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The $\gamma/\sigma1$ and $\alpha/\sigma2$ Hemicomplexes of Clathrin Adaptors AP-1 and AP-2 Harbor the Dileucine Recognition Site

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The clathrin adaptors AP-1 and AP-2 bind cargo proteins via two types of motifs: tyrosine-based Yxxϕ and dileucine-based [DE]XXXL[LI]. Although it is well established that Yxxϕ motifs bind to the μ subunits of AP-1 or AP-2, dileucine motifs have been reported to bind to either the μ or β subunits of these adaptors as well as the $\gamma/\sigma1$ hemicomplex of AP-1. To clarify this controversy, the various subunits of AP-1 and AP-2 were expressed individually and in hemicomplex form in insect cells, and they were used in glutathione *S*-transferase pull-down assays to determine their binding properties. We report that the $\gamma/\sigma1$ or $\alpha/\sigma2$ hemicomplexes bound the dileucine-based motifs of several proteins quite strongly, whereas binding by the $\beta1/\mu1$ and $\beta2/\mu2$ hemicomplexes, and the individual β or μ subunits, was extremely weak or undetectable. The $\gamma/\sigma1$ and $\alpha/\sigma2$ hemicomplexes displayed substantial differences in their preference for particular dileucine-based motifs. Most strikingly, an aspartate at position −4 compromised binding to the $\gamma/\sigma1$ hemicomplex, whereas minimally affecting binding to $\alpha/\sigma2$. There was an excellent correlation between binding to the $\alpha/\sigma2$ hemicomplex and in vivo internalization mediated by the dileucine-based sorting signals. These findings provide new insights into the trafficking mechanisms of D/EXXXL[LI]-mediated sorting signals.

INTRODUCTION

The plasma membrane and the *trans*-Golgi network (TGN) represent key sorting sites for many transmembrane proteins, mediated via sorting signals present in the cytosolic domain of these proteins. One mechanism for sorting involves the assembly of clathrin-coated vesicles at the sorting site that subsequently pinch off and uncoat before fusing with their target membranes, thereby delivering cargo to their appropriate destinations. The heterotetrameric adaptor proteins AP-1 and AP-2 are major components of clathrin-coated vesicles originating at the TGN and plasma membrane, respectively. Each of these adaptors is composed of two large subunits (γ and $\beta1$ for AP-1, α and $\beta2$ for AP-2), a medium subunit ($\mu1$ and $\mu2$), and a small subunit ($\sigma1$ and $\sigma2$) (see Figure 1A for a schematic of the subunit organization of AP-1 and AP-2). The interactions of AP-1 and AP-2 with transmembrane proteins occur mainly via two types of sorting signals: tyrosine-based YXXϕ (ϕ-bulky hydrophobic) and dileucine-based [DE]XXXL[LI] motifs (Bonifacino and Traub, 2003). Biochemical and structural studies have unequivocally determined that YXXϕ motifs engage the μ subunits of adaptor proteins (Ohno *et al*., 1995; Owen and Evans, 1998). However, the binding site for dileucine-based motifs has been the subject of debate. It has been reported by various groups that this motif binds to the β subunits of AP-1 and AP-2 (Rapoport *et al*., 1998; Greenberg *et al*., 1998; Geyer *et al*., 2002; Schmidt *et al*., 2006), to the μ subunits of AP-1 and AP-2 (Rodionov and Bakke, 1998; Hofmann *et al*., 1999; Craig *et al*., 2000; Rodionov *et al*., 2002; Hinners *et al*., 2003), and, more recently, to the $\gamma/\sigma1$ hemicomplex of AP-1 (Janvier *et al*., 2003; Coleman *et al*., 2005, 2006; Theos *et al*., 2005). The latter studies involved the use of the yeast three-hybrid technique. However, this assay failed to detect an interaction between dileucine motifs and the $\alpha/\sigma2$ hemicomplex of AP-2 (Janvier *et al*., 2003; Coleman *et al*., 2005).

Because an understanding of how dileucine-based motifs bind AP-1 and AP-2 is essential to explain the sorting process at the molecular level, we sought to address the issue in a more direct manner. To do so, we have expressed each of the subunits of AP-1 and AP-2 individually as well as the $\gamma/\sigma1$, $\beta1/\mu1$, $\alpha/\sigma2$, and $\beta2/\mu2$ hemicomplexes in Sf9 insect cells. These recombinant proteins were then used in glutathione *S*-transferase (GST) pull-down assays involving various dileucine-based motifs. Here, we report that the $\gamma/\sigma1$ and $\alpha/\sigma2$ hemicomplexes bind dileucine signals strongly, whereas binding to the $\beta1/\mu1$ and the $\beta2/\mu2$ hemicomplexes, or the isolated β or μ subunits, is extremely weak or undetectable by comparison. However, the $\gamma/\sigma1$ and $\alpha/\sigma2$ hemicomplexes exhibit substantial differences in their preference for particular dileucine-based motifs. In addition, we present evidence for a strong correlation between AP-2 $\alpha/\sigma2$ hemicomplex binding to various dileucine sequences and internalization mediated by these sequences.

MATERIALS AND METHODS

DNA Constructs, Plasmids, and Antibodies

Full-length cDNA clones encoding the γ subunit of human AP-1; the $\beta1$, $\mu1$, and $\sigma1$ subunits of mouse AP-1; and the α(C), $\beta2$, $\mu2$, and $\sigma2$ subunits of human AP-2 were purchased from American Type Culture Collection (Manassas, VA). The cDNAs were amplified by polymerase chain reaction (PCR) and inserted into the vector pFastBac-Dual (Invitrogen, Carlsbad, CA), either individually or as $\gamma/\sigma1$, $\beta1/\mu1$, $\alpha/\sigma2$, and $\beta2/\mu2$ bicistronic coexpression constructs (Figure 1B). In addition, the γ/α and $\beta1/\beta2$ cDNAs had sequences

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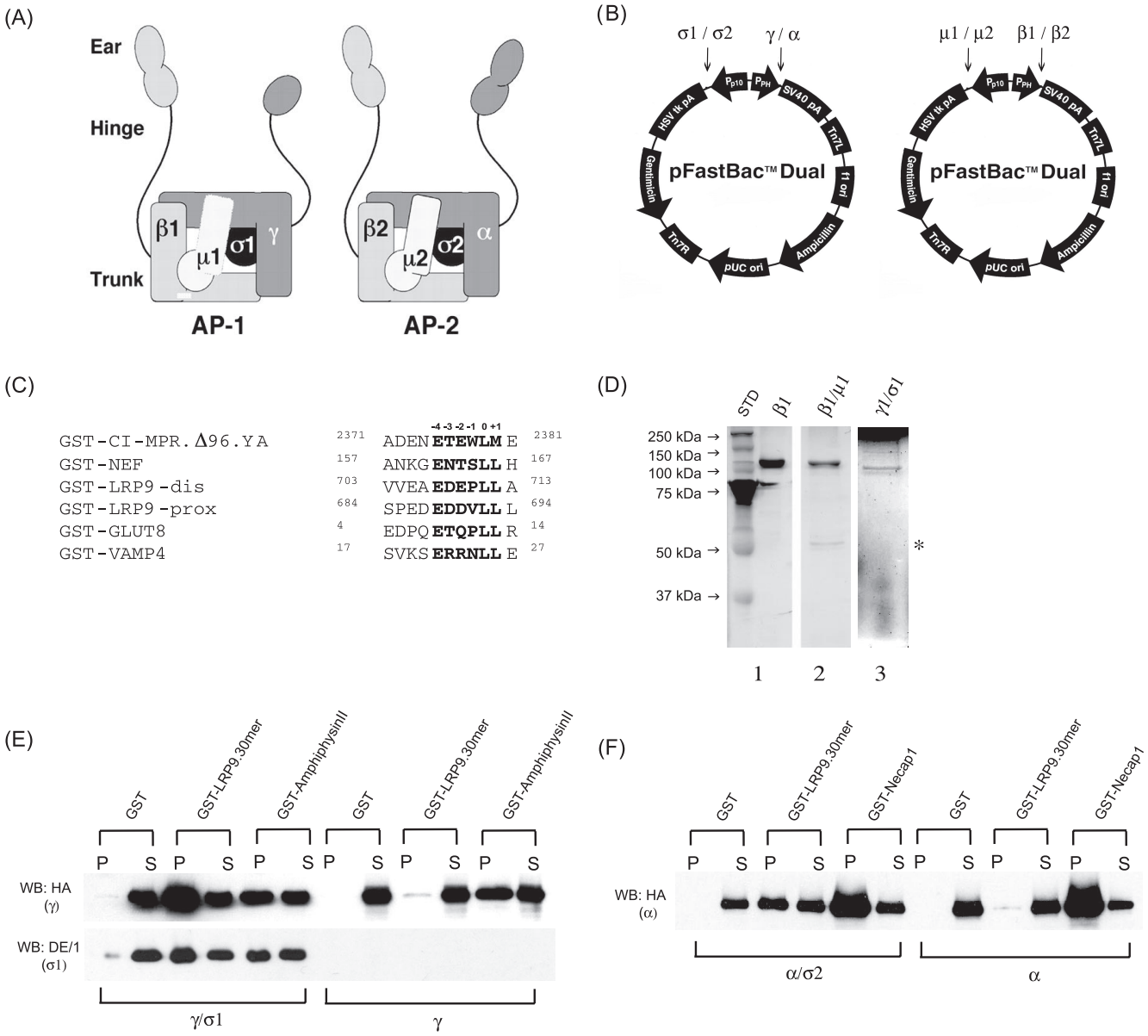

Figure 1. Dileucine-based motifs bind to the σ plus γ/α subunits of AP-1 and AP-2. (A) Schematic of the subunit organization of clathrin adaptors AP-1 and AP-2 (taken from Janvier *et al*., 2003) (B) cDNAs corresponding to human γ1, mouse β1/μ1/σ1, and human α(C)/β2/μ2/σ2 adaptins were cloned into the pFastBact-dual vectors as indicated. Recombinant bacmids isolated from *E. coli* DH10Bac cells were transfected into Sf9 insect cells to generate recombinant viruses for protein expression. (C) Sequences of the various dileucine motifs used in this study. Proteins were expressed as GST-fusions and purified from *E. coli* BL21 cells. (D) Silver stained gels of adaptin subunits affinity purified from Sf9 lysates as described in *Materials and Methods*. Asterisk (*) indicates position of μ1 adaptin. (E and F) GST pull-down assays were performed with insect cell-expressed γ/σ1 or free γ (C), and α/σ2 or free α (D) as described in *Materials and Methods*.

appended at their 3′ ends that encoded peptides for detection by the hemagglutinin (HA) and FLAG antibodies, respectively.

Full-length mouse low-density lipoprotein receptor-related protein (LRP)9 (Sugiyama *et al*., 2000) was cloned into the vector pCDNA3.1 (Invitrogen) by PCR from an American Type Culture Collection clone encoding the complete cDNA. The HA tag was inserted after codon 28, downstream of the sequence encoding the signal peptide as predicted by SignalP (The Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark). The mLRP4-LRP9 tail chimera was constructed by cloning in frame the full-length cytoplasmic tail of LRP9 downstream of the extracellular (HA-tagged mini receptor with ligand-binding repeat domain 4, hence mLRP4) and transmembrane region of LRP1 (Li *et al*., 2000). Similarly, the LRP9-CI-MPR tail chimera was constructed by cloning in-frame the bovine cation-independent mannose 6-phosphate receptor (CI-MPR) tail sequence downstream of the complete extracellular domain and transmembrane segment of LRP9.

GST-CI-MPRΔ96.YA was constructed from the plasmid encoding the 163-amino acid bovine CI-MPR tail fusion protein (Zhu *et al*., 2001) by inserting a stop codon at amino acid K2403 downstream of the internal dileucine-based sequence (ETEWLM), and mutating the YSKV sequence to ASKA, effectively eliminating any contribution from the tyrosine-based sorting motif. GST-YSKV was constructed by mutating E2373, upstream of the ETEWLM sequence, to a stop codon. GST-Nef (full-length) was kindly provided by Warner Greene (University of California, San Francisco, CA), whereas GST-Glut8 (1-25) was a generous gift from Kelle Moley (Washington University, St. Louis, MO). GST-clathrin terminal domain (TD 1-579), GST-SIPWDLWEPT, the distal amphiphysin II sequence, and GST-NECAP1 (full-length) have been described previously (Doray and Kornfeld, 2001; Drake and Traub, 2001; Bai *et al*., 2004). A GST fusion construct encoding the full-length tail of mouse LRP9 was made by PCR from the American Type Culture Collection clone. GST-Vamp4 (full-length) was kindly contributed by Matthew Drake (Mayo Clinic, Rochester MN). GST-LRP9-30mer encoding the C-terminal 30 amino

acids of mouse LRP9, including the proximal and distal dileucines, GST-LRP9-p17mer (proximal 17 amino acids), GST-LRP9-d17mer (distal 17 amino acids), GST-Vamp4-P41stop, and GST-Vamp4-S30stop were made from their respective full-length tail fusion proteins. A list of the GST fusion proteins and the sequences of the various dileucine-based motifs used in this study is shown in Figure 1C. All mutant constructs were made using primers incorporating the desired mutations with the QuikChange system (Stratagene, La Jolla, CA). All constructs and mutations were confirmed to be correct by dideoxynucleotide sequencing.

The anti-μ1 polyclonal antibody RY/1, and the anti-σ1 polyclonal antibody DE/1 were kindly provided by Linton Traub (University of Pittsburgh School of Medicine, Pittsburgh, PA). The anti-μ2 polyclonal antibody R11-29 was a generous contribution of Juan Bonifacino (National Institutes of Health, Bethesda, MD). The anti-HA monoclonal antibody (mAb) was purchased from Covance (Berkeley, CA), whereas the anti-FLAG tag and anti-γ-adaptin 100/3 monoclonal antibodies were from Sigma-Aldrich (St. Louis, MO). Affinity-purified rabbit anti-bovine CI-MPR antibody was prepared by Walter Gregory in our laboratory. Glutathione-Sepharose 4B was from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). All peptides were synthesized by Biomolecules Midwest (Waterloo, IL). The amino acid sequences of peptides used are as follows: ExxxLM, CADEN**E**TEW**LM**EEI; AxxxAA, CADEN**A**TEW**AA**EEI.

Protein Expression and Purification

All GST-fusion proteins (Figure 1C) were expressed in the *Escherichia coli* strain BL-21 (RIL) (Stratagene) and purified essentially as described previously (Doray and Kornfeld, 2001).

For expression in Sf9 insect cells, the various pFastBac-Dual constructs were transformed into *E. coli* DH10Bac-competent cells to generate recombinant bacmids per manufacturer's protocol (Invitrogen). Bacmid DNAs were prepared and transfected using standard protocol into Sf9 cells (Invitrogen) to produce recombinant baculoviruses that were amplified and used to express or coexpress the different AP-1 and AP-2 subunits in insect cells.

Immunoprecipitation of β1 and β1/μ1 from Sf9 cell lysates was performed using anti-FLAG agarose, whereas immunoprecipitation of γ/σ1 was carried out using the anti-γ-adaptin 100/3 monoclocal antibody with protein G-Sepharose. Washed beads were competitively eluted with either the FLAG peptide or the 100/3 epitope peptide. Eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and the protein band was visualized using the Silver Stain Plus kit (Bio-Rad, Hercules, CA).

Binding Assays

GST pull-down assays were performed essentially as described previously (Doray and Kornfeld, 2001). For insect cell-expressed proteins, typically 100–150 μl of total cell lysates (5–10 mg/ml) was used for each GST pull-down assay. Peptide competition experiments were performed in the same manner except that the indicated peptides were added to a final concentration of 200 μM from a buffered stock solution. Typically, 20% of pellet fractions and 3% of unbound fractions were analyzed by SDS-PAGE and Western blotting. Nitrocellulose membranes were routinely stained with Ponceau solution to ascertain equal loadings of fusion proteins.

Cell Culture and Transfection

The LRP1-null Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 medium as described previously (FitzGerald *et al.*, 1995). Transient transfection into LRP-null CHO cells was achieved by transfection of 1 μg of plasmid DNA in 12-well plates by using the Mirus Trans-IT CHO kit according to the manufacture's instructions (Mirus, Madison, WI). Microscopy, cell surface biotinylation, and endocytosis assays were generally performed 24 h posttransfection.

Immunofluorescence and Confocal Microscopy

CHO cells on glass coverslips transiently expressing wild-type HA-LRP9, or the mutants were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS), washed with PBS, and blocked and permeabilized with 0.4% Triton X-100, 2% IgG-free bovine serum albumin (BSA) (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS. Anti-HA mouse mAb (1:1000 dilution) or anti-CI-MPR rabbit polyclonal antibody (1:1000 dilution) were applied in antibody dilution buffer (0.1% Triton X-100, 0.5% IgG-free BSA in PBS). After washing with PBS, secondary antibodies Alexa 488-anti-mouse and Alexa 568-anti-rabbit (Invitrogen) were applied in antibody dilution buffer. The coverslips were washed and mounted on slides with mounting medium (Biomeda, Foster City, CA). Images were obtained with a Fluoview 500 confocal microscope (Olympus, Tokyo, Japan).

Cell Surface Biotinylation

Cell surface biotinylation experiments were performed using the EZ-Link Sulfo-NHS-LC Biotin reagent (Pierce Chemical, Rockford, IL). Transfected cells were washed three times with ice-cold PBS + 1 mM calcium chloride and 0.5 mM magnesium chloride (PBSc), and 1 ml of the cell-impermeable biotin solution (2 mg/ml) in PBSc was added to each well. Cells were incubated on ice with gentle agitation for 15 min and then washed once with cold 50 mM Tris, pH 8, to quench the reaction, followed by three washes with PBSc. Cells were lysed in 250 μl/well PBS + 1% Triton-X-100, 1X complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), and 1 mM phenylmethylsulfonyl fluoride with rocking at 4°C for 30 min. An aliquot of the postnuclear supernatant was reserved, and the remaining lysate was combined with PBSc and 50 μl of streptavidin-agarose beads in a total volume of 1 ml. After an overnight incubation of the beads and lysate at 4°C with tumbling, the beads were washed 4 times with ice-cold PBSc and resuspended in reducing sample buffer. One percent of the lysates and 5% of the pull-down products were run on 8% SDS-PAGE and transferred to nitrocellulose membranes, followed by Western blot with anti-HA.

Kinetic Analysis of Endocytosis

Kinetic analysis of endocytosis was performed essentially as described previously (Li *et al.*, 2000) except iodinated anti-HA IgG was used as ligand instead of iodinated receptor-associated protein, and PBS, pH 2.0, was used to strip antibody from the cell surface. The sum of ligand that was internalized plus that which remained on the cell surface after each assay was used as the maximum potential internalization. The fraction of internalized ligand after each time point was calculated and plotted.

RESULTS

Dileucine-based Motifs Bind Selectively to the γ/σ1 and α/σ2 Hemicomplexes of AP-1 and AP-2

In our initial experiments, we used the GST pull-down technique to examine the binding of individual subunits of AP-1 and AP-2 as well as the γ/σ1, β1/μ1, α/σ2, and β2/μ2 hemicomplexes to dileucine-based motifs. The various subunits were expressed in Sf9 insect cells with the hemicomplexes being coexpressed from a bicistronic vector. Cell lysates served as the source of the proteins. The γ/α and β1/β2 subunits were tagged with HA and FLAG epitopes, respectively, to facilitate detection. To rule out the possibility that the overexpressed mammalian adaptin subunits were coassembling with the orthologous subunits of Sf9 cells, affinity-purified β1, β1/μ1, and γ/σ1 were analyzed by silver staining of SDS gels. As shown in Figure 1D, the presence of μ1 adaptin was only evident in the β1/μ1 lane, indicating that the individually expressed β1 subunit was unable to form a hemicomplex with the insect cell μ1 subunit. The lack of μ1 in the purified γ/σ1 lane likewise indicates that this hemicomplex is unable to form a heterotetramer with the endogenous β1/μ1 hemicomplex of Sf9 cells.

As ligands, we used GST peptide fusions (or the full-length protein in the case of NEF) encoding dileucine motifs derived from various proteins as shown in Figure 1C. We initially tested the binding by γ/σ1 and α/σ2 hemicomplexes as well as free γ and α subunits to GST-LRP9-30mer, which contains two acidic dileucine motifs, each of which mediates rapid endocytosis (Knisely and Kornfeld, unpublished data). As shown in Figure 1, E and F, both hemicomplexes bound well to the GST-LRP9 dileucine-based motifs, whereas only trace binding occurred with the individual γ and α subunits. However, the free γ and α subunits bound robustly to GST-amphiphysin and GST-NECAP1, respectively. This binding, previously shown to be mediated by the interaction of the appendage domains of γ and α to WXXF/W motifs present in these proteins (Drake and Traub, 2001; Ritter *et al.*, 2003) indicates that the γ and α subunit appendage domains had folded properly in the insect cells. When σ1 was expressed individually in the Sf9 cells, the yield was very poor compared with γ/σ1 coexpression and the protein that was recovered was almost completely in a high-molecular-weight aggregate. This prevented us from testing the σ1 subunit directly in the pull-down assays.

We next tested the ability of the γ/σ1 and α/σ2 hemicomplexes to bind to a panel of dileucine-based motifs derived

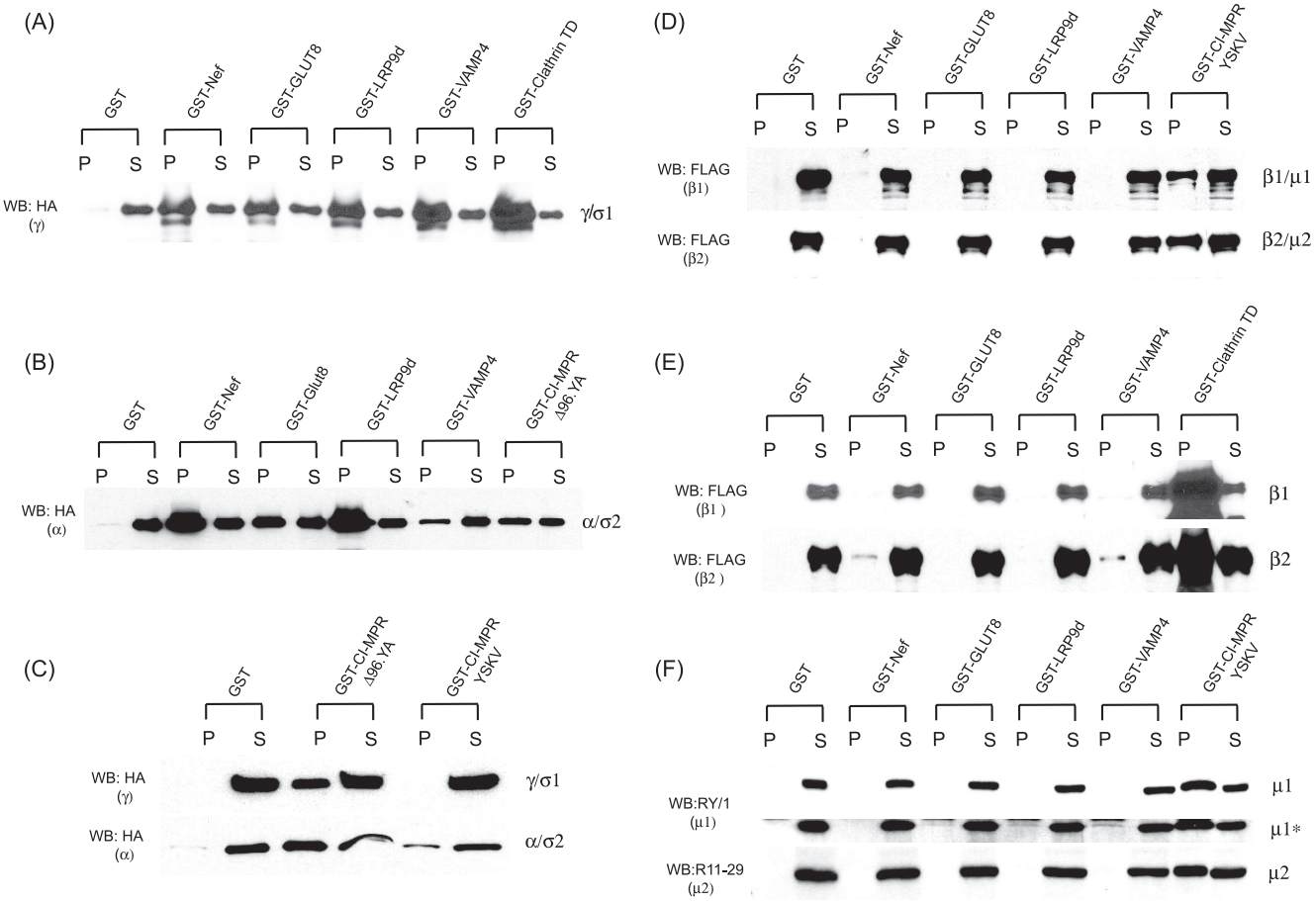

Figure 2. Dileucine-based motifs bind selectively to the $\gamma/\sigma1$ and the $\alpha/\sigma2$ hemicomplex. (A–F) The different subunits of AP-1 and AP-2 were tested for their ability to bind to a panel of dileucine-based motifs derived from the indicated proteins. GST-clathrin TD and GST-YSKV served as positive controls in the binding assays for the β and μ subunits, respectively. Binding assays were performed as described in *Materials and Methods*, and Western blots were probed with the indicated antibodies. $\mu1$* indicates a 5X longer exposure of the $\mu1$ blot.

from proteins where these motifs have been implicated in AP-1 and/or AP-2 binding. As shown in Figure 2, A and B, both hemicomplexes bound to each of the dileucine-based motifs, although there were differences in the degree of the binding. Thus, the $\gamma/\sigma1$ hemicomplex bound the Vamp4 dileucine-based motif much better than it bound the NEF motif, whereas the opposite occurred in the case of $\alpha/\sigma2$. The basis for these differences and the implication for protein trafficking are explored in more detail in a later section of this article under the subheading *Roles of the Residues Adjacent to the Position 0 Leucine in Hemicomplex Binding*. Although the $\gamma/\sigma1$ hemicomplex bound to multiple dileucine-based motifs, it exhibited no binding to a typical Y-based motif (Figure 2C). We consistently observed that the $\alpha/\sigma2$ hemicomplex displayed a low level of binding above background levels for the CI-MPR Y-based motif in our pull-down assays, but this binding was significantly weaker relative to the dileucine-based motif (Figure 2C).

In contrast to the findings with the $\gamma/\sigma1$ and $\alpha/\sigma2$ hemicomplexes, the $\beta1/\mu1$ and $\beta2/\mu2$ hemicomplexes as well as the individual β and μ subunits exhibited either undetectable binding to the panel of GST-dileucine–based motifs or trace binding that could at times only be detected after prolonged exposure of the blots (Figure 2, D–F). As a positive control, both β/μ hemicomplexes as well as the individual μ subunits bound well to the YSKV motif of the CI-MPR. This motif has been shown to be important for the interaction of the CI-MPR tail with cytosolic AP-1 (Ghosh and Kornfeld, 2004) and for internalization of the receptor from the cell surface (Jadot *et al*., 1992), presumably through its association with the μ subunits of AP-1 and AP-2. There was no binding above background level of the isolated β subunits to the GST-YSKV (data not shown), indicating that the β/μ hemicomplexes bound via their μ subunits. The β subunits, instead, bound well to the GST-clathrin TD as expected (Shih *et al*., 1995), demonstrating that the peptide sequences in the hinge of the β subunits responsible for binding to the terminal domain were exposed. Mutations of the dileucine sequences to dialanine in both GST-CIMPR-Δ96.YA and GST-LRP9-d17mer abolished binding by the $\gamma/\sigma1$ hemicomplex, indicating the specificity of the interaction (Figure 3, A and B). Similar results were obtained with the $\alpha/\sigma2$ hemicomplex (Figure 3, B and C).

Together, these results clearly demonstrate that the $\gamma/\sigma1$ and $\alpha/\sigma2$ hemicomplexes bind well to dileucine-based motifs, whereas the $\beta1/\mu1$ and $\beta2/\mu2$ hemicomplexes as well as the individual γ, α, β, and μ subunits bind poorly, or not at all, to these motifs.

The Appendage and Hinge Regions of γ and α Are Not Required for Hemicomplex Binding to Dileucine-based Signals

The crystal structure of the AP-1 core reveals that the $\sigma1$ subunit interacts with the N-terminal half of the γ subunit

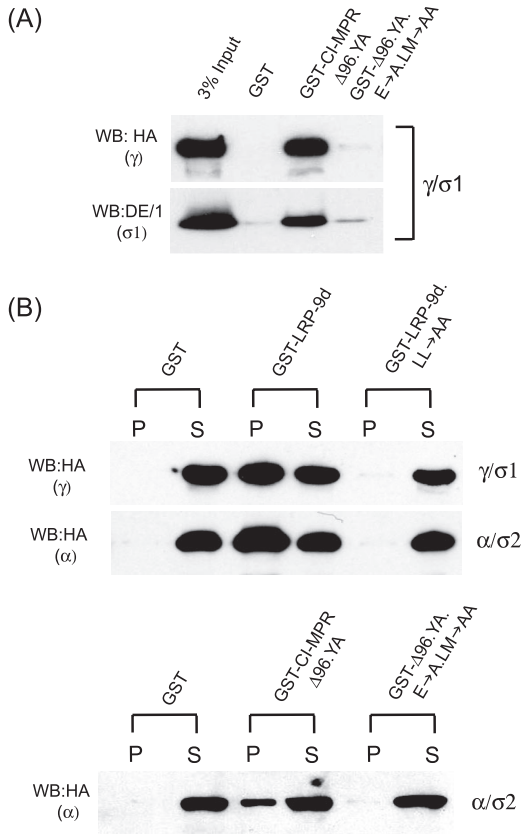

Figure 3. Mutation of dileucine sequence to dialanine abolishes hemicomplex binding. (A–C) The specificity of the interaction of the γ/σ1 and α/σ2 hemicomplexes for either the CI-MPR internal dileucine-based sequence or the LRP9 distal dileucine-based sequence was tested by mutation of the CI-MPR **E**TEW**LM** sequence to **A**TEW**AA** and the LRP9-d17mer EDEP**LL** sequence to EDEP**AA**.

(Heldwein *et al*., 2004). Furthermore, it has been reported that the γ/σ1 hemicomplex does not require the hinge and appendage domains of γ for interacting with dileucine-based motifs (Janvier *et al*., 2003). However, in that study, the 595 amino acid trunk domain was left intact. By expressing a series of γ/σ1 hemicomplexes with progressive deletions from the C-terminal portion of γ trunk, we have determined that the N-terminal 341 amino acids of the γ trunk are sufficient to form a competent dileucine-binding hemicomplex with σ1 (Figure 4, A and B). This constitutes the portion of the trunk that interacts with the σ1 subunit (Heldwein *et al*., 2004). This result is also in agreement with the findings of Page and Robinson (1995), showing that the N-terminal 331 amino acids of the γ trunk are sufficient to target a γ-α chimeric protein to the TGN. Our own observation that the γ subunit is incapable of forming a competent dileucine-binding hemicomplex with the σ2 subunit in Sf9 cells (data not shown), together with the results of previous yeast two- and three-hybrid studies (Page and Robinson, 1995; Janvier *et al*., 2003), indicate that the ability of either the γ or α subunit to form a hemicomplex with its cognate σ adaptin subunit is critical for both proper targeting (TGN or plasma membrane) and binding to sorting signals.

In our pull-down experiment, we also examined the effect of a soluble 14-amino acid peptide containing the dileucine motif of the CI-MPR (**E**TEW**LM**) on the binding of the truncated γ/σ1 hemicomplex to two GST-fusions. A peptide with the same sequence except for E → A and LM → AA substitutions was used a control. As shown in Figure 4B, the wild-type peptide, at 200 μM, strongly inhibited binding to both GST-fusion proteins, whereas the mutant peptide at the same concentration was without effect. These findings provide additional evidence that the γ/σ1 hemicomplex is actually binding to dileucine-based motifs.

We also prepared a α/σ2 hemicomplex with the α subunit truncated at residue 623, resulting in loss of the hinge and appendage domains of that subunit. Figure 4C, shows that this mutant hemicomplex bound well to the GST-LRP9-30mer, whereas the truncated α subunit by itself exhibited only trace binding over the GST control. Thus, the hinge and appendage of the α subunit is not required for binding of the α/σ2 hemicomplex to dileucine-based motifs.

Role of the Residues Adjacent to the Position 0 Leucine in Hemicomplex Binding

During the course of these experiments, we noted several instances where individual dileucine-based motifs bound differently to the γ/σ1 and α/σ2 hemicomplexes. An example of this involving the binding of the Vamp4 and NEF motifs was pointed out in an earlier section of the article under the subheading *Dileucine-based Motifs Bind Selectively to the γ/σ1 and α/σ2 Hemicomplexes of AP-1 and AP-2* (Figure 2, A and B). Another instance concerns the proximal (EDDVLL) and distal (EDEPLL) dileucine-based motifs of LRP9. The α/σ2 hemicomplex bound both motifs well, whereas the γ/σ1 hemicomplex only bound well to the distal motif (Figure 5A). An alignment of these sequences shows only two amino acid differences, at the −1 and −2 positions. Hence, we mutated the aspartate and valine of the proximal motif, either individually or together, to glutamate and proline, respectively, to see whether binding to the γ/σ1 hemicomplex could be achieved. As shown in Figure 5B, the individual substitution of the aspartate to glutamate at position −2 had a modest impact on γ/σ1 binding, whereas the valine-to-proline substitution at position −1 had only a very small effect. However, when the two residues were simultaneously mutated, strong binding was achieved, equivalent to that obtained with the distal dileucine-based motif. These results demonstrate that amino acids immediately upstream of the dileucine sequence can significantly impact the specificity of the interaction with different hemicomplexes.

In contrast to the dileucine-based motifs of LRP9, both of which bind well to the α/σ2 hemicomplex, the internal dileucine motif of the CI-MPR (ETEWLM) binds better to the γ/σ1 hemicomplex than to the α/σ2 hemicomplex (Figure 5C). Mutation of the −1 residue from W to P, as occurs in the LRP9 distal dileucine motif, had no substantial impact on binding (Figure 5C). However, when the LM sequence was changed to LL, there was a major increase in binding by the α/σ2 hemicomplex (Figure 5C) with no alteration in binding by γ/σ1. This indicates that the two hemicomplexes differ in their preference for the +1 residue.

We have reported that rapid internalization of the CI-MPR is dependent on the tyrosine-based YSKV motif in the cytosolic tail of the protein (Jadot *et al*., 1992). Because the mutant CI-MPR tail internal dileucine-based sequence (LM → LL) bound the α/σ2 hemicomplex much better than did the native sequence, we asked whether this mutant sequence could compensate for the tyrosine mutation in the context of the LRP9-CI-MPR tail chimeric fusion in the in vivo setting. To answer this question, we performed cell surface biotinylation experiments on cells transfected with the chimera containing either the wild-type CI-MPR tail, the YSKV → ASKA mutation, or the YSKV → ASKA and ETEWLM → ETEPLL double mutation. The results presented in Figure

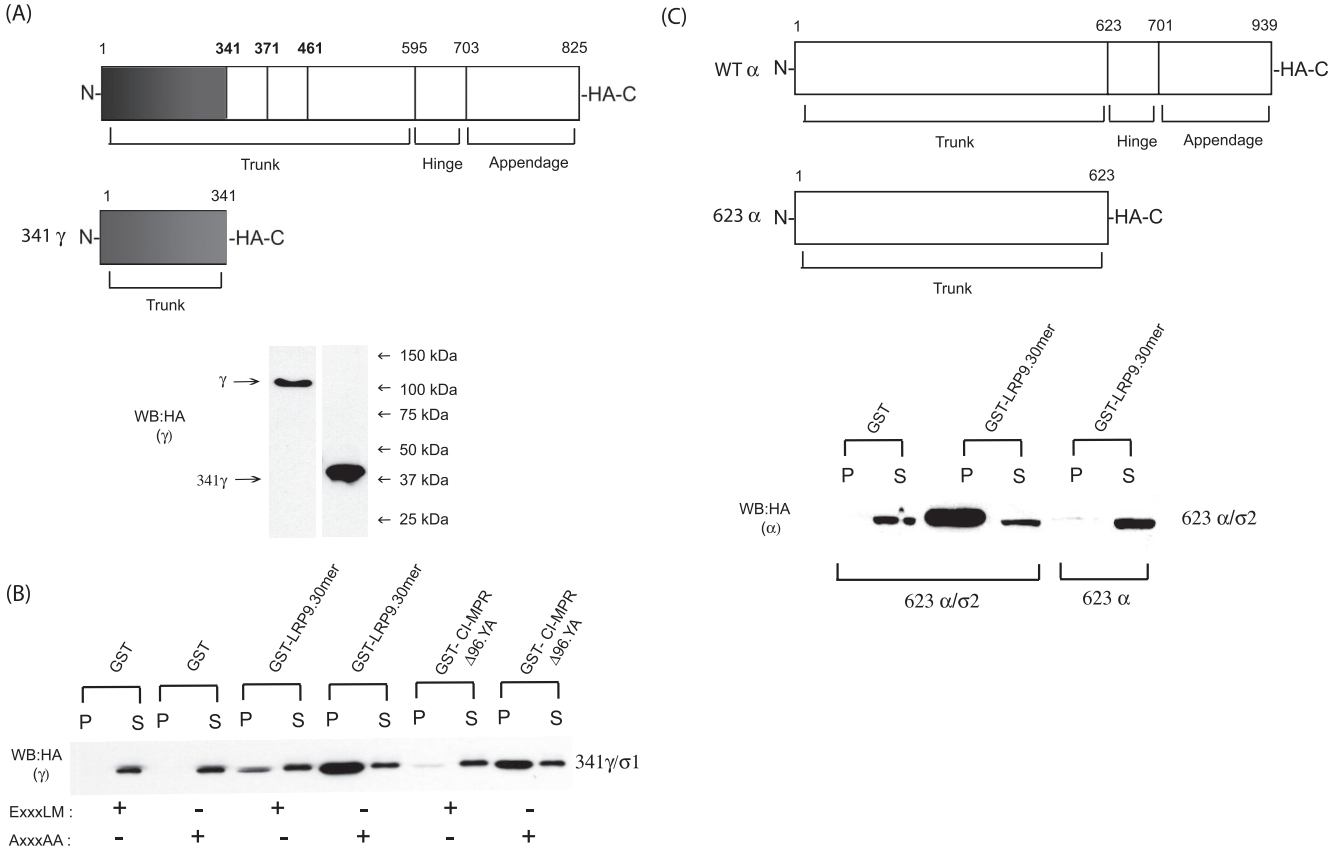

Figure 4. Hemicomplex binding to dileucine-based motifs does not require the appendage and hinge regions of AP-1 and AP-2. (A) The N-terminal 341 amino acids of γ trunk are sufficient to form a hemicomplex with σ1. (B) Competition experiments with the γ341/σ1 hemicomplex were performed with either wild-type (**E**TEW**LM**) or mutant (**A**TEW**AA**) soluble peptides at 200 μM concentration as described in *Materials and Methods*. (C) α Adaptin(1-623) was expressed in Sf9 cells with or without the σ2 and tested for its ability to bind the LRP9 dileucine-based motifs by using the GST pull-down assay.

5D show that the tyrosine mutation, as expected, strikingly increased the level of chimeric protein on the cell surface. The ETEWLM → ETEPLL mutation, which significantly enhanced binding of the α/σ2 hemicomplex in our in vitro binding assays, reversed the effect of the tyrosine mutation, indicating that improved binding to the α/σ2 hemicomplex is recapitulated in in vivo trafficking events.

Role of the Residue at the −4 Position of Dileucine-based Motifs

Aside from a few exceptions, the dileucine-based motifs reported to date have either a glutamate or aspartate at the −4 position (Bonifacino and Traub, 2003). Because all the dileucine sequences tested thus far in this study had a glutamate residue at that position, we asked whether interaction with the two different hemicomplexes would be perturbed by substituting the relevant glutamate for either an aspartate, alanine, or arginine. We initially substituted the −4 glutamate in GST-CIMPR-Δ96.YA and determined the effect on γ/σ1 and α/σ2 hemicomplex binding in GST pull-down assays. As shown in Figure 6A, the E → A substitution greatly decreased the binding of both hemicomplexes, as expected. Surprisingly, the E → D substitution almost completely abrogated binding by the γ/σ1 hemicomplex, whereas only having a modest effect on α/σ2 binding. Mutation of the −4 glutamate of the Vamp4 dileucine motif to an aspartate also led to loss of γ/σ1 binding (Figure 6A). Similarly, the E → D substitution of the GST-LRP9-d17mer construct resulted in a significant loss of binding by the γ/σ1 hemicomplex with only a minimal effect on α/σ2 binding (Figure 6B). An E → R mutation at the −4 position of GST-LRP9d17mer resulted in complete loss of binding by both γ/σ1 and α/σ2, indicating that a basic residue cannot be tolerated at this position for hemicomplex binding (Figure 6B).

To validate that the pull-down assays correlated with in vivo protein trafficking, we examined the consequence of the LL → AA and E → D substitutions on the distribution and internalization of either LRP9 or the mLRP4-LRP9 chimera, respectively, transfected into LRP1-null CHO cells. The latter is presumably a measure of interaction with AP-2. The LRP9 cytosolic tails used in these experiments had the proximal dileucine-based motif mutated, so that its trafficking was solely dependent on the distal dileucine sequence (Knisely and Kornfeld, unpublished data). Similar to wild-type LRP9 (data not shown), this protein was predominantly localized to intracellular vesicles that had substantial overlap with the CI-MPR (Figure 7A). When the distal dileucines were changed to alanines, the mutant protein accumulated on the cell surface, confirming that its internalization was dependent on the distal dileucine-based motif (Figure 7B). Like the LL → AA mutation, the −4 E → R substitution within the distal dileucine-based sequence also resulted in a predominantly cell surface distribution (data not shown), indicating that substitution of a basic amino acid for the acidic residue at this position is detrimental to the internalization of LRP9. A similar observation has been made with the glutamate to argi-

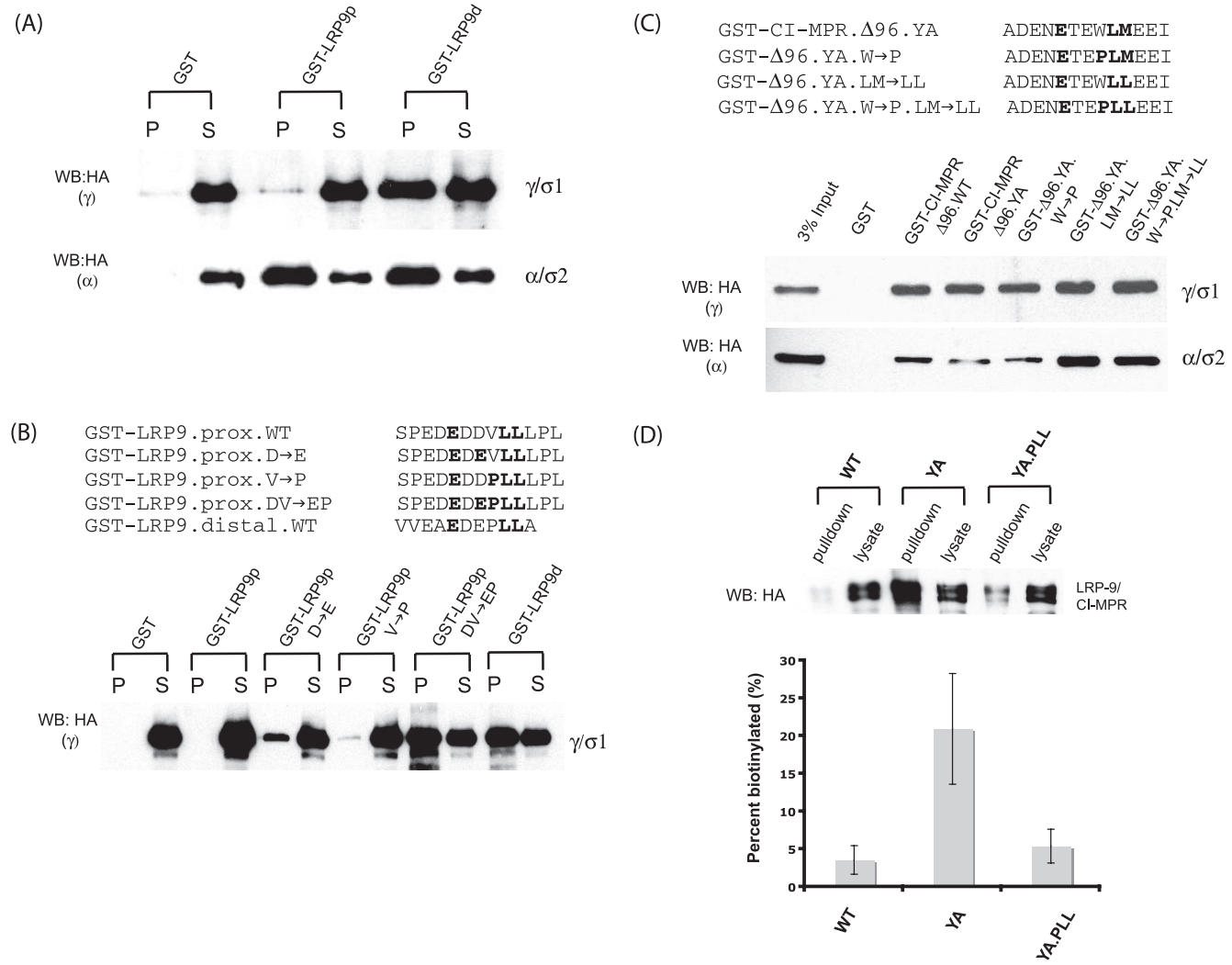

Figure 5. Residues adjacent to the leucine at position 0 can dictate avidity and specificity of hemicomplex binding to particular dileucine-based motifs. (A) The ability of the γ/σ1 and α/σ2 hemicomplexes to bind to the LRP9 distal and proximal dileucine-based motifs was assessed by GST pull-down assays performed simultaneously. (B) Mutations of residues at the −1 and −2 positions were introduced singly or together within the LRP9 proximal dileucine-based sequence to see whether binding of the γ/σ1 hemicomplex could be achieved. (C) Substitutions within the CI-MPR internal dileucine-based sequence were assessed for changes in avidity for the γ/σ 1or α/σ2 hemicomplex binding by using the GST pull-down assay. (D) Cell surface biotinylation was performed to test the ability of the altered CI-MPR dileucine-based sequence (ETEWLM → ETEPLL) to rescue the tyrosine mutation as described in *Materials and Methods*.

nine mutation within the LIMPII dileucine-based sequence (Sandoval *et al*., 2000). In contrast, LRP9 with an E → D substitution at the −4 position achieved a distribution indistinguishable from the protein with the wild-type sequence (Figure 7C), consistent with the substitution having only a minor effect on the interaction with AP-2 (Figure 6B). As a complement to these morphological assays, we performed kinetic studies to determine the rate of internalization of these constructs. In these assays, we used mLRP4-LRP9 chimeras consisting of the extracellular (ligand-binding repeat domain 4) and transmembrane domains of LRP1 fused to the cytoplasmic tail of LRP9. Iodinated antibodies against the HA epitope within the mLRP4 domain on the cell surface were bound at 4°C, and after warming the cells to 37°C, the rate of internalization of the antibody followed for 10 min (Figure 7D). These assays showed that the E → D substitution had no effect on the rate of endocytosis, whereas the LL → AA mutation abolished rapid internalization. Together, these studies establish an excellent correlation between the pull-down assays involving the α/σ2 hemicomplex and the in vivo behavior of the protein in AP-2–mediated processes. Unfortunately, there are no assays available at this time that would allow us to determine in a selective manner the interaction of the various dileucine-based motifs with AP-1 in intact cells.

DISCUSSION

The data presented in this study provide strong evidence that it is the γ/σ1 and α/σ2 hemicomplexes of AP-1 and AP-2, respectively, rather than the β/μ hemicomplexes that bind dileucine-based motifs. The findings with the γ/σ1 hemicomplex confirm the results obtained with the yeast three-hybrid assay (Janvier *et al*., 2003; Coleman *et al*., 2005, 2006; Theos *et al*., 2005). However, that assay failed to detect any interaction between dileucine motifs and the α/σ2 hemicomplex, leaving unresolved the question of how AP-2 binds dileucine-based motifs. The results of our binding experiments with the individual subunits and hemicomplex

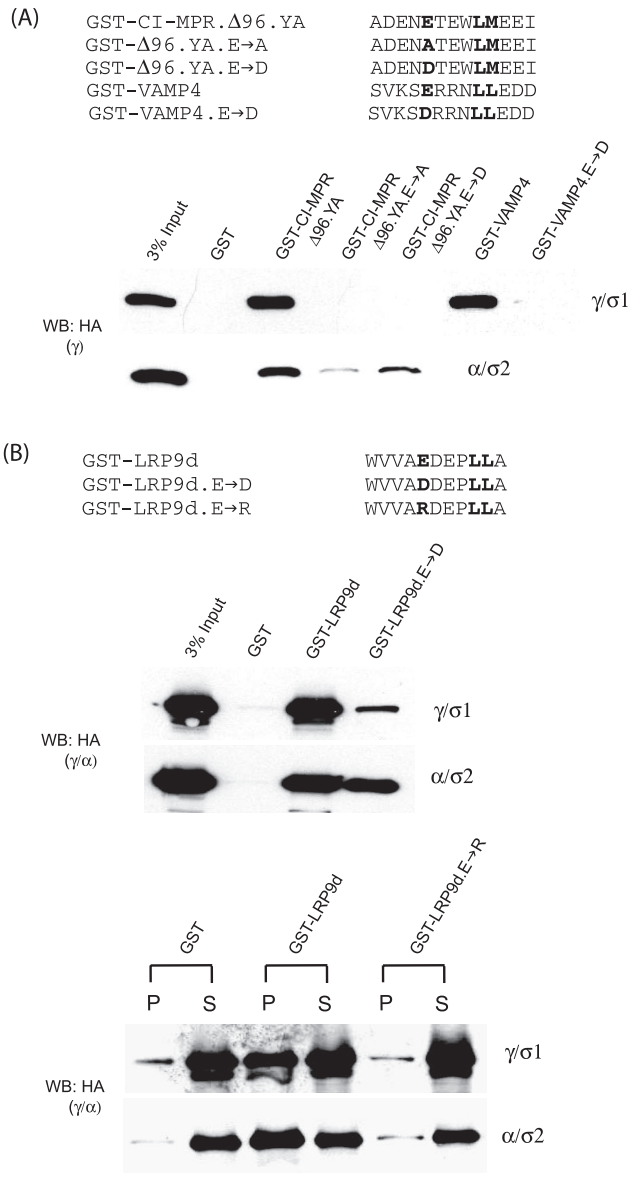

Figure 6. An aspartate cannot substitute for the −4 glutamate for $\gamma/\sigma1$ binding but can do so for $\alpha/\sigma2$ binding. (A–C) GST pull-down assays were performed with either the wild-type dileucine-based motifs of CI-MPR, Vamp4, and LRP9, or the −4 glutamate of these sequences mutated to an alanine or an aspartate (A and B), or an arginine (C). Binding assays were performed with the $\gamma/\sigma1$ and $\alpha/\sigma2$ hemicomplexes as described in *Materials and Methods*.

forms of AP-1 and AP-1 expressed in Sf9 insect cells establish that both AP-1 andAP-2 recognize dileucine-based motifs via a similar mechanism. Furthermore, our demonstration that the exogenously expressed mammalian adaptin subunits do not associate with endogenous insect cell adaptins gives us confidence that the sorting signal–adaptin interactions we report in this study are highly specific for the adaptin subunits in question (Figure 1D).

In our experiments the β/μ hemicomplexes as well as the individual μ subunits exhibited good binding to a known tyrosine-based signal (YSKV of the CI-MPR), but they failed to display appreciable binding to any of the dileucine-based sequences tested in the GST pull-down assays. This is in contrast to previous studies showing either the β subunit (Greenberg *et al*., 1998; Rapoport *et al*., 1998; Geyer *et al*., 2002; Schmidt *et al*., 2006) or the μ subunit (Rodionov and Bakke, 1998; Hofmann *et al*., 1999; Craig *et al*., 2000; Rodionov *et al*., 2002; Hinners *et al*., 2003) as being the AP complex subunit responsible for recognition of dileucine-based sorting motifs. In our experiments with the free β and μ subunits, we did detect very low levels of binding to selected dileucine-based motifs upon longer exposure of the blots (Figure 2, E and F). Perhaps these weak interactions may account for the results obtained in the previous yeast two-hybrid and surface plasmon resonance studies, because these assays are more sensitive than the GST pull-down technique. Nevertheless, because our assays tested all four hemicomplexes as well as the individual subunits (with the exception of $\sigma1$ and $\sigma2$) in side-by-side comparisons, we believe that our findings strongly favor the conclusion that it is the $\gamma/\sigma1$ and $\alpha/\sigma2$ hemicomplexes that mediate binding of dileucine-based motifs, whereas the μ subunits bind tyrosine-based motifs.

Although both the $\gamma/\sigma1$ and $\alpha/\sigma2$ hemicomplexes bound dileucine-based motifs, they exhibited considerable differences in their preferences for the various sequences that were tested. Based on numerous reports in the literature, most of which deal with the function of dileucine-based motifs in endocytosis, a general sequence of $^{-4}$D/E^{-3}X^{-2}X^{-1}X^{0}L^{+1}[LMI] has been defined (Bonifacino and Traub, 2003). Our data show that residues upstream and downstream of the leucine at position 0 can selectively influence binding to the AP-1 $\gamma/\sigma1$ and AP-2 $\alpha/\sigma2$ hemicomplexes. The most striking example of this was seen when we compared the effect of having an aspartate as opposed to a glutamate at the −4 position. Both hemicomplexes bound well when a glutamate was present, but an aspartate at this position severely compromised binding by the $\gamma/\sigma1$ hemicomplex, whereas only having a modest effect on $\alpha/\sigma2$ hemicomplex binding. This result was obtained with three dileucine motifs that differed in the residues in the −1 to −3 positions (Figure 6). In agreement with the lack of effect on $\alpha/\sigma2$ hemicomplex binding, the E → D mutation at position −4 did not alter the rate of endocytosis or cellular distribution of LRP9 (Figure 7, A and B). Unfortunately, the lack of an in vivo assay system that is specific for AP-1 precluded us from testing the effect of this mutation in the in vivo setting. Nonetheless, these findings demonstrate that $\gamma/\sigma1$, unlike $\alpha/\sigma2$, is much less tolerant of an aspartate in the −4 position compared with a glutamate in the same position. This observation may provide an explanation for why the DKQTLL sequence of CD3-γ failed to bind to $\gamma/\sigma1$ in the yeast three-hybrid assay, unlike the other dileucine-based sequences that were tested, all of which had a glutamate at the −4 position (Coleman *et al*., 2005). Our results also suggest, as has been alluded to previously (Rodionov *et al*., 2002), that the $\alpha/\sigma2$ hemicomplex of AP-2 may display a broader specificity in terms of recognizing dileucine-based motifs to ensure the efficient internalization of proteins that missort to the cell surface.

The LRP9 proximal and distal dileucine-based sequences illustrate how specificity of hemicomplex binding is influenced by residues in the −1 and −2 positions. These two sequences are identical except for the residues at these two positions (Figure 1C). In this case, the $\gamma/\sigma1$ hemicomplex binds only the distal sequence, whereas the $\alpha/\sigma2$ hemicomplex binds both sequences equally well (Figure 5, A and B), in agreement with our finding that either sequence can function efficiently in endocytosis (Knisely and Kornfeld, unpublished data).

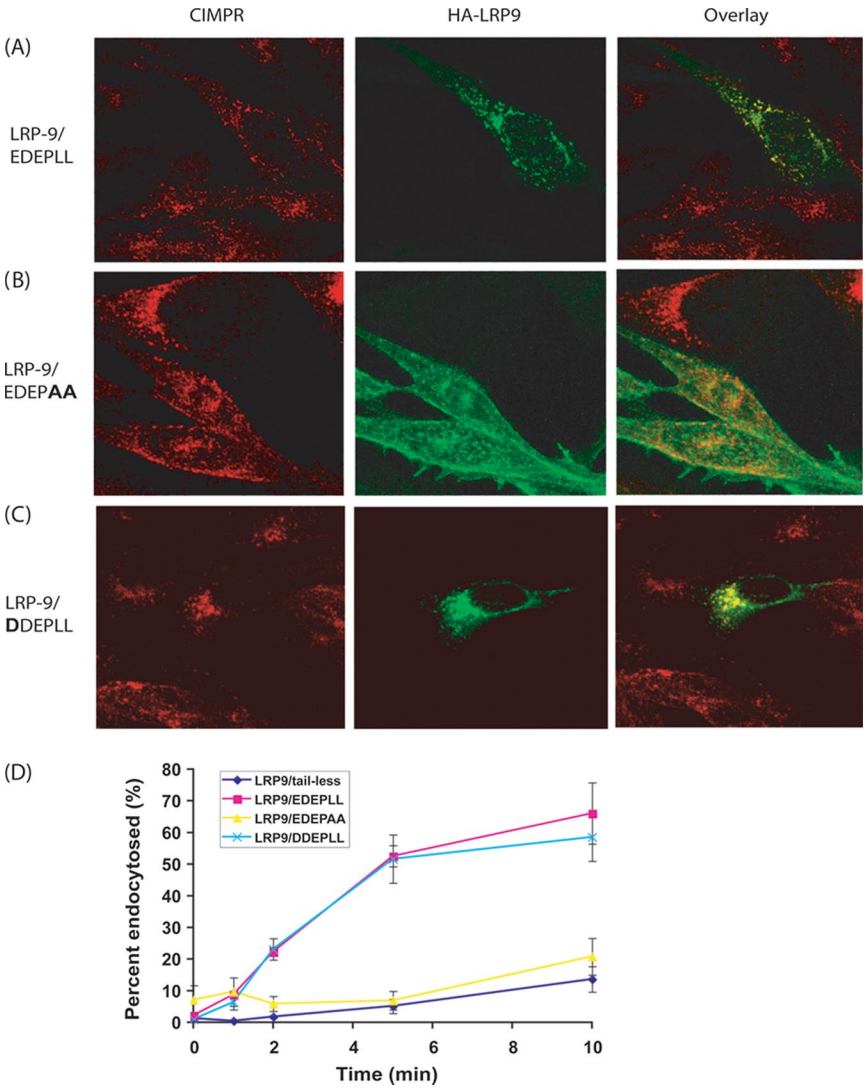

Figure 7. $\alpha/\sigma 2$ binding to the variant LRP9 distal dileucine-based sequences correlates strongly with in vivo internalization mediated by these sequences. (A) The subcellular distribution of transiently expressing LRP9 (green) with the proximal dileucine-based motif mutated was analyzed along with endogenous CI-MPR (red) in CHO cells. Colocalization (yellow) is observed by merging the green and red signals. The distribution of the distal LL → AA and −4 E → D mutants in the context of the LRP9 proximal LL → AA construct is shown in B and C, respectively. (D) Kinetic analysis of endocytosis of the mLRP4-LRP9 tail constructs was performed as described in *Materials and Methods*. The mLRP4 tail-less construct served as the negative control in these assays.

An example of the role of the residue at position +1 in determining specificity for the hemicomplexes is illustrated by our mutagenesis study whereby a single substitution within the CI-MPR internal dileucine-based sequence (ETEWLM → ETEWLL) resulted in a significant increase in binding of the $\alpha/\sigma 2$ hemicomplex to the altered sequence (Figure 5C). That this change in hemicomplex binding in vitro is physiologically relevant in vivo is shown by the ability of the altered dileucine sequence, but not the original sequence, to compensate for the tyrosine mutation in the CI-MPR tail in internalization (Figure 5D). This process is presumably mediated by AP-2.

Together, these results show that the interaction of any particular D/EXXXL[LMI] sequence with either the AP-1 $\gamma/\sigma 1$ or the AP-2 $\alpha/\sigma 2$ hemicomplex will be dictated by the nature of the amino acids at the −1 to −4 positions, and the +1 position. The nature of these residues can determine whether a functional interaction will occur at the various sorting stations within the cell. Interestingly, Bakke and colleagues have made similar observations, although their reported interactions of the dileucine-based sequences were with the μ subunits of AP-1 and AP-2 (Hofmann *et al.*, 1999; Rodionov *et al.*, 2002).

Although our results establish that both the $\gamma/\sigma 1$ and $\alpha/\sigma 2$ hemicomplexes bind well to dileucine-based motifs, the question remains as to precisely how these motifs engage the hemicomplexes. The studies using various truncations of the γ subunit revealed that the N-terminal 341 amino acids of γ, representing the amino half of the trunk domain, when coexpressed with $\sigma 1$, are sufficient to form a hemicomplex that binds dileucine-based motifs just as well as the complex containing the full-length γ subunit. This finding localizes the binding site to this region of the $\gamma/\sigma 1$ hemicomplex. The γ and α subunits by themselves exhibited only trace binding to the dileucine-based motifs. These subunits bound strongly to WXXW/F motifs that are known to interact with the appendage domains of the protein, showing that the appendages had folded correctly in the insect cells. However, this does not prove that the trunk domains had also folded properly, leaving open the possibility that dileucine-based motifs interact with elements of the N-terminal half of the γ trunk. Unfortunately, the $\sigma 1$ subunit, when expressed alone, formed an insoluble aggregate that prevented us from testing this subunit for its ability to bind the dileucine-based motifs. This suggests that the $\sigma 1$ subunit requires the N-terminal region of γ to fold correctly, consis-

tent with the reported structure of the AP-1 core, which revealed that σ1 interacts with this portion of the γ subunit (Heldwein *et al*., 2004). At this point, three models could explain our findings. The binding pocket for dileucine-based motifs could either reside on the σ subunits or the trunk regions of the γ and α subunits, or they could be formed by elements derived from both the σ subunits and the N-terminal trunk regions of γ and α that interact with σ. Efforts to characterize the precise binding site for dileucine-based motifs within the γ/σ1 hemicomplex are currently underway in our laboratory. Nevertheless, we think that the experimental evidence presented in this study establish that the σ subunits with their cognate partners comprise the bona fide dileucine binding sites of the AP-1 and AP-2 complexes.

While this article was under revision, it was reported by Bonifacino and colleagues (Chaudhuri *et al*., 2007; published online ahead of print on Jan. 31, 2007, in the Journal of Virology) that the Nef EXXXLL motif binds to the α/σ2 hemicomplex, in agreement with our data.

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