind to AP-2 at least 4-fold more avidly (Morris
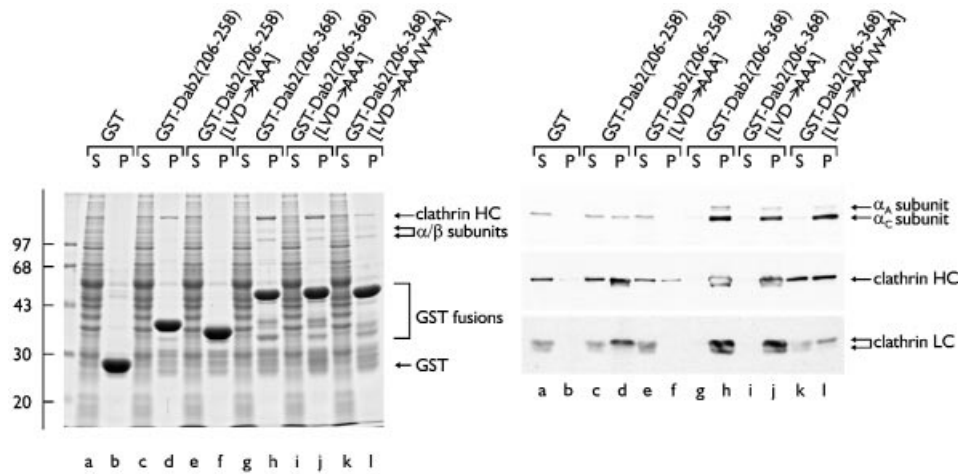
Fig. 2. A clathrin-binding region within Dab2. Approximately 50 µg of either GST (lanes a and b) or GST–Dab2(206–258) (lanes c and d), GST–Dab2(206–258) (LVD→AAA) (lanes e and f), GST–Dab2(206–368) (lanes g and h), GST–Dab2(206–368) (LVD→AAA) (lanes i and j) or GST–Dab2(206–368) (LVD→AAA/W→A) (lanes k and l) immobilized on GSH–Sepharose were incubated with rat brain cytosol. After centrifugation, aliquots corresponding to 1/50 of each supernatant (S) and 1/5 of each washed pellet (P) were resolved by SDS–PAGE and either stained with Coomassie Blue or transferred to nitrocellulose. Portions of the blots were probed with the anti-AP-2 α-subunit mAb 100/2, anti-AP-2 µ2-subunit antiserum, the anti-clathrin HC mAb TD.1 or the anti-clathrin LC mAb Cl 57.3.

and Cooper, 2001) (Figure 1B, lanes h), indicating that this distal region also contributes to the interaction of Dab2 with AP-2. We recently characterized an alternate α-appendage-binding sequence, the FXDXF motif (Brett *et al*., 2002), and Dab2 contains the sequence [480]FLDLF within the distal segment. To explore the distal AP-2-binding determinant(s) further, we prepared a limited series of truncated Dab2 fusions (Figure 1A). Amino acids 206–368 is the minimal sequence required for optimal AP-2 binding in pull-down-type assays (Figure 1B, lanes f). Surprisingly, the removal of the FXDXF motif has no discernible effect on AP-2 binding, indicating that this sequence is not critical for the α-appendage association measured by these assays. The additional appendage-binding sequences in Dab2 remain to be characterized.

Dab2 interacts directly with clathrin triskelia

The central region of murine Dab2 also binds to a very prominent ~180 kDa polypeptide in the pull-down assays (Figure 1B). Appropriate antibodies identify this protein as the clathrin heavy chain (Figure 1B). The smallest GST–Dab2 fusion tested, encoding only residues 206–258, binds to clathrin (Figure 2, lanes d). Longer Dab2 segments fused to GST bind to soluble clathrin with noticeably greater affinity (Figure 2, lanes h) and remove trimers from cytosol more completely (lanes g compared with a and c). As little AP-2 binds to the GST–Dab2 (206–258) protein (lanes d), the interaction with clathrin is largely independent of the adaptor complex and may well be direct. Indeed, isolated GST–Dab2(206–258) binds to pre-assembled clathrin *in vitro* and, upon centrifugation, is recovered together with the polyhedral cages in the pellet (Figure 3, lane j). GST (Figure 3, lanes e and f) does not sediment appreciably with the cages (lane f) under the same conditions. In the absence of the clathrin, both GST (Figure 3, lane c) and the GST–Dab2 fusion (lane g) remain soluble and appear in the supernatant fraction, together with the carrier BSA. Similarly, a larger GST–Dab2 fusion (residues 206–350) binds to the assembled
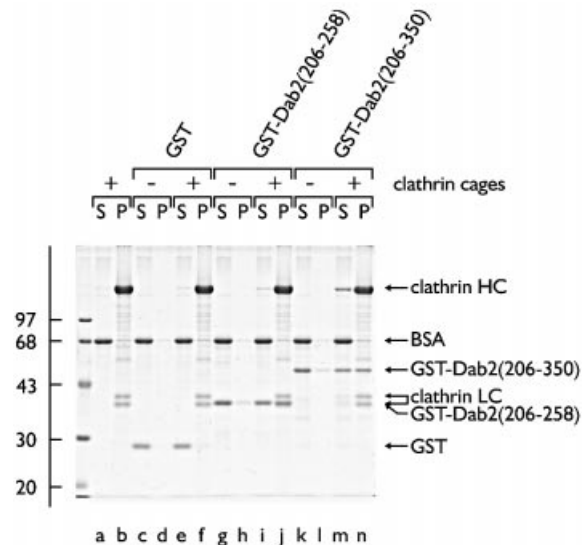

Fig. 3. Dab2 associates directly with assembled clathrin cages. Pre-assembled clathrin cages (~0.5 µM), GST, GST–Dab2(206–258), GST–Dab2(206–350) (each ~1 µM) or combinations thereof were incubated in MES–OH buffer on ice. After centrifugation, aliquots corresponding to 1/10 of each supernatant (S) or 1/8 of each pellet (P) were analyzed by SDS–PAGE and stained with Coomassie Blue.

cages (Figure 3, lane n), but does not sediment significantly in the absence of clathrin (lane l).

Sequence analysis reveals a putative type I clathrin-box sequence, [236]LVDLN, located within the minimal segment of Dab2 (residues 206–258) that binds clathrin efficiently. To assess the contribution of this motif to clathrin binding, the first three residues of the sequence were mutated to Ala (LVDLN→AAALN). In the context of GST–Dab2(206–258), the smallest clathrin-binding fragment, disruption of this type I sequence almost completely ablates clathrin association (Figure 2, lanes f compared with d). Introducing the LVDLN→AAALN substitution within a

larger GST– Dab2(206–368) fusion has little effect on clathrin binding, however (Figure 2, lane j). The sequence [363]PWPYP is similar to the type II clathrin-binding sequence [381]PWDLW found in amphiphysin I and II (Ramjaun and McPherson, 1998; Slepnev *et al.*, 2000). In amphiphysin, substitution of the proximal Trp with Ala completely inactivates the binding properties (Drake and Traub, 2001). Simultaneous mutation (LVD→AAA/ W→A) of both the type I LVDLN and putative type II PWPYP sequences in GST–Dab2(206–368) reduces clathrin binding 4- to 5-fold in pull-down assays. The interaction of this Dab2 fusion with AP-2 is not similarly affected (Figure 2, lane l). These experiments clearly indicate that Dab2 contains multiple, tandemly arrayed clathrin-binding sequences. When Dab2 is immunoprecipitated from cytosolic extracts, clathrin trimers do not co-precipitate in significant amounts (not shown). However, we also fail to detect significant co-immunoprecipitation of clathrin with either epsin 1 (not shown) or AP-2 (Arneson *et al.*, 1999), two proteins known to engage clathrin at coat assembly sites using clathrin-binding sequences related to those present in Dab2 (Drake *et al.*, 2000; Dell'Angelica, 2001; Kalthoff *et al.*, 2002).

Previously, we have shown that tandemly arranged type I and type II sequences allow amphiphysin to bind to the terminal domain of the clathrin heavy chain and polymerize trimers into sedimentable assemblies (Drake and Traub, 2001). Similarly, if a GST–Dab2(1–368) fusion is mixed with soluble clathrin trimers, a portion of both proteins is recovered in a high-speed pellet after dialysis into either MES–OH buffer (pH 6.8; not shown) or a more physiological assay buffer (pH 7.2; Figure 4A, lane o). Substantially lower levels of the clathrin alone (Figure 4A, lane c) or the GST–Dab2(1–368) alone (lane l) sediment under the same conditions. Electron microscope (EM) examination of the Dab2 assembled population after negative staining reveals regular assemblies of polyhedral clathrin cages (Figure 4B). Despite binding to pre-assembled cages (Figure 3), the minimal clathrin-binding Dab2 fusion GST–Dab2(206–258) does not increase clathrin recovery in the high-speed pellet above background (lane i compared with c). GST is also unable to induce clathrin-coat assembly (data not shown). Compared with the GST–dimer form, thrombin-cleaved monomeric Dab2(206–350) shows a reduced capacity to assemble clathrin cages (not shown). However, monomeric Dab2(1–368) effectively recruits soluble clathrin trimers onto a membrane surface (see Figure 8D). Together, we believe our data show that in addition to AP-2 (Morris and Cooper, 2001), Dab2 binds to clathrin directly, interacts with pre-assembled clathrin cages and, by utilizing multiple binding sites, engages soluble clathrin trimers to productively assemble closed clathrin cages.

Dab2 binds to phosphoinositides via the N-terminal PTB domain

The capacity of the central region of Dab2 to interface with the core endocytic machinery is reminiscent of other so-called endocytic accessory proteins, including AP180, amphiphysin, epsin and HIP1. Indeed, in HeLa cells, epsin 1 and Dab2 show extensive co-localization at steady state (Figure 5), indicating that the two proteins are probably present within common clathrin-coated struc-
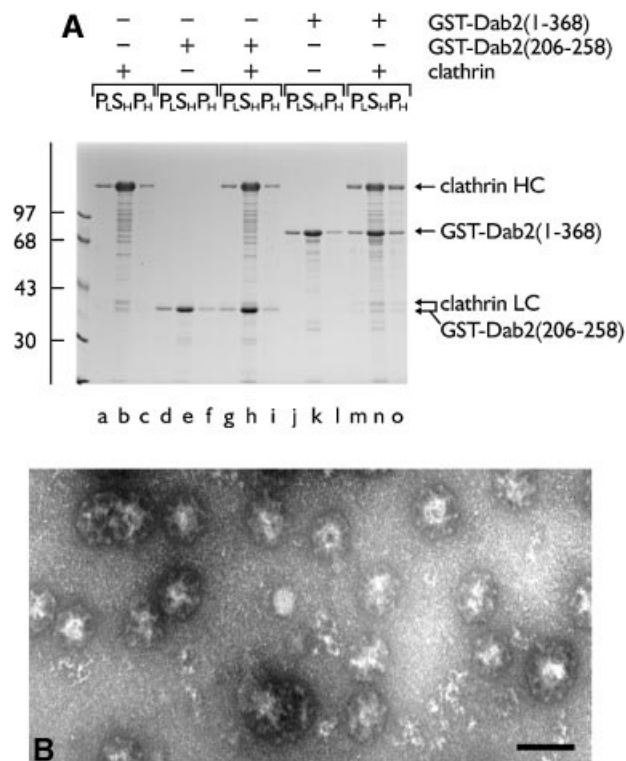

Fig. 4. Dab2 assembles soluble clathrin trimers into spherical poly-hedral cages. (**A**) Clathrin (~0.2 µM), GST–Dab2(206–258) (~1 µM), GST–Dab2(1–368) (~1 µM) or combinations thereof were mixed in 0.5 M Tris–HCl pH 7.0 and then dialyzed overnight against assay buffer. The samples were subject to differential centrifugation generating a low-speed pellet (P_L) containing aggregated material, a high-speed supernatant (S_H) containing soluble protein, and a high-speed pellet (P_H) with the sedimentable assemblies. (**B**) Representative EM micrograph of a negatively stained aliquot of the high-speed pellet obtained from an incubation of clathrin with GST–Dab2(1–368) after resuspension in assay buffer on ice. Bar = 100 nm.

tures. AP180, epsin and HIP1 utilize an ENTH domain to interact with PtdIns(4,5)P_2 and, tethered to a phospho-inositide-containing membrane surface, each protein can assist AP-2 in the assembly of clathrin-coated buds (Ford *et al.*, 2001; Mishra *et al.*, 2001). In Dab2, a PTB domain replaces an ENTH domain (Xu *et al.*, 1995). PTB domains are structurally related to the pleckstrin homology (PH) domain, a known phosphoinositide-binding fold. In fact, the PTB domains of Shc (Ravichandran *et al.*, 1997), IRS-1 (Takeuchi *et al.*, 1998), Dab1 (Howell *et al.*, 1999) and numb (Dho *et al.*, 1999) all bind to phosphoinositides, leading us to test whether the Dab2 PTB domain similarly associates with phosphoinositides. In binding assays with mixed lipid liposomes, the Dab2 PTB domain (amino acids 1–205) fused to GST binds robustly to phospho-inositide-containing membranes in a dose-dependent fashion (Figure 6A, lanes d and f). The extent of the association with phosphoinositides is similar to an epsin 1 ENTH domain–GST fusion (Figure 6A, lane h) (Itoh *et al.*, 2001; Mishra *et al.*, 2001), while PTB-domain binding to liposomes devoid of phosphoinositides (lane b), or GST association with inositide-containing liposomes (lane j), is extremely poor. The monomeric PTB domain, cleaved from GST with thrombin, binds ~25-fold better to PtdIns(4,5)P_2 liposomes, rising from ~2% without phosphoinositide to >50% bound (Figure 6B, lanes a, b
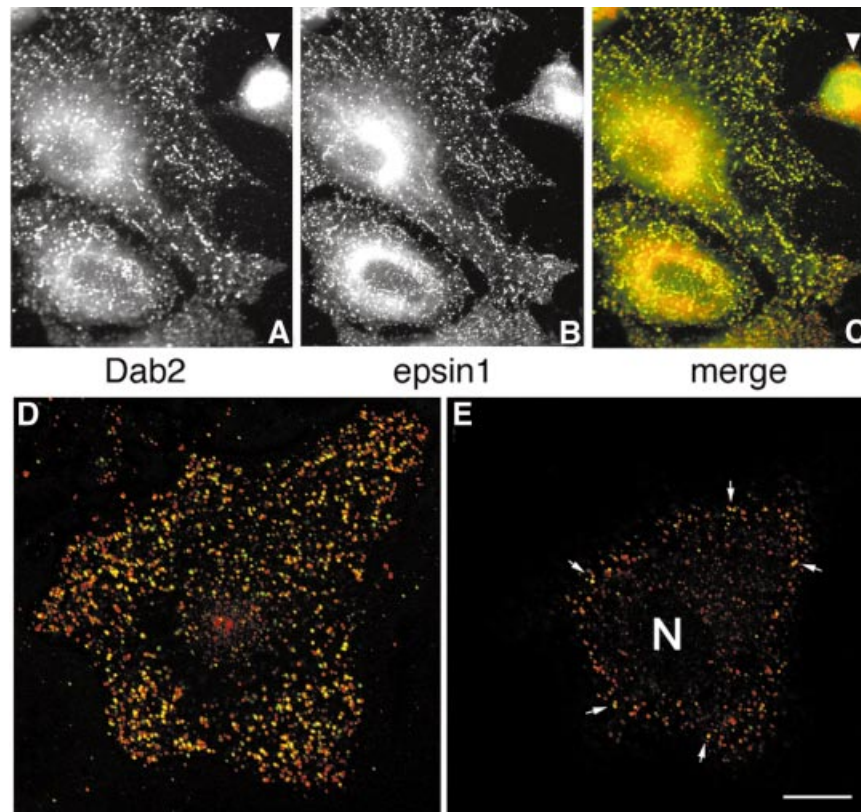
Fig. 5. Dab2 co-localizes with epsin 1 at the plasma membrane. Saponin-permeabilized HeLa cells were fixed and probed with antibodies against Dab2 (**A**) and epsin 1 (**B**). The merged color images (**C–E**) show that areas of overlap are extensive (yellow), but there are clear regions containing each protein alone. Single 0.3 µm confocal sections of the ventral surface (D) and the medial region of the cell (E) through the nucleus (N) show Dab2 co-localized with epsin at the cell surface (arrows). The arrowhead indicates a pre/post-mitotic cell where the Dab2 staining pattern is principally nuclear, although some epsin is still visible at the cell surface; we note that the intracellular distribution of Dab2 appears to alter with the cell cycle. Importantly, the anti-Dab2 mAb used here detects all three splice isoforms of Dab2 (p67, p93 and p96), so we cannot conclude whether the different locations represent distinct Dab2 isoforms. Bar = 25 µm in (A–C) and 10 µm in (D) and (E).

compared with e, f). This suggests that the affinity of the Dab2 PTB domain for phosphoinositides is not massively increased by artificial GST-mediated dimerization. Protein–lipid overlays reveal that the Dab2 PTB domain displays a preference for PtdIns(4,5)P_2, although there is binding to several phosphoinositides in this type of assay (Figure 6C).

PTB domains are known to bind directly to the sequence NP*X*pY (Margolis, 1996), but for Dab1 and Dab2, the PTB designation is a misnomer as both bind to F*X*NP*X*Y preferentially in the non-tyrosine-phosphorylated state (Howell *et al*., 1999; Morris and Cooper, 2001). Dab2 PTB association with phosphoinositide-containing mixed liposomes does not preclude NP*X*Y sequence engagement. Addition of a 3- to 5-fold molar excess of a peptide corresponding to residues 2–22 of the LDL receptor cytosolic domain ([814]NWRLKNINSIFD**NPVY**QKTT) does not diminish the extent of monomeric PTB domain association with the liposomes (Figure 6D, lane d compared with b). Rather, the wild-type peptide is recovered in the liposome pellet, along with PTB domain (lane d). On standard SDS–PAGE, the bound peptide migrates at the dye front but Tris–Tricine gels confirm the identity of the 2.7 kDa peptide (not shown). The Dab1 PTB domain also binds lipid and NPXY peptides simultaneously (Howell *et al*., 1999) and, like Dab1, mutation of an invariant Phe

(F166V) involved in F*X*NP*X*Y engagement (Howell *et al*., 1999; Hocevar *et al*., 2001) does not prevent phosphoinositide binding (not shown). If an altered peptide, NWRLKNINSIFD<u>A</u>PV<u>A</u>QKTT, is included, negligible amounts of the NP*X*Y-sequence-altered peptide sediment with the liposome/PTB domain pellet (Figure 6D, lane f). Because alteration of the Asn and Tyr side chains in the LDL receptor F*X*NP*X*Y internalization sequence disrupts endocytic uptake (Chen *et al*., 1990), the sequence determinants necessary for the LDL receptor peptide to engage the membrane-bound Dab2 PTB domain are the same as those required for LDL receptor internalization.

If the Dab2 PTB domain can engage the F*X*NP*X*Y internalization sequence *in vivo*, overexpression of this segment of Dab2 might produce a selective block in LDL receptor internalization. Transient transfection of COS-7 cells with myc epitope-tagged Dab2(1–205) (PTB domain) had little effect on LDL receptor distribution (not shown). The phosphatidylinositol 3-phosphate-binding FYVE domain only targets efficiently to the appropriate intracellular site when expressed in tandem (Gillooly *et al*., 2000), so we also examined the effect of myc-Dab2 PTBx2 overexpression. Two populations of labeled cells are seen after a 15 min pulse with diI-LDL (Figure 7). The majority of the cells display bright perinuclear punctae, corresponding to endosomes (Figure 7A). A minority of
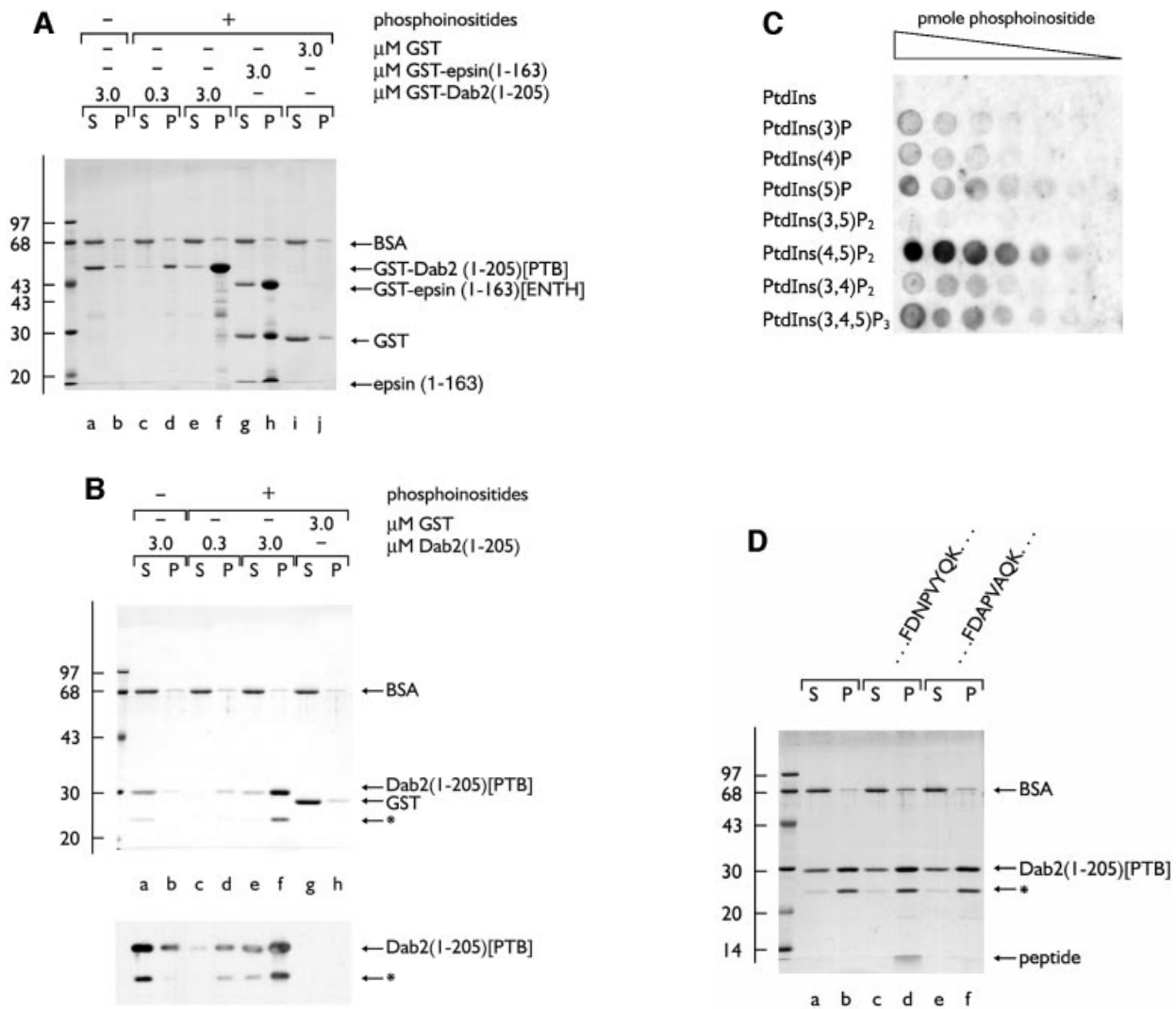
Fig. 6. The PTB domain of Dab2 binds to polyphosphoinositides. (**A**) GST–Dab2(1–205) (lanes a–f), GST–epsin 1(1–163) (lanes g and h) or GST (lanes i and j), as indicated, were mixed with either control (lanes a and b) or phosphoinositide-containing (lanes c–j) liposomes and incubated together on ice for 60 min. After centrifugation, aliquots of 1/25 of each supernatant (S) or 1/4 of each pellet (P) were analyzed by SDS–PAGE and stained with Coomassie Blue. (**B**) Thrombin-cleaved Dab2(1–205) (lanes a–f) or GST (lanes g and h) was mixed with either control (lanes a and b) or phosphoinositide-containing (lanes c–j) liposomes and incubated on ice. After centrifugation, aliquots of 1/25 of each supernatant (S) or 1/4 of each pellet (P) were analyzed by SDS–PAGE and stained with Coomassie Blue or transferred to nitrocellulose. The blot was probed with an anti-Dab2 mAb. The asterisk denotes a stable ~25 kDa thrombin degradation product that binds phosphoinositides and reacts with the anti-Dab2 mAb 52. (**C**) Nitrocellulose-immobilized phosphoinositides (100, 50, 25, 12.5, 6.2, 3.1, 1.5 pmol/spot) were incubated with GST–Dab2(1–205) PTB domain and then bound protein visualized with GSH-derivatized HRP. (**D**) The PTB domain of Dab2 binds to phosphoinositides and FXNPXF internalization motifs synchronously. Thrombin-cleaved Dab2(1–205) (PTB domain) was added to 3 μM, together with 0.4 mg/ml phosphoinositide-containing liposomes alone (lanes a and b) or liposomes plus either 15 μM NWRLKNINSIFDNPVYQKTT (lanes c and d) or NWRLKNINSIFDAPVAQKTT (lanes e and f) peptide and incubated on ice for 60 min. After centrifugation, aliquots of 1/25 of each supernatant (S) or 1/4 of each pellet (P) were analyzed by SDS–PAGE and stained with Coomassie Blue. The bound peptide migrates at the dye front and the asterisk denotes the stable ~25 kDa Dab2 degradation product of the PTB domain.

cells display diffuse surface diI-LDL labeling with little or no evidence of accumulation within intracellular structures. These cells invariably express the myc-PTBx2 construct (Figure 7B–D), which also appears, at least in part, associated with the plasma membrane.

Importantly, the PTB domain only affects LDL receptor uptake, and does not produce a global perturbation in endocytic activity of transfected cells. PTBx2-expressing cells, unable to internalize diI-LDL during a 15 min pulse, nevertheless take up transferrin normally compared with the surrounding, untransfected cells (Figure 7E–H). These

results verify the capacity of the Dab2 PTB domain to engage the LDL receptor family F*X*NP*X*Y internalization motif *in vivo*.

Dab2 as an endocytic adaptor protein

We interpret our findings to indicate that the N-terminal PTB domain of Dab2 can engage both PtdIns(4,5)P$_2$ and select cargo molecules simultaneously. Concurrent AP-2 and clathrin association with the central portion of the protein might promote clathrin-coat assembly and drive cargo incorporation. To investigate this idea further, we
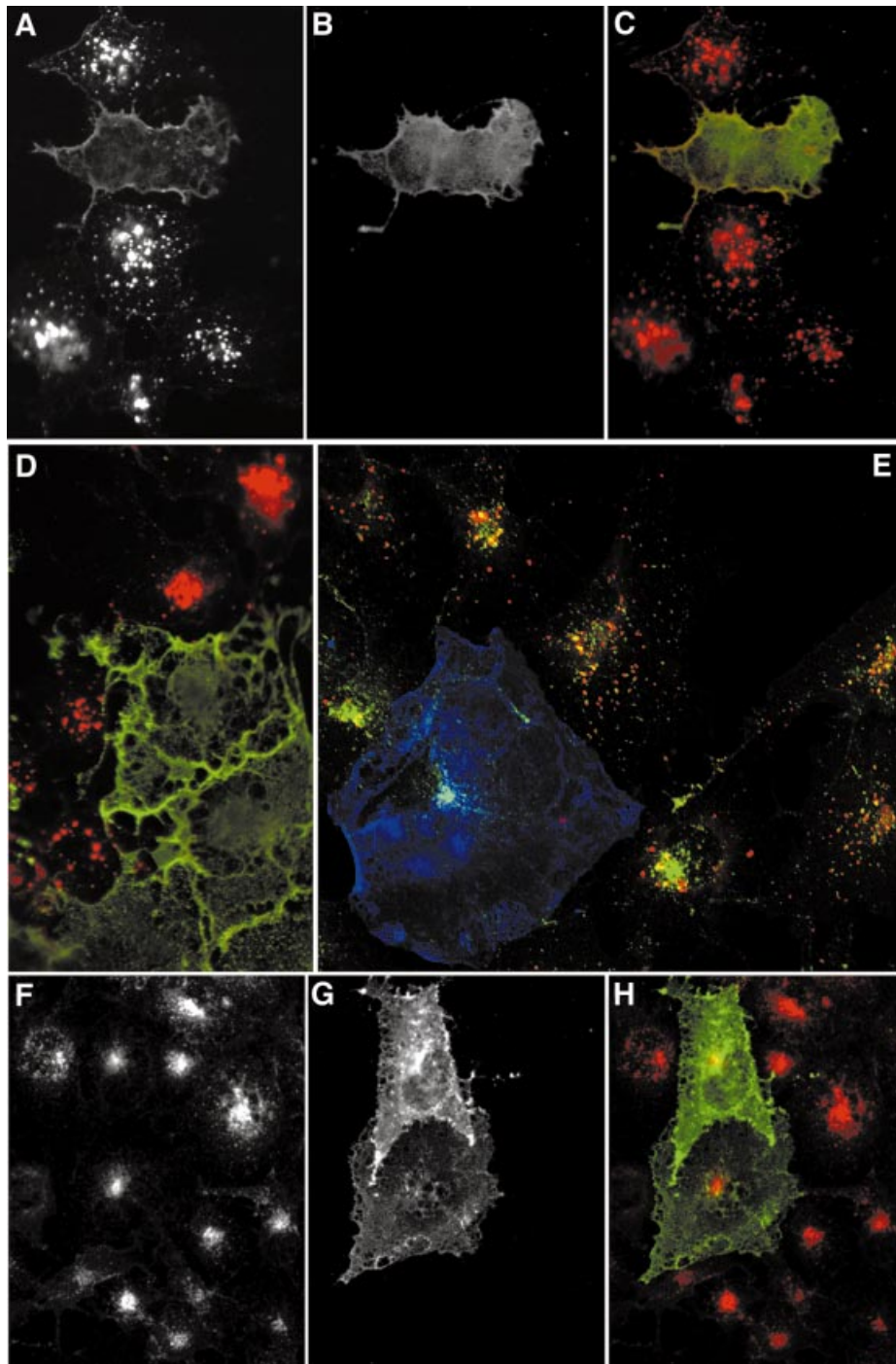
Fig. 7. Dab2 PTB domain overexpression selectively interferes with LDL uptake. COS-7 cells transiently transfected with myc-tagged Dab2 PTBx2 were pulsed for 15 min with 20 μg/ml diI-LDL alone (**A–D**), both diI-LDL and 25 μg/ml biotinylated transferrin (**E**) or transferrin alone (**F–H**) for 15 min prior to fixation. Transfected cells were identified using mAb 9E10 (B–D, G and H, in green in C, D and H) and transferrin with Alexa 488– (E) or Alexa 594–streptavidin (F and H). A single confocal section of triple-labeled cells is shown in (E) (myc epitope, blue; transferrin, green; LDL, red).

used the larger GST–Dab2 fusion encompassing both the PTB and coat machinery-binding segments. Like the PTB domain alone, GST–Dab2(1–368) binds efficiently to mixed liposomes containing low levels of phosphoinositides (see Figure 8C, lane f). Oriented in this way upon a 10% PtdIns(4,5)P$_2$-containing monolayer of mixed lipids (Ford *et al.*, 2001), the protein is able to recruit soluble clathrin and drive the formation of regular polyhedral structures (Figure 8A and B), which, alone, does not

associate with the monolayer (Figure 8A, inset, see C, lane b). Similar results are obtained in biochemical assays (Figure 8C, lanes a, b and e, f). Again, the clathrin-recruiting properties of Dab2 do not depend upon GST-mediated dimerization as monomeric Dab2(1–368), tethered to a mixed liposome surface, effectively recruits clathrin in a dose-dependent fashion (Figure 8D). These data confirm the direct clathrin-binding properties of Dab2 (Figures 2–4) and further reveal that the adjacent PTB

domain does not negate clathrin interactions. In this type of assay, purified AP-2 adaptors can also bind the liposomes (Ford *et al.*, 2001; Mishra *et al.*, 2001) (Figure 8C, lane d), due to a basic cluster of residues located at the N-terminus of the α-subunit (Gaidarov and Keen, 1999). Clathrin recruitment by the membrane-bound AP-2 is only weak, however (lane d) (Ford *et al.*, 2001; Mishra *et al.*, 2001). Pre-incubating liposomes

Fig. 8. Dab2 co-operates with AP-2 to drive the assembly of invaginated clathrin buds upon lipid membranes. Clathrin was added to 10% PtdIns(4,5)P$_2$-containing lipid monolayers pre-incubated in the absence (**A**, inset) or presence (A and **B**) of GST–Dab2(1–368). An EM grid was used to remove each monolayer and then negatively stained. Bar = 50 nm. (**C**) Phosphoinositide-containing liposomes were first pre-incubated with AP-2 (lanes c, d, g–j and m, n), GST–Dab2(1–368) (lanes e–j) or GST–epsin 1(1–407) (lanes k–n) at 4°C for 60 min as indicated. After recovery by centrifugation, each liposome pellet was resuspended and then incubated at 4°C for 60 min with purified clathrin trimers in the presence of carrier BSA. After centrifugation, aliquots of 1/40 of each supernatant (S) and 1/4 of each pellet (P) were resolved by SDS–PAGE and stained with Coomassie Blue. Before centrifugation, Triton X-100 (1% final) was added in one reaction (lanes i and j). (**D**) PtdIns(4,5)P$_2$-containing liposomes were first pre-incubated with (lanes a–j) or without (lanes k and l) thrombin-cleaved Dab2(1–368) at 4°C for 60 min. After recovery by centrifugation, each liposome pellet was resuspended and then incubated at 4°C for 60 min with increasing amounts of purified clathrin trimers in the presence of carrier BSA. After centrifugation, aliquots of 1/25 of each supernatant (S) and 1/5 of each pellet (P) were resolved by SDS–PAGE and stained with Coomassie Blue.

together with a mixture of AP-2 and the GST–Dab2 fusion increases the recovery of both proteins in the liposome pellet (Figure 8C, lane h), validating the AP-2–Dab2 interactions seen in pull-down assays (Figures 1 and 2) (Morris and Cooper, 2001). When clathrin is then added, substantially more (~50% of total) assembles onto the sedimenting liposomes (Figure 8C, lane h compared with d and f).

Approximately one-quarter of the liposome-bound coat components are resistant to extraction with 1% Triton X-100 (Figure 8C, lane j), which has been shown by others to correlate well with extensive assembly into curved polyhedral coats (Ford *et al.*, 2001). Both AP-2 and the GST–Dab2 fusion protein remain with the pelleted clathrin after Triton X-100 addition. The behavior of GST–Dab2 closely parallels that of a GST–epsin 1(1–407) fusion in these assays (Figure 8C, lanes k–n), showing that Dab2 can function analogously to known endocytic accessory proteins (Chen *et al.*, 1998; Yamabhai *et al.*, 1998; Drake *et al.*, 2000; Mishra *et al.*, 2001). Importantly, and in contrast to eps15 (Cupers *et al.*, 1998), extensive clathrin recruitment does not disrupt the interaction between AP-2 and either Dab2 or epsin (Figure 8) (Mishra *et al.*, 2001), AP180 (Ford *et al.*, 2001) or HIP1 (Mishra *et al.*, 2001), such that these proteins are now released from the membrane surface. The difference might reflect the fact that eps15 has no known intrinsic clathrin-binding sequence. The common properties of Dab2, epsin, AP180 and HIP1 suggest that they can play functionally similar roles during clathrin-coat assembly, distinct from eps15.

Discussion

There is little question that the EGF, transferrin and LDL receptors all utilize the same basic endocytic elements to enter the cell. All three receptors can be found in common endosomes (Ajioka and Kaplan, 1987) and, if clathrin-mediated endocytosis is grossly perturbed, as in stonin 2 overexpression, which prevents proper recruitment of AP-2 to the plasma membrane, transferrin, EGF and LDL internalization are all impaired (Martina *et al.*, 2001). Yet these three transmembrane proteins each appear to use
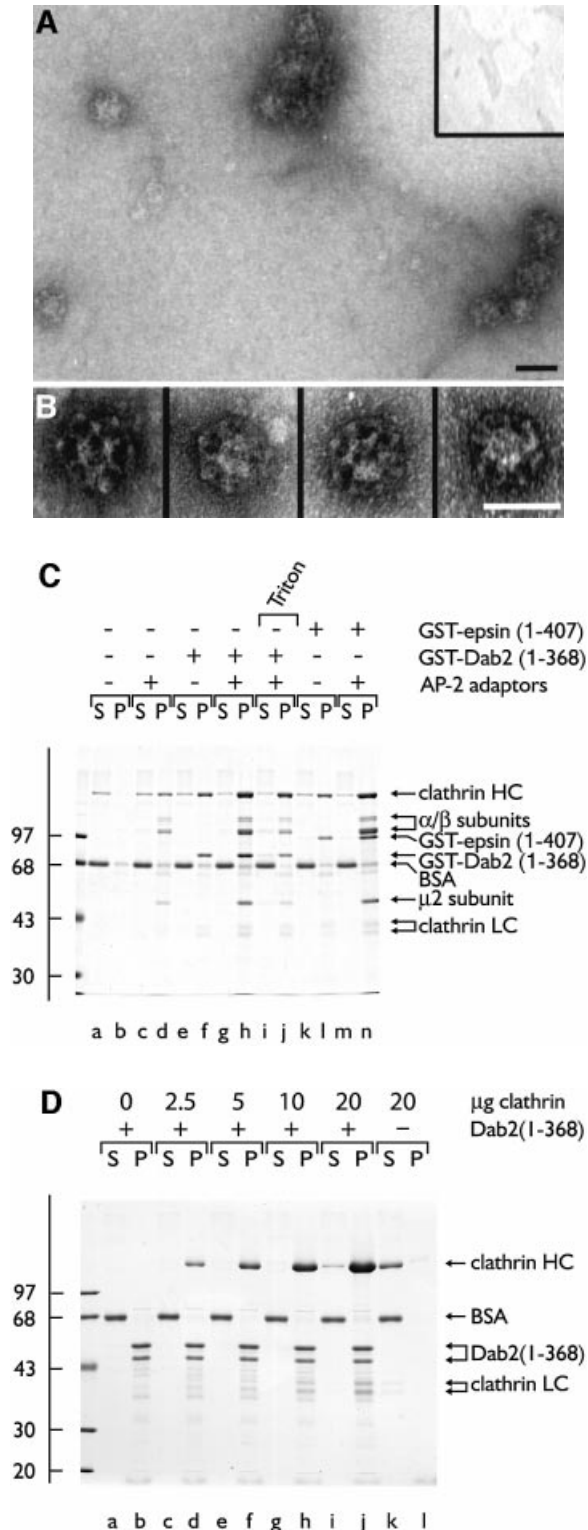
different mechanisms for selective entry into clathrin-coated buds. Four-fold overexpression of the LDL receptor in HeLa cells does not inhibit the rate of transferrin uptake (Warren *et al*., 1998) and cells from hypercholesterolemic patients with no mutations in the LDL receptor, apolipoprotein (apo) B or AP-2 μ2-subunit fail to internalize LDL while transferrin endocytosis proceeds normally (Norman *et al*., 1999). This suggests that LDL receptor uptake might depend on alternate endocytic machinery.

Dab2 and cargo recognition

A large body of data links the NP*XY* internalization sequence present in LDL receptor family members to PTB domain recognition. The PTB domains of Dab1 (Trommsdorff *et al*., 1998; Howell *et al*., 1999; Gotthardt *et al*., 2000) and the rodent CED-6 ortholog, GULP (Su *et al*., 2002), bind directly to NP*XY* sequence-bearing LDL receptor family members, and to amyloid precursor proteins (Trommsdorff *et al*., 1998; Homayouni *et al*., 1999; Howell *et al*., 1999), albeit with differing selectivity. Intact Dab2 associates directly with megalin (Oleinikov *et al*., 2000) and the isolated Dab2 PTB domain binds to LDL receptor family F*X*NP*XY* sequences (Morris and Cooper, 2001) (Figure 6). Compelling genetics ties Dab1, with ~65% identity to the Dab2 PTB domain, to the very low density lipoprotein (VLDL) receptor, apoE receptor-2 (apoER2), and the extracellular ligand reelin in the process of neural migration and spatial positioning in the brain (Cooper and Howell, 1999; Herz, 2001). Genetic disruption of both apoER2 and VLDL receptors in mice precisely phenocopies the mutant strains *reeler* (reelin defective) and *scrambler* (Dab1 defective) (Trommsdorff *et al*., 1999). The results indicate that a vectorial pathway of reelin→VLDL receptor/apoER2→Dab1 is vital for correct central nervous system formation.

Several types of mutation in the LDL receptor including Class 4, internalization defective due to alterations in the NP*XY* internalization sequence (Chen *et al*., 1990), cause familial hypercholesterolemia (Goldstein and Brown, 2001). Naturally occurring mutation of the major ligand, apoB, results in a similar, but milder, clinical phenotype termed familial defective apoB-100 (Goldstein and Brown, 2001). However, unlike the Dab1 signaling pathway paradigm, no mutations in Dab2 have been linked to hypercholesterolemia. This suggests that other PTB domain proteins might compensate or substitute for Dab2 loss, and thus Dab2 would not be singularly responsible for LDL receptor incorporation into clathrin-coated vesicles. This notion is also probable because Dab2 is not expressed ubiquitously, and many ovarian and breast carcinomas fail to express Dab2 at all (Sheng *et al*., 2000). siRNA knockdown of Dab2 levels by >90% in HeLa cells does not inhibit diI-LDL uptake (not shown). Proteins structurally related to Dab2 are, in fact, already known; numb is a PTB domain-containing protein that, like Dab2, binds to AP-2 and also exhibits several NPF triplets in the distal segment of the protein (Santolini *et al*., 2000). More interesting is the recent identification of the gene mutated in patients with a rare form of non-LDL receptor-dependent hypercholesterolemia (Garcia *et al*., 2001). Several different natural mutations in the encoded protein, ARH (<u>a</u>utosomal <u>r</u>ecessive <u>h</u>ypercholesterolemia), pheno-

copy LDL receptor-mutant familial hypercholesterolemia (Garcia *et al*., 2001). Primary sequence alignments of the ARH PTB domain show that numb, CED-6/GULP and Dab1/2 are the most closely related members of the PTB domain-containing superfamily, predicting that ARH will probably bind preferentially to non-phosphorylated NP*XY* motifs. It thus appears that several related PTB-type endocytic adaptors might function to cluster the LDL receptor and/or other NP*XY*-bearing proteins into clathrin-coated buds, possibly in tissue-specific combinations.

The phenotype of Dab2 nullizygous mice also supports a role for Dab2 in endocytosis. Targeted disruption of the Dab2 gene is lethal, but if expression is disrupted only in the embryo, the animals are viable and survive to adulthood (Morris *et al*., 2002b). These mice have a prominent kidney sorting dysfunction that mimics, in a milder form, megalin gene disruption (Morris *et al*., 2002b). The mice exhibit proteinuria and secrete several vitamin-binding proteins normally efficiently reabsorbed in the kidney proximal tubule by internalization of the scavenger receptor megalin within clathrin-coated vesicles (Morris *et al*., 2002b). Proximal tubules of Dab2–/– mice, like those of megalin-deficient animals, display decreased numbers of apical clathrin-coated pits and a generally diminished subapical endosome compartment (Leheste *et al*., 1999; Morris *et al*., 2002b). This links Dab2 to megalin endocytosis in the kidney *in vivo*. An additional link between Dab2 and clathrin-mediated endocytosis comes from the recent identification of myosin VI as a Dab2 interaction partner (Inoue *et al*., 2002; Morris *et al*., 2002a). Myosin VI is a component of some clathrin-coated vesicles (Buss *et al*., 2001) and Dab2 probably plays an important role in targeting this motor to the vesicle.

Overexpression of Dab1 in CHO cells results in ~2-fold more LDL receptor at the cell surface without affecting the rate of uptake (Gotthardt *et al*., 2000). The increased residency of the LDL receptor at the plasma membrane could be due to Dab1 PTB-mediated interference with receptor incorporation into endocytic coated buds, as Dab1 does not interface with the clathrin-coat machinery. This is in full accord with our demonstration that the PTBx2 potently, but selectively, prevents LDL uptake in COS cells. In addition to two NP*XY* motifs, LRP also contains a Y*XX*Ø sequence within the cytosolic domain that confers more rapid endocytosis than either the LDL, megalin, VLDL or apoER2 receptor cytosolic domain (Li *et al*., 2001). This suggests that NP*XY*-driven internalization might be augmented by also engaging the AP-2 complex at the bud site.

Dab2 as a member of a class of monomeric clathrin adaptors

As overexpression of a single Dab2 PTB domain has negligible effect on LDL uptake, we believe that Dab2 normally encounters F*X*NP*XY*-bearing cargo in the context of an assembling coated bud. The PtdIns(4,5)P$_2$-, AP-2- and clathrin-binding properties will facilitate Dab2 placement at the bud site, as do analogous determinants in AP180, epsin and HIP1 (Ford *et al*., 2001; Mishra *et al*., 2001). Although able to assemble clathrin cages *in vitro*, we do not contend that Dab2 plays an indispensable role in clathrin lattice assembly. Rather, like epsin and AP180, it potentiates AP-2-driven coat formation, perhaps also

determining vesicle size. Each of these proteins is likely to be found within a single forming bud (Figure 5). This distinguishes this group of proteins from β-arrestin, which, despite also containing PtdIns(4,5)P$_2$-, AP-2- and clathrin-binding determinants, appears to accompany heptahelical receptors into existing coated buds (Santini *et al.*, 2002; Scott *et al.*, 2002).

If the overall domain organization and binding properties of epsin, AP180, HIP1 and Dab2 predict a common function as dedicated endocytic sorting adaptors, what cargo might the other proteins be selecting? AP180 could actively cluster synaptobrevin/VAMP (Nonet *et al.*, 1999). A potential cargo for HIP1 is huntingtin, which interacts with the N-terminal segment of HIP1 (Wanker *et al.*, 1997) and has been localized to clathrin-coated structures and endosomes (Velier *et al.*, 1998). Epsin might be part of the long-sought-after machinery that recognizes ubiquitin as an endocytic signal (Polo *et al.*, 2002). Importantly, our identification of AP180, epsin, HIP1 and Dab2 as putative adaptors, each dedicated to selection of certain cargo to the bud site, clarifies our understanding of clathrin-coat assembly; we believe that these proteins can now be viewed as a general class of functionally similar cargo sorters.

Like epsin, Dab2 also contains multiple NPF triplets, the EH domain ligand (de Beer *et al.*, 2000). Mammalian EH domain-containing proteins include eps15, intersectin, POB-1 and EHD1 (Santolini *et al.*, 1999). Dab2 is, therefore, capable of establishing a complex web of protein–protein interactions at the clathrin bud site. These additional functional sequences argue that, like numb (Santolini *et al.*, 2000), Dab2 probably accompanies the clathrin-coated vesicle during budding and fission. These multifaceted connections may be a critical aspect of ensuring the fidelity of cargo incorporation into assembling coated buds.

Finally, Dab2 may participate in more sorting events than only those involving LDL receptor family members. The protein was initially identified as a prominent phosphoprotein accompanying colony-stimulating factor-1 (CSF-1) receptor activation (Xu *et al.*, 1995). The CSF-1 receptor utilizes ubiquitin as an endocytic signal (Lee *et al.*, 1999), but the NPF triplets in Dab2 could recruit eps15, which, like epsin, can bind ubiquitin directly (Polo *et al.*, 2002). Alternatively, the observed phosphorylation of Dab2 might possibly prevent incorporation into coated buds, favoring epsin/eps15 inclusion and possibly enhancing CSF-1 receptor internalization. Dab2 is also implicated in transforming growth factor β (TGF-β) receptor activation and downstream Smad activation (Hocevar *et al.*, 2001). Direct association of the Dab2 PTB domain with Smad2/3 has been reported (Hocevar *et al.*, 2001). Intriguingly, Smad2 and Smad3 activation by liganded TGF-β receptors occurs only post-endocytic internalization (Penheiter *et al.*, 2002), raising the possibility that Dab2, acting at the coated vesicle, coordinates aspects of TGF-β signaling.

Materials and methods

DNA constructs
Plasmids encoding murine Dab2 residues 206–350 and 206–492, each in pGSTag, were generously provided by Shelli Morris and Jonathon Cooper. The PTB domain of mouse Dab2 residues 1–205 [GST–Dab2(1–205)] was amplified using EST uf85g06.y1 (Research Genetics). GST–Dab2(1–368) was produced using the GST–Dab2(1–205) and GST–Dab2(206–492) plasmids as templates. The GST–epsin 1(1–163) ENTH domain was prepared using the rat epsin 1 cDNA provided by Pietro de Camilli and preparation of GST–epsin 1(1–407) has been described previously (Mishra *et al.*, 2001). All mutations were introduced into the appropriate vectors using the QuikChange (Stratagene) system with the required mutagenic primer pairs. All clones and mutations were verified by automated sequencing.

Antibodies
The monoclonal antibody (mAb) directed against a peptide from within the PTB domain of Dab2 (p96) was purchased from Transduction Laboratories. Anti-clathrin mAb TD.1 and Cl57.3, the AP-2 α-subunit mAb 100/2 and the affinity-purified rabbit anti-epsin antibodies have been described previously (Drake *et al.*, 2000). The rabbit anti-AP-2 μ2-subunit serum was generously provided by Juan Bonifacino and the anti-myc mAb 9E10 was from Covance.

Protein and peptide preparation
Synthetic peptides corresponding to amino acids 814–834 (NWRLKN-INSIFDNPVYQKTT) of the murine LDL receptor and a related peptide, NWRLKNINSIFD<u>A</u>PV<u>A</u>QKTT, were synthesized by the University of Pittsburgh peptide synthesis facility and both peptides purified by HPLC on a C-18 column to >98% purity. GST and the various GST fusion proteins, rat brain cytosol and purified AP-2 and clathrin were prepared exactly as described previously (Mishra *et al.*, 2001). Clathrin cages were prepared by first concentrating aliquots of the clathrin pool from the Sepharose CL-4B column by precipitation with 50% ammonium sulfate followed by dialysis overnight against 100 mM MES–OH pH 6.2, 0.5 mM magnesium chloride, 0.05% sodium azide and 3 mM calcium chloride. Assembled cages were collected by centrifugation at 42 000 r.p.m. in a TLA-55 rotor for 20 min at 4°C and resuspended gently in MES buffer on ice before use.

Binding assays
Pull-down-type assays, in 300 μl total volume containing 25 mM HEPES–KOH pH 7.2, 125 mM potassium acetate, 5 mM magnesium acetate, 2 mM EDTA, 2 mM EGTA, 1 mM DTT (assay buffer), were as described previously (Drake *et al.*, 2000; Drake and Traub, 2001). PTB domain binding to liposomes was performed using either 10% cholesterol, 45% PtdEth and 45% PtdCho, or 10% cholesterol, 30% PtdEth, 30% PtdCho and 30% phosphoinositides (Sigma), or 10% cholesterol, 35% PtdEth, 35% PtdCho 10% PtdSer and 10% PtdIns(4,5)P$_2$ prepared as described previously (Zhu *et al.*, 1999), at a final concentration of 0.4 mg/ml. Proteins were added in assay buffer with 0.1 mg/ml BSA as carrier and, after incubation at 4°C for 60 min, the tubes centrifuged at 20 000 g_{max} for 15 min at 4°C. Aliquots of each supernatant and pellet were analyzed by SDS–PAGE and immunoblotting where indicated. The two-stage liposome-binding assays were performed similarly, as described previously (Mishra *et al.*, 2001). Data quantification was with NIH Image 1.62.

The specificity of the Dab2 PTB domain was assayed using nitrocellulose-immobilized phospholipids (PIP-array; Echelon Research Laboratories). After blocking the nitrocelluose with 3% BSA in 10 mM Tris–HCl pH 7.5, 150 mM sodium chloride, 1 mM EDTA and 0.05% Tween-20, 1 μg/ml GST–Dab2(1–205) was added and, following a 30 min incubation at 25°C, 1 μg/ml GSH-derivatized horseradish peroxidase (HRP; Sigma) was added and the incubation continued for an additional 60 min. After thorough washing, the bound GST fusion was visualized by enhanced chemiluminescence. The monolayer binding assay upon a lipid mixture of 10% PtdIns(4,5)P$_2$, 10% PtdSer, 10% cholesterol, 35% PtdEth and 35% PtdCho was performed as described previously (Ford *et al.*, 2001).

Immunofluorescence analysis
HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine. Cells on glass coverslips were placed into assay buffer with 0.03% saponin at 25°C for 1 min before fixation in 3.7% formaldehyde in PBS. After permeabilization in 0.2% saponin, 10% normal goat serum in PBS, cells were incubated for 2–3 h at 25°C with anti-Dab2 and anti-epsin antibodies, followed by appropriate secondary reagents. Transfection of COS-7 cells with DEAE–dextran was as described previously (Mishra *et al.*, 2001), but with incubation in DMEM supplemented with 8% lipoprotein-deficient serum and 2 mM L-glutamine for ~40 h before LDL and transferrin uptake assays. After

fixation, cells were permeablized in 0.05% saponin, 10% normal goat serum in PBS.

Acknowledgements

References

Ajioka,R.S. and Kaplan,J. (1987) Characterization of endocytic compartments using the horseradish peroxidase–diaminobenzidine density shift technique. *J. Cell Biol.*, **104**, 77–85.

Arneson,L.S., Kunz,J., Anderson,R.A. and Traub,L.M. (1999) Coupled inositide phosphorylation and phospholipase D activation initiates clathrin-coat assembly on lysosomes. *J. Biol. Chem.*, **274**, 17794–17805.

Bonifacino,J.S. and Dell'Angelica,E.C. (1999) Molecular bases for the recognition of tyrosine-based sorting signals. *J. Cell Biol.*, **145**, 923–926.

Brett,T.J., Traub,L.M. and Fremont,D.H. (2002) Accessory protein recruitment motifs in clathrin-mediated endocytosis. *Structure*, **10**, 797–809.

Buss,F., Arden,S.D., Lindsay,M., Luzio,J.P. and Kendrick-Jones,J. (2001) Myosin VI isoform localized to clathrin-coated vesicles with a role in clathrin-mediated endocytosis. *EMBO J.*, **20**, 3676–3684.

Chen,H., Fre,S., Slepnev,V.I., Capua,M.R., Takei,K., Butler,M.H., Di Fiore,P.P. and De Camilli,P. (1998) Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature*, **394**, 793–797.

Chen,W.J., Goldstein,J.L. and Brown,M.S. (1990) NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.*, **265**, 3116–3123.

Cooper,J.A. and Howell,B.W. (1999) Lipoprotein receptors: signaling functions in the brain? *Cell*, **97**, 671–674.

Cupers,P., Jadhav,A.P. and Kirchhausen,T. (1998) Assembly of clathrin coats disrupts the association between Eps15 and AP-2 adaptors. *J. Biol. Chem.*, **273**, 1847–1850.

deBeer,T., Hoofnagle,A.N., Enmon,J.L., Bowers,R.C., Yamabhai,M., Kay,B.K. and Overduin,M. (2000) Molecular mechanism of NPF recognition by EH domains. *Nat. Struct. Biol.*, **7**, 1018–1022.

Dell'Angelica,E.C. (2001) Clathrin-binding proteins: got a motif? Join the network! *Trends Cell Biol.*, **11**, 315–318.

Dho,S.E., French,M.B., Woods,S.A. and McGlade,C.J. (1999) Characterization of four mammalian numb protein isoforms. Identification of cytoplasmic and membrane-associated variants of the phosphotyrosine binding domain. *J. Biol. Chem.*, **274**, 33097–33104.

Drake,M.T. and Traub,L.M. (2001) Interaction of two structurally-distinct sequence types with the clathrin terminal domain β-propeller. *J. Biol. Chem.*, **276**, 28700–28709.

Drake,M.T., Downs,M.A. and Traub,L.M. (2000) Epsin binds to clathrin by associating directly with the clathrin-terminal domain: evidence for cooperative binding through two discrete sites. *J. Biol. Chem.*, **275**, 6479–6489.

Ford,M.G., Pearse,B.M., Higgins,M.K., Vallis,Y., Owen,D.J., Gibson,A., Hopkins,C.R., Evans,P.R. and McMahon,H.T. (2001) Simultaneous binding of PtdIns(4,5)P_2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science*, **291**, 1051–1055.

Gaidarov,I. and Keen,J.H. (1999) Phosphoinositide–AP-2 interactions required for targeting to plasma membrane clathrin-coated pits. *J. Cell Biol.*, **146**, 755–764.

Garcia,C.K. *et al.* (2001) Autosomal recessive hypercholesterolemia caused by mutations in a putative LDL receptor adaptor protein. *Science*, **292**, 1394–1398.

Gillooly,D.J., Morrow,I.C., Lindsay,M., Gould,R., Bryant,N.J., Gaullier,J.M., Parton,R.G. and Stenmark,H. (2000) Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J.*, **19**, 4577–4588.

Goldstein,J.L. and Brown,M.S. (2001) Molecular medicine. The cholesterol quartet. *Science*, **292**, 1310–1312.

Goodman,O.B.,Jr, Krupnick,J.G., Santini,F., Gurevich,V.V., Penn,R.B., Gagnon,A.W., Keen,J.H. and Benovic,J.L. (1996) β-arrestin acts as a clathrin adaptor in endocytosis of the β2-adrenergic receptor. *Nature*, **383**, 447–450.

Gotthardt,M., Trommsdorff,M., Nevitt,M.F., Shelton,J., Richardson,J.A., Stockinger,W., Nimpf,J. and Herz,J. (2000) Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. *J. Biol. Chem.*, **275**, 25616–25624.

Herz,J. (2001) The LDL receptor gene family: (un)expected signal transducers in the brain. *Neuron*, **29**, 571–581.

Hocevar,B.A., Smine,A., Xu,X.X. and Howe,P.H. (2001) The adaptor molecule Disabled-2 links the transforming growth factor β receptors to the Smad pathway. *EMBO J.*, **20**, 2789–2801.

Homayouni,R., Rice,D.S., Sheldon,M. and Curran,T. (1999) Disabled-1 binds to the cytoplasmic domain of amyloid precursor-like protein 1. *J. Neurosci.*, **19**, 7507–7515.

Howell,B.W., Lanier,L.M., Frank,R., Gertler,F.B. and Cooper,J.A. (1999) The disabled 1 phosphotyrosine-binding domain binds to the internalization signals of transmembrane glycoproteins and to phospholipids. *Mol. Cell. Biol.*, **19**, 5179–5188.

Inoue,A., Sato,O., Homma,K. and Ikebe,M. (2002) DOC-2/DAB2 is the binding partner of myosin VI. *Biochem. Biophys. Res. Commun.*, **292**, 300–307.

Itoh,T., Koshiba,S., Kigawa,T., Kikuchi,A., Yokoyama,S. and Takenawa,T. (2001) Role of the ENTH domain in phosphatidyl-inositol-4,5-bisphosphate binding and endocytosis. *Science*, **291**, 1047–1051.

Kalthoff,C., Alves,J., Urbanke,C., Knorr,R. and Ungewickell,E.J. (2002) Unusual structural organization of the endocytic proteins AP180 and epsin 1. *J. Biol. Chem.*, **277**, 8209–8216.

Laporte,S.A., Oakley,R.H., Zhang,J., Holt,J.A., Ferguson,S.S., Caron, M.G. and Barak,L.S. (1999) The β2-adrenergic receptor/β-arrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc. Natl Acad. Sci. USA*, **96**, 3712–3717.

Laporte,S.A., Oakley,R.H., Holt,J.A., Barak,L.S. and Caron,M.G. (2000) The interaction of β-arrestin with the AP-2 adaptor is required for the clustering of β2-adrenergic receptor into clathrin-coated pits. *J. Biol. Chem.*, **275**, 23120–23126.

Lee,P.S., Wang,Y., Dominguez,M.G., Yeung,Y.G., Murphy,M.A., Bowtell,D.D. and Stanley,E.R. (1999) The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis and attenuates macrophage proliferation. *EMBO J.*, **18**, 3616–3628.

Leheste,J.R. *et al.* (1999) Megalin knockout mice as an animal model of low molecular weight proteinuria. *Am. J. Pathol.*, **155**, 1361–1370.

Li,Y., Lu,W., Marzolo,M.P. and Bu,G. (2001) Differential functions of members of the low density lipoprotein receptor family suggested by their distinct endocytosis rates. *J. Biol. Chem.*, **276**, 18000–18006.

Margolis,B. (1996) The PI/PTB domain: a new protein interaction domain involved in growth factor receptor signaling. *J. Lab. Clin. Med.*, **128**, 235–241.

Martina,J.A., Bonangelino,C.J., Aguilar,R.C. and Bonifacino,J.S. (2001) Stonin 2: an adaptor-like protein that interacts with components of the endocytic machinery. *J. Cell Biol.*, **153**, 1111–1120.

Miller,W.E. and Lefkowitz,R.J. (2001) Expanding roles for β-arrestins as scaffolds and adapters in GPCR signaling and trafficking. *Curr. Opin. Cell Biol.*, **13**, 139–145.

Mishra,S.K., Agostinelli,N.R., Brett,T.J., Mizukami,I., Ross,T.S. and Traub,L.M. (2001) Clathrin- and AP-2-binding sites in HIP1 uncover a general assembly role for endocytic accessory proteins. *J. Biol. Chem.*, **276**, 46230–46236.

Morris,S.M. and Cooper,J.A. (2001) Disabled-2 colocalizes with the LDLR in clathrin-coated pits and interacts with AP-2. *Traffic*, **2**, 111–123.

Morris,S.M., Arden,S.D., Roberts,R.C., Kendrick-Jones,J., Cooper,J.A., Luzio,J.P. and Buss,F. (2002a) Myosin VI binds to and localises with Dab2, potentially linking receptor-mediated endocytosis and the actin cytoskeleton. *Traffic*, **3**, 331–341.

Morris,S.M., Tallquist,M.D., Rock,C.O. and Cooper,J.A. (2002b) Dual roles for the Dab2 adaptor protein in embryonic development and kidney transport. *EMBO J.*, **21**, 1555–1564.

Nesterov,A., Carter,R.E., Sorkina,T., Gill,G.N. and Sorkin,A. (1999)

Inhibition of the receptor-binding function of clathrin adaptor protein AP-2 by dominant-negative mutant μ2 subunit and its effects on endocytosis. *EMBO J.*, **18**, 2489–2499.

Nonet,M.L. *et al.* (1999) UNC-11, a *Caenorhabditis elegans* AP180 homologue, regulates the size and protein composition of synaptic vesicles. *Mol. Biol. Cell*, **10**, 2343–2360.

Norman,D., Sun,X.M., Bourbon,M., Knight,B.L., Naoumova,R.P. and Soutar,A.K. (1999) Characterization of a novel cellular defect in patients with phenotypic homozygous familial hypercholesterolemia. *J. Clin. Invest.*, **104**, 619–628.

Ohno,H.,J.,S., Fournier,M.-C., Bosshart,H., Rhee,I., Miyatake,S., Saito,T., Galluser,A., Kirchhausen,T. and Bonifacino,J.S. (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science*, **269**, 1872–1875.

Oleinikov,A.V., Zhao,J. and Makker,S.P. (2000) Cytosolic adaptor protein Dab2 is an intracellular ligand of endocytic receptor gp600/megalin. *Biochem. J.*, **347 Pt 3**, 613–621.

Owen,D.J. and Evans,P.R. (1998) A structural explanation for the recognition of tyrosine-based endocytotic signals. *Science*, **282**, 1327–1332.

Penheiter,S.G., Mitchell,H., Garamszegi,N., Edens,M., Dore,J.J.,Jr and Leof,E.B. (2002) Internalization-dependent and -independent requirements for transforming growth factor β receptor signaling via the Smad pathway. *Mol. Cell. Biol.*, **22**, 4750–4759.

Polo,S., Sigismund,S., Faretta,M., Guidi,M., Capua,M.R., Bossi,G., Chen,H., De Camilli,P. and Di Fiore,P.P. (2002) A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature*, **416**, 451–455.

Ramjaun,A.R. and McPherson,P.S. (1998) Multiple amphiphysin II splice variants display differential clathrin binding: identification of two distinct clathrin-binding sites. *J. Neurochem.*, **70**, 2369–2376.

Ravichandran,K.S., Zhou,M.M., Pratt,J.C., Harlan,J.E., Walk,S.F., Fesik,S.W. and Burakoff,S.J. (1997) Evidence for a requirement for both phospholipid and phosphotyrosine binding via the Shc phosphotyrosine-binding domain *in vivo*. *Mol. Cell. Biol.*, **17**, 5540–5549.

Santini,F., Gaidarov,I. and Keen,J.H. (2002) G protein-coupled receptor/arrestin3 modulation of the endocytic machinery. *J. Cell Biol.*, **156**, 665–676.

Santolini,E., Salcini,A.E., Kay,B.K., Yamabhai,M. and Di Fiore,P.P. (1999) The EH network. *Exp. Cell Res.*, **253**, 186–209.

Santolini,E., Puri,C., Salcini,A.E., Gagliani,M.C., Pelicci,P.G., Tacchetti,C. and Di Fiore,P.P. (2000) Numb is an endocytic protein. *J. Cell Biol.*, **151**, 1345–1352.

Scott,M.G., Benmerah,A., Muntaner,O. and Marullo,S. (2002) Recruitment of activated G protein-coupled receptors to pre-existing clathrin-coated pits in living cells. *J. Biol. Chem.*, **277**, 3552–3559.

Sheng,Z., Sun,W., Smith,E., Cohen,C. and Xu,X.X. (2000) Restoration of positioning control following Disabled-2 expression in ovarian and breast tumor cells. *Oncogene*, **19**, 4847–4854.

Slepnev,V.I., Ochoa,G.C., Butler,M.H. and De Camilli,P. (2000) Tandem arrangement of the clathrin and AP-2 binding domains in amphiphysin 1 and disruption of clathrin coat function mediated by amphiphysin fragments comprising these sites. *J. Biol. Chem.*, **275**, 17583–17589.

Su,H.P., Nakada-Tsukui,K., Tosello-Trampont,A.C., Li,Y., Bu,G., Henson,P.M. and Ravichandran,K.S. (2002) Interaction of CED-6/GULP, an adapter protein involved in engulfment of apoptotic cells, with CED-1 and CD91/LRP. *J. Biol. Chem.*, **277**, 11772–11779.

Takeuchi,H., Matsuda,M., Yamamoto,T., Kanematsu,T., Kikkawa,U., Yagisawa,H., Watanabe,Y. and Hirata,M. (1998) PTB domain of insulin receptor substrate-1 binds inositol compounds. *Biochem. J.*, **334**, 211–218.

Trommsdorff,M., Borg,J.P., Margolis,B. and Herz,J. (1998) Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *J. Biol. Chem.*, **273**, 33556–33560.

Trommsdorff,M., Gotthardt,M., Hiesberger,T., Shelton,J., Stockinger,W., Nimpf,J., Hammer,R.E., Richardson,J.A. and Herz,J. (1999) Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. *Cell*, **97**, 689–701.

Velier,J., Kim,M., Schwarz,C., Kim,T.W., Sapp,E., Chase,K., Aronin,N. and DiFiglia,M. (1998) Wild-type and mutant huntingtins function in vesicle trafficking in the secretory and endocytic pathways. *Exp. Neurol.*, **152**, 34–40.

Wanker,E.E., Rovira,C., Scherzinger,E., Hasenbank,R., Walter,S., Tait,D., Colicelli,J. and Lehrach,H. (1997) HIP-I: a huntingtin interacting protein isolated by the yeast two-hybrid system. *Hum. Mol. Genet.*, **6**, 487–495.

Warren,R.A., Green,F.A., Stenberg,P.E. and Enns,C.A. (1998) Distinct saturable pathways for the endocytosis of different tyrosine motifs. *J. Biol. Chem.*, **273**, 17056–17063.

Xu,X.X., Yang,W., Jackowski,S. and Rock,C.O. (1995) Cloning of a novel phosphoprotein regulated by colony-stimulating factor 1 shares a domain with the *Drosophila* disabled gene product. *J. Biol. Chem.*, **270**, 14184–14191.

Yamabhai,M., Hoffman,N.G., Hardison,N.L., McPherson,P.S., Castagnoli,L., Cesareni,G. and Kay,B.K. (1998) Intersectin, a novel adaptor protein with two Eps15 homology and five Src homology 3 domains. *J. Biol. Chem.*, **273**, 31401–31407.

Zhu,Y., Drake,M.T. and Kornfeld,S. (1999) ADP-ribosylation factor 1 dependent clathrin-coat assembly on synthetic liposomes. *Proc. Natl Acad. Sci. USA*, **96**, 5013–5018.