The EMBO Journal Vol.17 No.8 pp.2148–2155, 1998

Dileucine-based sorting signals bind to the β chain of AP-1 at a site distinct and regulated differently from the tyrosine-based motif-binding site

Iris Rapoport, Yu Chen Chen[1], Philippe Cupers, Steven E.Shoelson[1] and Tomas Kirchhausen[2]

Department of Cell Biology, Harvard Medical School and The Center for Blood Research and [1]Joslin Diabetes Center and Department of Medicine, Harvard Medical School, Boston, MA 02115, USA

[2]Corresponding author
e-mail: kirchhausen@crystal.harvard.edu

In previous work, we showed that peptides from endocytosed proteins containing the tyrosine YXXφ sorting motif are recognized by the μ2 subunit of AP-2, the plasma membrane clathrin adaptor protein complex. This interaction is activated by phosphoinositide lipids that are phosphorylated at the D-3 position of the inositol ring, and is also enhanced by the formation of clathrin–AP-2 coats. Here, we describe the detection of a specific interaction between peptides containing a second sorting motif, the dileucine motif, and AP-1, the clathrin adaptor complex responsible for sorting proteins at the *trans*-Golgi network (TGN). Surprisingly, the site of dileucine binding is the β1 subunit, not μ1. A YXXφ-containing peptide from a protein trafficked within the TGN does bind to μ1, however. Phosphatidylinositol 3,4-diphosphate and 3,4,5-triphosphate did not activate the interaction between dileucine-containing peptides and AP-1 but instead inhibited it, and clathrin–AP-1 coat formation did not alter the interaction. Thus, there are at least two physically separate binding sites for sorting signals on APs, which are also regulated independently.

Keywords: coated vesicles/endocytosis/exocytosis/receptor sorting

Introduction

Vesicular traffic of membrane-bound proteins requires a set of recognition processes that differentiate the proteins to be moved from those that should remain in place. The best understood of these sorting processes is the incorporation of transmembrane proteins into clathrin-coated vesicles. The membrane proteins that form the cargo associate with clathrin coats through the recognition of short, variable cytoplasmic motifs by the clathrin adaptor protein (AP) complexes. Most sorted proteins contain sorting signals such as the tyrosine-based YXXφ motif (where X is any amino acid and φ has a bulky hydrophobic side chain) or the dileucine motif (reviewed recently in Trowbridge *et al*., 1993; Sandoval and Bakke, 1994; Kirchhausen *et al*., 1997; Marks *et al*., 1997). These observations hint at the biochemical mechanism of sorting: the clathrin AP complexes appear to bind directly to cytoplasmic tails containing these motifs (Pearse, 1988; Glickman *et al*., 1989; Sorkin and Carpenter, 1993; Sosa *et al*., 1993; Boll *et al*., 1995, 1996; Ohno *et al*., 1995; Sorkin *et al*., 1995; Heilker *et al*., 1996), selecting the protein for inclusion into the vesicle. We previously used photoactivated cross-linking to demonstrate the interaction between YXXφ-containing peptides and the μ2 chain of purified AP-2 complexes (the complexes responsible for plasma membrane sorting) (Rapoport *et al*., 1997). We also showed that this interaction is enhanced by the phosphoinositides phosphatidylinositol 3,4-diphosphate (PtdIns 3,4-P_2) and phosphatidylinositol 3,4,5-triphosphate (PtdIns 3,4,5-P_3), raising the possibility that internalization of this class of transmembrane proteins containing the YXXφ sorting signal can be modulated by signal transduction events, and that cross-linking is also enhanced by the formation of clathrin–AP-2 coats, suggesting that coat formation may 'trap' the membrane protein within the coated pit. There are other types of sorting signals, however; another common motif (the dileucine motif) contains a doublet of leucine residues within the cytosolic tail. Selective interactions between APs and peptides containing the dileucine motif have been harder to demonstrate.

We initially attempted to use the same cross-linking protocol to examine the interaction between the dileucine motif-containing peptides and APs, but found that it was difficult to detect specific binding. Here we describe results using a modified protocol in which samples were first frozen, and cross-linking was activated with the samples still in the frozen state. This technique allowed us to detect a specific interaction between dileucine-containing peptides and AP-1, the complex responsible for sorting proteins at the *trans*-Golgi network (TGN). Although we do not fully understand how this procedure enhances the signal, we believe that during the freezing process phase separation leads to high local concentrations of the AP-1 complexes and the peptides. In this sense, the freezing process may compensate to some extent for the fact that the binding event is occurring in solution, and not in the plane of the membrane.

Surprisingly, the site of dileucine binding is the β1 subunit, not μ1, whereas a YQTI-containing peptide from Lamp-1, a protein trafficked within the TGN, does bind to μ1. PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3 did not activate the interaction between dileucine-containing peptides and AP-1 but instead inhibited it, and the formation of clathrin–AP-1 coats did not have any effect on binding. Thus, the two binding sites for sorting signals on APs are physically separate and independently regulated. The emerging picture of membrane protein sorting is that the interactions between the sorting motif and the AP complexes 'filter' the membrane proteins that pass into the different membrane compartments of the cell. Our results suggest that the selectivity and efficiency of these filters are modulated in a complex way.

peptides:

TGN38-YQRL	KVTRRPKASD**YQRL**
TGN38-AQRL	KVTRRPKASD**AQRL**
Lamp1-YQTI	RKRSHAG**YQTI**
Lamp1-AQTI	RKRSHAG**AQTI**
CD3γ-LL	RQSRApSDKQT**LL**PN
CD3γ-AA	RQSRApSDKQT**AA**PN
CD4-LL	RMpSQIKR**LL**SEK
CD4-AA	RMpSQIKR**AA**SEK
GLUT4-LL	ISAAFRRTPS**LL**EQEVKPSTEL
M6PR	VSFHDDSDED**LL**HI
***TGN38-YQRL**	**biotin-**KVTRRPK(**benzoylphenylalanine**)SD**YQRL**
***Lamp1-YQTI**	**biotin-**KKRS(**benzoylphenylalanine**)AG**YQTI**
***CD3γ-LL**	**biotin-**KQS(**benzoylphenylalanine**)ApSDKQT**LL**PN

Fig. 1. Schematic representation of the synthetic peptides containing LL and YXXϕ sorting signals used in the study. The indicated YXXϕ and LL motifs have been defined in previous studies by analyzing the effect of point mutations on the traffic of these selected transmembrane proteins. The sequences represent portions of the corresponding cytoplasmic tails that surround these motifs. pS = phosphoserine.

Results

Dileucine motifs are important in the traffic of a wide variety of membrane proteins, including the invariant chain of MHC class II proteins, LIMP-II, a lysosomal protein, and VP165, a protein that co-purifies with vesicles containing the Glut4 glucose transporter. We studied dileucine motifs derived from four distinct proteins (Figure 1): CD3γ, the subunit of the T-cell receptor in which the dileucine motif was first identified (Letourneur and Klausner, 1992); CD4, the T-cell co-receptor, which is down-regulated upon T-cell activation and also in human immunodeficiency virus (HIV) infection (Aiken *et al*., 1994); Glut4, the glucose transporter, which traffics in response to insulin stimulation and which accumulates in secretory vesicles directly from the TGN (James and Piper, 1994; Verhey and Birnbaum, 1994); and M6PR, the cation-independent mannose-6-phosphate receptor, which delivers mannose-6-phosphate-tagged proteins to the lysosome (Johnson *et al*., 1990).

The freezing protocol does not affect the specificity of AP-2–peptide interactions

As a control for changes in binding due to freezing, we studied the interaction between the photoreactive peptide *TGN38-YQRL (Rapoport *et al*., 1997) bearing the tyrosine-based YQRL endocytic motif of the cytosolic tail of TGN38 and the μ2 chain of AP-2 (Figure 2A). Freezing increases the efficiency of cross-linking but has no effect on the specificity of this interaction. We have shown previously that the YQRL motif interacts with AP-2 and not AP-1 (Rapoport *et al*., 1997). This selectivity is maintained using the freezing protocol (Figure 2B). Some background cross-linking can be seen between the tyrosine-containing peptide and most of the subunits of AP-2, both with and without freezing. Under the freezing protocol, this background increases, but the only specific (competable) band is the cross-linked μ2 chain. It is difficult to measure IC_{50}s with confidence using these conditions, however, since freezing probably results in areas of frozen liquid in which the concentrations of peptide and AP complex are high relative to those of the starting solution.

The β1 chain is the subunit responsible for dileucine motif recognition in AP-1

A photoreactive peptide *CD3γ-LL, corresponding to a 13 amino acid segment spanning from Q122 to P134 of CD3γ and including the dileucine motif, cross-links to the AP-1 complex under freezing conditions (Figure 3A, lane 3). The labeled ~100 kDa component of AP-1 was identified readily as the β1 chain by Western blot (lane 2). Tryptic digestion of the cross-linked AP-1 complex (Kirchhausen *et al*., 1989; Schroder and Ungewickell, 1991) localized the binding site to the ~63 kDa N-terminal trunk domain of β-1 (Figure 3B, lane 4). Only weak non-competable cross-linking of the *CD3γ-LL peptide to AP-2 complexes was seen, on only one subunit (the β2 chain) (Figure 3A, lane 4, and C, lanes 9 and 10).

The interaction between β1 and *CD3γ-LL is specific, since addition of the corresponding non-photoactivatable CD3γ-LL peptide to the cross-linking reaction blocks labeling of β1 in a dose-dependent manner (Figure 3C, lanes 3–6). In contrast, addition of the CD3γ-AA peptide, containing a double alanine instead of the double leucine [a mutation known to prevent CD3γ sorting (Letourneur and Klausner, 1992)], fails to inhibit the cross-linking reaction by *CD3γ-LL (Figure 3C, lane 2). The CD3γ-LL peptides used here all carry a phosphoserine at position –5. There is some evidence that phosphorylation on this serine enhances the down-regulation of the CD3γ chain (Dietrich *et al*., 1994); however, in these *in vitro* assays, using short peptides, we see no difference in AP-1 binding due to phosphorylation (data not shown). A similar lack of dependence on phosphorylation has been described for the *in vitro* recruitment of APs from cytosol by beads covalently linked to CD3γ peptides (Dietrich *et al*., 1997).

The dileucine-binding site does not overlap with the tyrosine-binding site on AP-1

To investigate whether the tyrosine motif-binding site on AP-1 is distinct from the dileucine-binding site, we examined the interaction between AP-1 and the tyrosine-based motif present in Lamp-1 (YQTI). This motif is believed to be recognized preferentially by AP-1, and the interaction with AP-1 is postulated to be responsible for the traffic of Lamp-1 between the TGN and the endosomal/

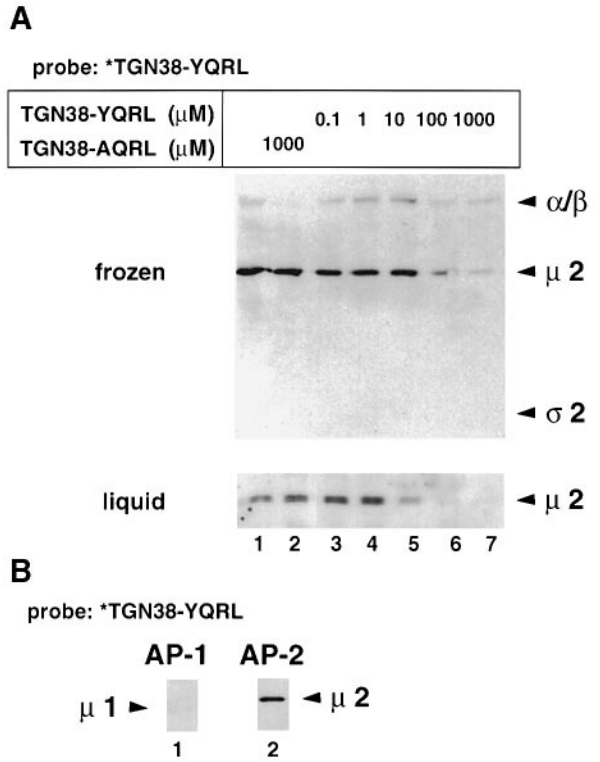

Fig. 2. The specificity of TGN38-YQRL for μ1 and μ2 is not affected by freezing. (**A**) AP-2 complexes (~0.2 mg/ml) purified from brain coated vesicles were incubated with 0.2 μM of photoreactive cross-linking peptide [*TGN38-YQRL: biotin-KVTRRPK(benzoylphenyl-alanine)SD**YQRL**] for 20 min at 4°C in total darkness in the absence (lane 1) or presence of the peptides TGN38-AQRL (KVTRRPK-ASD**AQRL**) at 1000 μM (lane 2) or TGN38-YQRL (KVTRRPK-ASD**YQRL**) at concentrations of 0.1, 1, 10, 100 and 1000 μM (lanes 3–7). Samples (in a final volume of 20 μl) frozen on dry ice (frozen) or held at 4°C (liquid) were exposed to UV light for 3 min to trigger the cross-linking reaction, then fractionated by SDS–PAGE, transferred to nitrocellulose and blotted with streptavidin–HRP. Cross-linked species were detected by enhanced chemiluminescence. The interaction with *TGN38-YQRL is specific, since cross-linking to μ2 is inhibited by co-incubation with the non-modified TGN38-YQRL peptide but not by the mutant peptide TGN38-AQRL. (**B**) Equivalent amounts (0.2 mg/ml) of AP-1 and AP-2 complexes were incubated with *TGN38-YQRL for 20 min at 4°C in the dark. After freezing, photocross-linking was triggered as above. The freezing protocol does not alter the specificity of the interaction: YQRL is known to bind to μ2 better than to μ1 in solution, and this pattern is maintained here.

lysosomal compartment (Honing *et al*., 1996; Rohrer *et al*., 1996). The peptide *Lamp1-YQTI with the complete 11 amino acid cytoplasmic tail of Lamp-1 preferentially cross-links to μ1 of AP-1 rather than to μ2 of AP-2 (Figure 4A, lanes 1 and 9), consistent with the *in vivo* trafficking observations. Labeling of μ1 is also specific since it is blocked by the non-modified peptide Lamp1-YQTI (Figure 4A, lanes 3–7) but not by the mutant peptide Lamp1-AQTI (the change from tyrosine to alanine is known to prevent Lamp-1 sorting) (Figure 4A, lane 2). Thus, there may be 'AP-1 directed' tyrosine-based signals that interact more strongly with AP-1 than with AP-2. We next examined whether the Lamp1-YQTI peptide could block the interaction of *CD3γ-LL to β1 of AP-1 (Figure 4B, lane 1). No inhibition of binding was observed, suggesting that the AP-1-binding sites for these peptides, present on μ1 and β1 respectively, do not overlap.

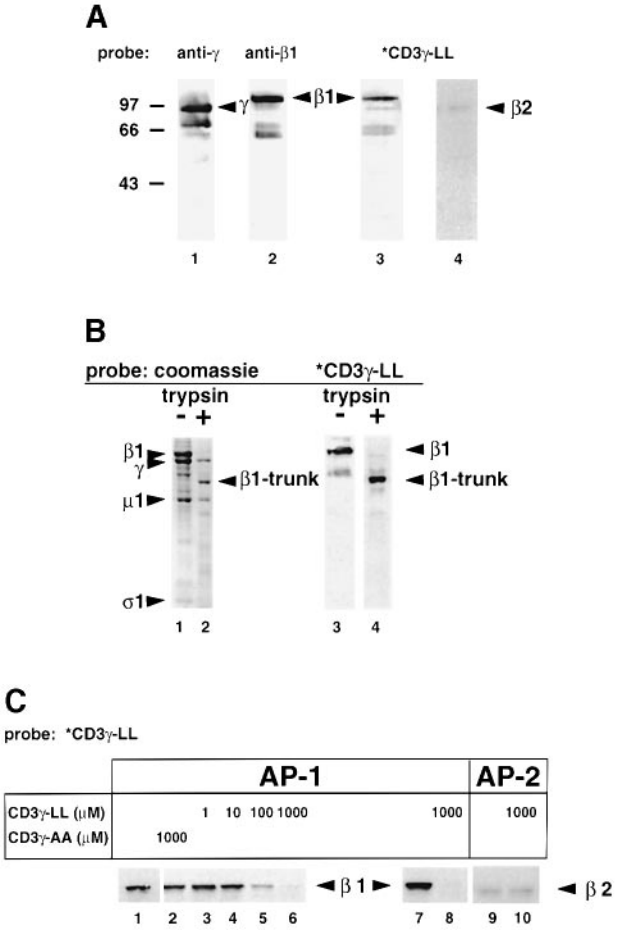

Fig. 3. The N-terminal trunk of β1 is the specific target for *CD3γ-LL in AP-1 complexes. Frozen solutions of AP-1 or AP-2 complexes (~0.2 mg/ml) were exposed to UV light for 12 min in the presence of 0.2 μM *CD3γ-LL [biotin-KQS(benzoylphenylalanine)ApSDKQ-T**LL**PN] to demonstrate the specific labeling of the β1 chain in AP-1. Peptides carrying phosphorylated serine were produced to mimic the motif that previously has been shown to be internalized most efficiently. (**A**) The most prominent labeled band (lane 3) was identified as β1 (lane 2) by Western blot analysis using the monoclonal antibody 6A, which is specific for β1/β2 subunits. The weaker set of bands near 70 kDa contain Hsc70, a contaminant in the preparation, as well as β1 degradation products. Cross-linking to Hsc70 cannot be competed by unmodified peptide. A CD3γ-LL peptide in which the benzoylphenylalanine cross-linker was located at position +2 was also tested in the cross-linking assay but gave no specific labeling (not shown). The same cross-linking reaction performed with AP-2 results in a weakly labeled band of the electrophoretic mobility of β2 (lane 4). (**B**) The interaction site within β1 for CD3γ-LL was mapped by limited tryptic proteolysis of AP-1, and found to reside in the N-terminal trunk portion of β1. AP-1 complexes were subjected to photocross-linking with *CD3γ-LL before proteolysis. Lanes 1 and 2 show the Coomassie blue staining pattern of AP-1 before and after a 30 min incubation at room temperature with 1:5 (w/w) trypsin. Lanes 3 and 4 (visualized by enhanced chemiluminescence) demonstrate that the label is attached to the fragment corresponding to the N-terminal β1 trunk. (**C**) The interaction between *CD3γ-LL and AP-1 is specific to the dileucine motif, as shown by the fact that CD3γ-LL (RQSRApSDKQT**LL**PN) inhibits binding (100 and 1000 μM, lanes 5–6), but CD3γ-AA (RQSRApSDKQT**AA**PN) does not (1000 μM, lane 2). Binding in the absence of competitor is shown in lane 1. Equal amounts (0.2 mg/ml) of frozen AP-1 (lanes 7 and 8) and AP-2 (lanes 9 and 10) were subjected to the cross-linking reaction to show that the cross-linking of CD3γ-LL to β2 in AP-2 is weak and non-competable.

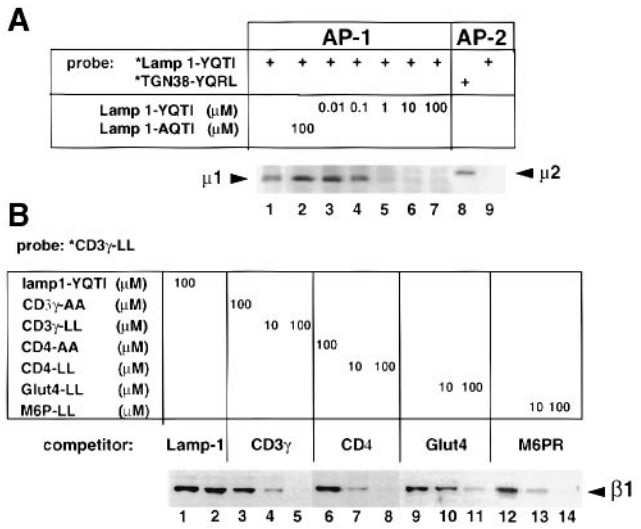

Fig. 4. The binding site for dileucine-containing peptides on AP-1 does not overlap with the binding site for tyrosine motif-containing peptides. (**A**) *Lamp1-YQTI binds specifically to μ1 on AP-1. The biotin-labeled, photoreactive peptide *Lamp1-YQTI [biotin-KKRS(benzoylphenylalanine)AG**YQTI**] (0.2 μM) was incubated with ~0.2 mg/ml AP-1 or AP-2 and cross-linked using the freezing protocol. Specific labeling was seen only with AP-1; the labeled band was identified as μ1 by Coomassie blue staining (not shown). Lane 1 shows cross-linking in the absence of competitor peptides, lane 2 cross-linking in the presence of 100 μM Lamp1-AQTI (RKRSHA-G**AQTI**); competition with unlabeled Lamp1-YQTI (RKRSHA-G**YQTI**) is shown in lanes 3–7 (at 0.01, 0.1, 1, 10 and 100 μM, respectively). Comparison of these data with the results in lanes 8 and 9 shows that the binding of *Lamp1-YQTI to AP-1 (lane 1) is stronger than its interaction with AP-2 (lane 9), and apparently comparable with the binding of *TGN38-YQRL to μ2 of AP-2 (lane 8). (**B**) Lamp-1 does not compete with dileucine-containing peptides for binding to AP-1. To determine whether the binding sites for the tyrosine motif-containing peptide Lamp1-YQTI and CD3γ-LL overlap, we used *CD3γ-LL (0.2 μM) as the cross-linking probe and Lamp1-YQTI as the competitor. No inhibition was observed at 100 μM of competing peptide (lane 1). To test whether all dileucine-containing peptides bind to the same site on AP-1, we examined the competition between *CD3γ-LL (0.2 μM) and CD3γ-LL (at 10 and 100 μM, lanes 4 and 5), CD4-LL (RMpSQIKR**LL**SEK) (lanes 7 and 8), Glut4-LL (ISAAFRRTPS**LL**EQEVKPSTEL) (lanes 10 and 11) and M6PR (VSFHDDSDED**LL**HI) (lanes 13 and 14). CD3γ-AA (lane 3) and CD4-AA (RMSQIKR**AA**SEK) (lane 6) peptides were used as controls to check that the inhibition seen was due to the presence of the dileucine motif.

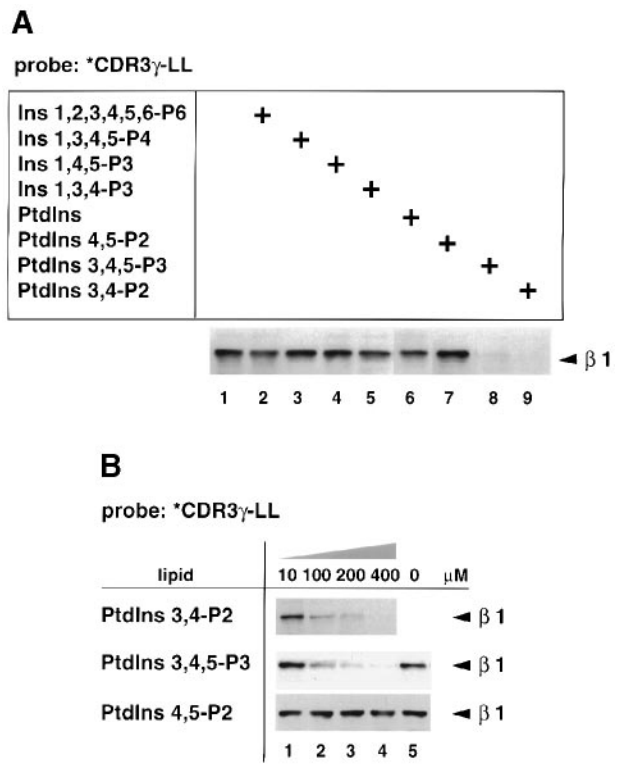

Fig. 5. PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3 inhibit the recognition of the CD3γ-LL dileucine motif by AP-1. (**A**) Similar amounts of frozen AP-1 (~0.2 mg/ml) were subjected to the cross-linking reaction with *CD3γ-LL (0.2 μM) in the presence of 400 μM of the indicated lipids. The concentration of lipid used here corresponds to the highest value tested in our earlier work on AP-2 (Rapoport *et al*., 1997). Inositol phosphates (lanes 2–5), the non-phosphorylated PtdIns (lane 6) and PtdIns 4,5-P_2 (lane 7) do not significantly affect the efficiency of cross-linking of β1 by *CD3γ-LL. The phosphorylated phosphoinositides PtdIns 3,4,5-P_3 (lane 8) and PtdIns 3,4-P_2 (lane 9) containing a phosphate at position D-3 of the inositol ring were effective in inhibiting the cross-linking of β1 by *CD3γ-LL. (**B**) Similar cross-linking experiments were carried out using the indicated amounts (0–400 μM) of PtdIns 3,4-P_2, PtdIns 3,4,5-P_3 and PtdIns 4,5-P_2. The inhibitory effect of PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3 was apparent at 100 μM of lipid.

The dileucine-binding site is unique

To test whether other peptides containing dileucine motifs bind the same site on AP-1 as CD3γ, competition assays were performed using *CD3γ-LL as cross-linker and synthetic peptides bearing the dileucine sorting signals of CD4, Glut4 and M6PR as competitors. All of these peptides competed with the *CD3γ peptide for binding to β1 (Figure 4B). The LL→AA-substituted peptides CD3γ-AA and CD4-AA did not compete with *CD3γ-LL for binding, even when used at a concentration of 1 mM (not shown). Thus, the β1 site revealed by the cross-linking reaction appears to be a general dileucine-binding site.

Phosphoinositide lipids inhibit, not activate, the recognition of dileucine motifs

Phosphoinositides have been implicated in the regulation of vesicular traffic (Schu *et al*., 1993; Brown *et al*., 1995; Joly *et al*., 1995; De Camilli *et al*., 1996; Spiro *et al*., 1996). We previously found that phosphoinositides containing a phosphate at the D-3 position of the inositol ring enhance recognition of the YXXϕ motif by AP-2 (Rapoport *et al*., 1997). We therefore tested whether PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3 might also affect the binding of dileucine motifs to AP-1. PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3 significantly inhibited the efficiency of cross-linking of the dileucine motif to the β1 chain (Figure 5A, lanes 8 and 9); PtdIns and PtdIns 4,5-P_2 did not (lanes 6 and 7). The relative efficacies of PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3 were approximately equal, as shown by the titration in Figure 5B. The inhibitory effect required the lipid portion, since addition of phosphorylated inositol rings, such as inositol (Ins) 1,3,4-P_3, Ins 1,4,5-P_3, Ins 1,3,4,5-P_4 or Ins 1,2,3,4,5,6-P_6 had no detectable effect (Figure 5A, lanes 2–5). The interaction between the Lamp-1 motif and AP-1, like the interaction between TGN38 and AP-2 (Rapoport *et al*., 1997), is enhanced by PtdIns 3,4-P_2 (data not shown), although the freezing technique appears to reduce the degree of stimulation of binding for both tyrosine motifs.

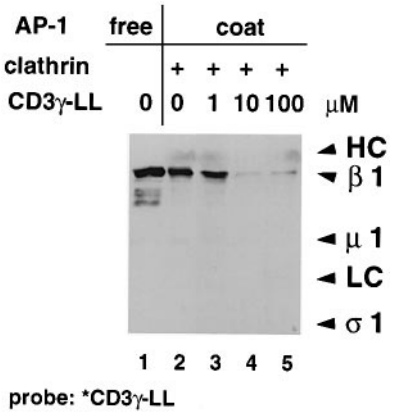

Fig. 6. Clathrin coat formation does not affect the interaction between AP-1 and *CD3γ-LL. Similar amounts (~0.2 mg/ml) of AP-1, either free (lane 1) or co-assembled with clathrin to form coats (lanes 2–5), were mixed with 0.2 μM *CD3γ-LL and subjected to the cross-linking reaction under freezing conditions. Both samples were kept in coat buffer (Gallusser and Kirchhausen, 1993). Comparison of lanes 1 and 2 shows that the intensity of the cross-linking reaction of β1 by *CD3γ-LL is not affected by the formation of coats. The cross-linking to β1 in coats is specific, as shown by the inhibition of the signal seen upon addition of increasing amounts of CD3γ-LL (lanes 3–5). The weaker set of bands cross-linked by *CD3γ-LL seen in lane 1 correspond to degradation products of β1 and Hsc70, not present in the clathrin coat samples (lanes 2–5). The positions of the β1, μ1 and σ1 subunits of AP-1 and the heavy (HC) and light chains (LC) of clathrin are indicated.

Clathrin–AP-1 coat formation does not affect dileucine motif binding

In our previous work, we found that the formation of clathrin–AP-2 coats enhanced the interaction between tyrosine motif-containing peptides and the μ2 chain, indicating that coat formation may 'trap' tyrosine motif-containing membrane proteins within the forming coated pit. We therefore examined the effect of coat formation on the interaction between AP-1 and dileucine motif-containing peptides; no enhancement was found (Figure 6, compare lanes 1 and 2) and the cross-linking by *CD3γ-LL was competable. Although it is possible that the freezing protocol might somehow inhibit clathrin–AP-1 interactions, this protocol does not prevent the enhancement of the interaction between tyrosine motif-containing peptides and AP-2 by coat formation (data not shown). Coat formation also does not appear to alter the interaction between AP-1 and the Lamp-1 YQTI peptide (data not shown), indicating that AP-1 complexes and AP-2 complexes differ in their response to clathrin.

Discussion

Our demonstration that the binding sites for dileucine and tyrosine motifs are distinct is fully consistent with previous observations that overexpression of a dileucine motif-containing membrane protein can inhibit the internalization of other dileucine motif-containing proteins, but not the internalization of tyrosine motif-containing proteins, and vice versa (Marks *et al.*, 1996). These observations originally were interpreted to indicate that the sorting machinery for the two motifs consisted of two entirely separate entities. Our results show that although the recognition sites are indeed distinct, they are carried on the same multi-functional protein complex, resolving the apparent contradiction between the previous work and the observations that both dileucine motifs (Heileker *et al.*, 1996; Honing *et al.*, 1997) and tyrosine motifs (Ohno *et al.*, 1995; Boll *et al.*, 1996) bind to AP complexes.

It is interesting that the cytoplasmic motifs of both CD3γ and CD4 bind AP-1. Our results predict that these proteins may be sorted from the TGN into post-Golgi compartments. Glut4 is already known to sort into secretory vesicles directly from the TGN (James and Piper, 1994; Verhey and Birnbaum, 1994), and it has been shown recently that the dileucine motif of the M6PR is active in post-Golgi sorting and AP-1 binding (Johnson and Kornfeld, 1992; Le Borgne *et al.*, 1993). Although dileucine motifs are known to facilitate receptor internalization, careful scrutiny of the published data shows that the internalization rates for proteins containing these motifs are low (Pelchen-Matthews *et al.*, 1989). Therefore, it is not surprising that the interaction of the dileucine motifs with AP-2s is hard to detect.

The observation that sorting signals are recognized separately, and that their recognition is regulated separately by lipids, is particularly interesting in the light of recent results showing the existence of 'non-classical' adaptor protein complexes such as AP-3 (Cowles *et al.*, 1997; Dell'Angelica *et al.*, 1997; Panek *et al.*, 1997; Simpson *et al.*, 1997). Although these adaptor protein complexes do not appear to interact with clathrin, their subunits are highly related in sequence and presumably in function to AP-1 and AP-2. It is not yet clear whether they compete with AP-1 and AP-2 for the same sorting signals, or whether they recognize a distinct set of peptide motifs. The situation is complicated yet further by the fact that many membrane proteins like, for example, the M6PR, contain more than one sorting motif in their cytoplasmic tails. This apparent redundancy may reflect a need for higher avidity of binding, or a combinatorial method to allow the specification of a larger variety of target sites.

One possibility is that different motifs may be exposed in different circumstances, for example by phosphorylation of the tail or by the loss of a masking molecule. Indeed, this method of regulation of sorting need not be restricted to cytoplasmic tails that contain more than one motif; it is equally applicable to tails containing only one motif. In the case of tyrosine motifs, the tyrosine may also be phosphorylated directly, as in the case of CTLA-4, and this modification may inhibit the interaction of the motif with APs (Boll *et al.*, 1996; Shiratori *et al.*, 1997). Phosphorylation of Ser126 in the tail of the CD3γ chain has been suggested to enhance the down-regulation of this protein from the plasma membrane (Dietrich *et al.*, 1997), presumably by preventing the association of the cytoplasmic tail with a masking protein or by opening up the tail to allow recognition of the motif. The M6PR appears to behave similarly; phosphorylation on the serine at position –4 from the LL motif appears to increase the efficiency of sorting to a post-Golgi compartment (Mauxion *et al.*, 1996). In the CD3γ-derived peptides we studied here, no effect of serine phosphorylation was seen, indicating that either these short peptides have no need to be opened up for AP-1 recognition, or the masking protein is not present in these assays.

A model for membrane traffic

AP-1, AP-2 and AP-3 are closely related molecular complexes (Robinson, 1992; Kirchhausen, 1993; Dell'Angelica *et al*., 1997; Kirchhausen *et al*., 1997; Simpson *et al*., 1997). The results presented here, taken together with our earlier studies of tyrosine-based motif recognition (Rapoport *et al*., 1997), suggest that these complexes act like differential filters for receptors with one or more sorting signals on their cytoplasmic tails (see Marks *et al*., 1997). The TGN, the compartment where most AP-1 is found (Ahle *et al*., 1988), is the initial sorting station for newly synthesized membrane proteins. The precise location of AP-3 is not well defined yet, although, like AP-1, it is mostly located in the perinuclear region. Transmembrane proteins with AP-1- or AP-3-directed dileucine or tyrosine signals will be captured by AP-1 (or AP-3) and be sorted to an endosomal/lysosomal compartment for further traffic, e.g. to specialized secretory vesicles or the lysosome. Other transmembrane proteins will pass on to the plasma membrane. Those with AP-2-directed tyrosine signals (and to a lesser extent with dileucine motifs) will be re-internalized. None of these steps are absolute: their efficiencies depend on relative affinities for the various signals.

There is ample evidence that phosphoinositides modulate vesicular traffic (Joly *et al*., 1994; Brown *et al*., 1995; De Camilli *et al*., 1996; Seaman *et al*., 1996; Spiro *et al*., 1996). These lipids are generally thought to recruit proteins to membranes or to activate specific enzymes. We previously have demonstrated a positive effect of these lipids in tyrosine signal recognition by AP-2 (Rapoport *et al*., 1997). We demonstrate here that the effect of phosphoinositides on AP-1 varies dramatically depending on the target motif. Lipid kinases also follow routes of membrane traffic, and the pattern of AP modulation by lipids may therefore be an intricate one. It will be interesting to determine how selective local generation of phosphatidyl inositides affects the traffic of proteins bearing different cytoplasmic signals in the various membrane compartments. The dileucine motif recognition site is in the core of the AP-1 complex (on the trunk of the β chain, as opposed to the ear–hinge portion), as is the tyrosine motif recognition site (on the μ chain). This relative proximity makes it easier to understand how a single allosteric binding event, such as binding of a phosphatidylinositide lipid molecule or vesicle, could simultaneously open up one of the two recognition sites and close the other.

It is somewhat surprising that the formation of a clathrin coat does not affect the interaction between AP-1 and sorting signals, given the clear enhancement of AP-2 binding to tyrosine motifs by clathrin–AP-2 coat formation (Rapoport *et al*., 1997). AP-2 is known to undergo a conformational change on coat formation that increases the sensitivity of the μ2 chain to protease digestion (Matsui and Kirchhausen, 1990). It is not known whether a similar conformational change occurs for AP-1, and it is not known whether the conformational change in AP-2 affects subunits other than the μ2 chain; no effect on the other AP-2 chains is seen by proteolysis. Our results suggest that the μ1 chain does not undergo a conformational change in response to clathrin coat formation, at least under the freezing conditions, and that β1 is also unaffected.

Although AP-1 and AP-2 are highly related, it is clear that there is some difference in the way that they behave on membranes; for example, the presence of a membrane protein that traffics via AP-1 will cause the recruitment of AP-1 to that membrane (Le Borgne *et al*., 1996; Salamero *et al*., 1996), whereas the presence of a membrane protein that traffics via AP-2 does not induce recruitment of AP-2 (Santini and Keen, 1996). The molecular basis of this difference in regulation is unclear, but an understanding of the interactions between the cytoplasmic tails of such proteins and the AP complexes, and how they are controlled, may eventually lead to clarification of these issues.

Materials and methods

Purification of APs and limited proteolysis

AP complexes were prepared by standard procedures by isolation from calf brain coated vesicles (Gallusser and Kirchhausen, 1993) followed by purification by ion exchange chromatography on a hydroxyapatite column (Econo-Pac, BioRad) (Pearse and Robinson, 1984; Boll *et al*., 1996). For the cross-linking experiments described here, AP-1 and AP-2 complexes were transferred into AP buffer [100 mM NaMES, 1 mM EDTA, 150 mM NaCl, 0.02% NaN_3, 0.5 mM dithiothreitol (DTT), 0.1% Triton X-100, pH 7.0] by overnight dialysis. Limited tryptic proteolysis of AP-1 was done in AP buffer. This treatment results in the release of the C-terminal domains (the ear and hinge) of the large β1 and γ subunits of the AP-1 (Zaremba and Keen, 1985; Ahle and Ungewickell, 1989; Kirchhausen *et al*., 1989).

Synthesis of peptides

Several peptides corresponding to portions of cytoplasmic tails of transmembrane proteins were synthesized (Figure 1). Details of their synthesis have been described elsewhere (Rapoport *et al*., 1997). Some of the peptides contained the photoreactive probe benzoylphenylalanine at the indicated positions and biotin added to the N-terminal lysine to permit identification of the cross-linked protein species. Stock solutions of peptides were dissolved in water (2–10 mM) and kept at –20°C.

Preparation of lipids

The phosphoinositides PtdIns, PtdIns 3,4-P_2, PtdIns 4,5-P_2 and PtdIns 3,4,5-P_3 were suspended (1 mg/ml final concentration) in 10 mM HEPES, 1 mM EGTA, pH 7.0 and sonicated in a cup water-bath sonicator (Branson) at 40% full power for 5 min at room temperature (Rapoport *et al*., 1997). The micelles were kept on ice and used within 1 h after formation. The phosphoinositols Ins 1,3,4-P_3, Ins 1,4,5-P_3 and Ins 1,3,4,5-P_4 were dissolved at 10–30 mM in 10 mM HEPES, 1 mM EGTA, pH 7.0. Ins 1,2,3,4,5,6-P_6 (Sigma Co.) was dissolved at 20 mM in AP buffer titrated to pH 7.0.

UV-induced cross-linking reaction

For the cross-linking studies, 10–18 μl of a solution containing the APs (~0.2 mg/ml final concentration) was mixed with 2 μl of the indicated photoreactive peptide (0.2 μM final concentration) and, when appropriate, with one of the competing peptides (final concentration in the range of 0–1000 μM). When necessary, 1–8 μl of phosphoinositols or phosphoinositides (0–400 μM final concentration) were also added to the reaction mixture.

The cross-linking experiments were carried out in 96-well microtiter plates (Falcon 3911 microtest III) in a final volume of 20–22 μl. Samples were frozen rapidly by placing part of the 96-well plate containing the samples on a bed of dry ice. The samples (still on dry ice) were then exposed to UV light for 3–12 min to trigger the cross-linking reaction. The UV light was generated by a mercury lamp (H44GS-100, Osram Sylvania Inc.) placed at a distance of 10 cm from the top of the plate. In some cases, the samples were frozen by placing the plate on top of liquid nitrogen instead of dry ice; no differences in the cross-linking results were observed (not shown). To control for the effects of freezing, samples containing AP-2 were kept at 4°C during the entire procedure (Rapoport *et al*., 1997). Following the cross-linking reaction, 5 μl of 5× Laemmli sample buffer containing β-mercaptoethanol was added and the samples were boiled for 3 min.

Detection of cross-linked products and Western blot analysis.
The cross-linked species were identified following SDS–PAGE fractionation, transfer to nitrocellulose, blotting with horseradish peroxidase-conjugated streptavidin (streptavidin–HRP) (Boehringer Mannheim) and detection by enhanced chemiluminescence (Rapoport *et al*., 1997). Western blot analysis was performed using enhanced chemiluminescence with the monoclonal antibodies 6A specific for β1 and β2 and 110/3 specific for γ. The antibody 6A was generated using recombinant rat brain β2 made in *Escherichia coli* as an immunogen.

Acknowledgements

We thank members of the laboratory for discussions and S.C.Harrison, M.Roth, R.Ward and M.Wessling-Resnick for reviewing the manuscript. We thank E.Ungewickell for kindly providing the monoclonal antibody 110/3 specific for γ. The mAb 6A specific for β1/β2 was generated in collaboration with Dr K.Clairmont (Bayer). T.K. is supported by a National Institutes of Health grant (GM36548) and by Special Funds from The Center for Blood Research and the Department of Cell Biology, H.M.S. S.E.S. is supported by a National Institutes of Health grant (DK43123).

References

Ahle,S. and Ungewickell,E. (1989) Identification of a clathrin binding subunit in the HA2 adaptor protein complex. *J. Biol. Chem.*, **264**, 20089–20093.

Ahle,S., Mann,A., Eichelsbacher,U. and Ungewickell,E. (1988) Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO J.*, **7**, 919–929.

Aiken,C., Konner,J., Landau,N.R., Lenburg,M.E. and Trono,D. (1994) Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell*, **76**, 853–864.

Boll,W., Gallusser,A. and Kirchhausen,T. (1995) Role of the regulatory domain of the EGF-receptor cytoplasmic tail in selective binding of the clathrin-associated complex AP-2. *Curr. Biol.*, **5**, 1168–1178.

Boll,W., Ohno,H., Songyang,Z., Rapoport,I., Cantley,L.C., Bonifacino, J.S. and Kirchhausen,T. (1996) Sequence requirements for the recognition of tyrosine-based endocytic signals by clathrin AP-2 complexes. *EMBO J.*, **15**, 5789–5795.

Brown,W.J., DeWald,D.B., Emr,S.D., Plutner,H. and Balch,W.E. (1995) Role for phosphatidylinositol 3-kinase in the sorting and transport of newly synthesized lysosomal enzymes in mammalian cells. *J. Cell Biol.*, **130**, 781–796.

Cowles,C.R., Odorizzi,G., Payne,G.S. and Emr,S.D. (1997) The Ap-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. *Cell*, **91**, 109–118.

De Camilli,P., Emr,S.D., McPherson,P.S. and Novick,P. (1996) Phosphoinositides as regulators in membrane traffic. *Science*, **271**, 1533–1539.

Dell'Angelica,E.C., Ohno,H., Ooi,C.E., Rabinovich,E., Roche,K.W. and Bonifacino,J.S. (1997) AP-3: an adaptor-like protein complex with ubiquitous expression. *EMBO J.*, **16**, 917–928.

Dietrich,J., Xiaohone,H., Wegener,A.-M.K. and Geisler,C. (1994) CD3γ contains a phosphoserine-dependent di-leucine motif involved in down-regulation of the T cell receptor. *EMBO J.*, **13**, 2156–2166.

Dietrich,J., Kastrup,J., Nielsen,B.L., Odum,N. and Geisler,C. (1997) Regulation and function of the Cd3-gamma Dxxxll motif—a binding site for adaptor protein-1 and adaptor protein-2 *in vitro*. *J. Cell Biol.*, **138**, 271–281.

Gallusser,A. and Kirchhausen,T. (1993) The β1 and β2 subunits of the AP complexes are the clathrin coat assembly components. *EMBO J.*, **12**, 5237–5244.

Glickman,J.N., Conibear,E. and Pearse,B.M.F. (1989) Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. *EMBO J.*, **8**, 1041–1047.

Heilker,R., Manning-Krieg,U., Zuber,J.F. and Spiess,M. (1996) *In vitro* binding of clathrin adaptors to sorting signals correlates with endocytosis and basolateral sorting. *EMBO J.*, **15**, 2893–2899.

Honing,S., Griffith,J., Geuze,H.J. and Hunziker,W. (1996) The tyrosine based lysosomal targeting signal in lamp-1 mediates sorting into Golgi-derived clathrin-coated vesicles. *EMBO J.*, **15**, 5230–5239.

James,D.E. and Piper,R.C. (1994) Insulin resistance, diabetes, and the insulin-regulated trafficking of glut-4. *J. Cell Biol.*, **126**, 1123–1126.

Johnson,K.F. and Kornfeld,S. (1992) The cytoplasmic tail of the mannose 6-phosphate/insulin-like growth factor-II receptor has two signals for lysosomal enzyme sorting in the Golgi. *J. Cell Biol.*, **119**, 249–257.

Johnson,K.F., Chan,W. and Kornfeld,S. (1990) Cation-dependent mannose 6-phosphate receptor contains two internalization signals in its cytoplasmic domain. *Proc. Natl Acad. Sci. USA*, **87**, 10010–10014.

Joly,M., Kazlauskas,A., Fay,F.S. and Corvera,S. (1994) Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding sites. *Science*, **263**, 684–687.

Joly,M., Kazlauskas,A. and Corvera,S. (1995) Phosphatidylinositol 3-kinase activity is required at a postendocytic step in platelet-derived growth factor receptor trafficking. *J. Biol. Chem.*, **270**, 13225–13230.

Kirchhausen,T. (1993) Coated pits and coated vesicles—sorting it all out. *Curr. Opin. Struct. Biol.*, **3**, 182–188.

Kirchhausen,T., Nathanson,K.L., Matsui,W., Vaisberg,A., Chow,E.P., Burne,C., Keen,J.H. and Davis,A.E. (1989) Structural and functional division into two domains of the large (100- to 115-kDa) chains of the clathrin-associated protein complex AP-2. *Proc. Natl Acad. Sci. USA*, **86**, 2612–2616.

Kirchhausen,T., Bonifacino,J.S. and Riezman,H. (1997) Linking cargo to vesicle formation—receptor tail interactions with coat proteins. *Curr. Opin. Cell Biol.*, **9**, 488–495.

Le Borgne,R., Schmidt,A., Mauxion,F., Griffiths,G. and Hoflack,B. (1993) Binding of AP-1 Golgi adaptors to membranes requires phosphorylated cytoplasmic domains of the mannose 6-phosphate/insulin-like growth factor II receptor. *J. Biol. Chem.*, **268**, 22552–22556.

Le Borgne,R., Griffiths,G. and Hoflack,B. (1996) Mannose 6-phosphate receptors and ADP-ribosylation factors cooperate for high affinity interaction of the AP-1 Golgi assembly proteins with membranes. *J. Biol. Chem.*, **271**, 2162–2170.

Letourneur,F. and Klausner,R.D. (1992) A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. *Cell*, **69**, 1143–1157.

Marks,M.S., Woodruf,L., Ohno,H. and Bonifacino,J.S. (1996) Protein targeting by tyrosine- and di-leucine-based signals: evidence for distinct saturable components. *J. Cell Biol.*, **135**, 341–354.

Marks,M.S., Ohno,H., Kirchhausen,T. and Bonifacino,J.S. (1997) Protein sorting by tyrosine-based signals: adapting to the Ys and wherefores. *Trends Cell Biol.*, **7**, 124–128.

Matsui,W. and Kirchhausen,T. (1990) Stabilization of clathrin coats by the core of the clathrin-associated protein complex AP-2. *Biochemistry*, **29**, 10791–10798.

Mauxion,F., Le Borgne,R., Munier-Lehmann,H. and Hoflack,B. (1996) A casein kinase II phosphorylation site in the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor determines the high affinity interaction of the AP-1 Golgi assembly proteins with membranes. *J. Biol. Chem.*, **271**, 2171–2178.

Ohno,H. *et al*. (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science*, **269**, 1872–1875.

Panek,H.R., Stepp,J.D., Engle,H.M., Marks,K.M., Tan,P.K., Lemmon,S.K. and Robinson,L.C. (1997) Suppressors of Yck-encoded yeast casein kinase 1 deficiency define the four subunits of a novel clathrin Ap-like complex. *EMBO J.*, **16**, 4194–4204.

Pearse,B.M.F. (1988) Receptors compete for adaptors found in plasma membrane coated pits. *EMBO J.*, **7**, 3331–3336.

Pearse,B.M. and Robinson,M.S. (1984) Purification and properties of 100-kd proteins from coated vesicles and their reconstitution with clathrin. *EMBO J.*, **3**, 1951–1957.

Pelchen-Matthews,A., Armes,J. and Marsh,M. (1989) Internalization and recycling of CD4 transfected into HeLa and NIH3T3 cells. *EMBO J.*, **8**, 3641–3649.

Rapoport,I., Miyazaki,M., Boll,W., Duckworth,B., Cantley,L.C., Shoelson,S. and Kirchhausen,T. (1997) Regulatory interactions in the recognition of endocytic sorting signals by AP-2 complexes. *EMBO J.*, **16**, 2240–2250.

Robinson,M.S. (1992) Adaptins. *Trends Cell Biol.*, **2**, 293–297.

Rohrer,J., Schweizer,A., Russell,D. and Kornfeld,S. (1996) The targeting of Lamp1 to lysosomes is dependent on the spacing of its cytoplasmic tail tyrosine sorting motif relative to the membrane. *J. Cell Biol.*, **132**, 565–576.

Salamero,J., Le Borgne,R., Saudrais,C., Goud,B. and Hoflack,B. (1996) Expression of major histocompatibility complex class II molecules in HeLa cells promotes the recruitment of AP-1 Golgi-specific assembly proteins on Golgi membranes. *J. Biol. Chem.*, **271**, 30318–30321.

Sandoval,I.V. and Bakke,O. (1994) Targeting of membrane proteins to endosomes and lysosomes. *Trends Cell Biol.*, **4**, 292–297.

Santini,F. and Keen,J.H. (1996) Endocytosis of activated receptors and clathrin-coated pit formation: deciphering the chicken or egg relationship. *J. Cell Biol.*, **132**, 1025–1036.

Schroder,S. and Ungewickell,E. (1991) Subunit interaction and function of clathrin-coated vesicle adaptors from the Golgi and the plasma membrane. *J. Biol. Chem.*, **266**, 7910–7918.

Schu,P.V., Takegawa,K., Fry,M.J., Stack,J.H., Waterfield,M.D. and Emr,S.D. (1993) Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science*, **260**, 88–91.

Seaman,M.N., Burd,C.G. and Emr,S.D. (1996) Receptor signalling and the regulation of endocytic membrane transport. *Curr. Opin. Cell Biol.*, **8**, 549–556.

Shiratori,T., Miyatake,S., Ohno,H., Nakaseko,C., Isono,K., Bonifacino, J.S. and Saito,T. (1997) Tyrosine phosphorylation controls internalization of CTLA-4 by regulating its interaction with clathrin-associated adaptor complex AP-2. *Immunity*, **6**, 583–589.

Simpson,F., Peden,A.A., Christopoulou,L. and Robinson,M.S. (1997) Characterization of the adaptor-related protein complex, AP-3. *J. Cell Biol.*, **137**, 835–845.

Sorkin,A. and Carpenter,G. (1993) Interaction of activated EGF receptors with coated pit adaptins. *Science*, **261**, 612–615.

Sorkin,A., McKinsey,T., Shih,W., Kirchhausen,T. and Carpenter,G. (1995) Stoichiometric interaction of the epidermal growth factor receptor with the clathrin-associated protein complex AP-2. *J. Biol. Chem.*, **270**, 619–625.

Sosa,M.A., Schmidt,B., von Figura,K. and Hille-Rehfeld,A. (1993) *In vitro* binding of plasma membrane-coated vesicle adaptors to the cytoplasmic domain of lysosomal acid phosphatase. *J. Biol. Chem.*, **268**, 12537–12543.

Spiro,D.J., Boll,W., Kirchhausen,T. and Wessling-Resnick,M. (1996) Wortmannin alters the transferrin receptor endocytic pathway *in vivo* and *in vitro*. *Mol. Biol. Cell*, **7**, 355–367.

Trowbridge,I.S., Collawn,J.F. and Hopkins,C.R. (1993) Signal-dependent membrane protein trafficking in the endocytic pathway. *Annu. Rev. Cell Biol.*, **9**, 129–161.

Verhey,K.J. and Birnbaum,M.J. (1994) A Leu-Leu sequence is essential for COOH-terminal targeting signal of Glut4 glucose transporter in fibroblasts. *J. Biol. Chem.*, **269**, 2353–2356.

Zaremba,S. and Keen,J.H. (1985) Limited proteolytic digestion of coated vesicle assembly polypeptides abolishes reassembly activity. *J. Cell Biochem.*, **28**, 47–58.

Received November 25, 1997; revised January 28, 1998; accepted January 30, 1998