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A role for the hinge/ear domain of the β chains in the incorporation of AP complexes into clathrin-coated pits and coated vesicles

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Abstract. The clathrin-associated adaptor protein (AP) complexes drive the polymerization of clathrin in coated pits to form coated vesicles. It has previously been shown that the carboxyl-terminal hinge/ear domain of the $\beta 2$ chain contains a binding site for clathrin and that removal of this domain from APs or from isolated $\beta 2$ chains abrogates their ability to form clathrin coats in vitro. We show here that the hinge/ear domain is necessary for efficient incorporation of AP complexes into coated pits and coated vesicles in cells, a result that is consistent with the view that the β chains indeed provide an important interaction between the AP complexes and clathrin.
Key words. Coated pits; vesicles; endocytosis; adaptors; coat assembly.

Clathrin coated vesicles represent the main pathway for the vesicular traffic to the endosomal compartment originating from the plasma membrane or the trans-Golgi network. It has been estimated that formation of a single coated vesicle takes less than a minute. This very rapid process demands a highly coordinated interaction of hundreds of molecules, including formation of the clathrin coat structure and recognition and selection of the cargo molecules.

The clathrin-associated adaptor protein (AP) complexes are large heterotetrameric structures [1, 2] located between the membrane and the clathrin lattice [3, 4]. There is growing evidence that these assemblies are responsible both for clathrin recruitment and for cargo selection. Two major AP complexes are known, AP-1 and AP-2, which selectively localize to the trans-Golgi network and to the plasma membrane, respectively (for review see ref. 5). Their role in coat assembly is exemplified by the capacity of APs to facilitate the formation of coats by a co-assembling with clathrin in vitro [2, 6, 7] and by their stimulatory effect in the formation of coated pits in a broken cell system [8]. APs also fulfil a role as the sorting component in the clathrin-dependent traffic pathway recognizing membrane proteins targeted for internalization. This function of APs was originally suggested by in vitro binding studies [9–13] and more recently by the demonstration that AP-2 associates with the epidermal growth factor receptors in cells [12–17]. Indeed, the $\mu 2$ chain of AP-2 'reads' the endocytic Y-motif found in the cytoplasmic tail of membrane proteins internalized by coated vesicles [18–20].

The composition of APs has been defined, based on the biochemical characterization of AP complexes isolated from bovine brain coated vesicles, as γ, $\beta 1$, $\mu 1$ and $\sigma 1$ for AP-1 and α, $\beta 2$, $\mu 2$ and $\sigma 2$ for AP-2 (reviewed in ref. 21). The α, $\beta 1/\beta 2$ and γ chains, collectively known as the 'large chains' or 'adaptins', each have two major domains, the amino-terminal trunk and the carboxyl-terminal ear, connected by a hinge region [2, 22, 23]. We have recently shown by in vitro experiments that the hinge/ear region of the $\beta 2$ chain is necessary and sufficient to drive clathrin coat formation [24, 25] and that the clathrin binding site is located in a 50-amino acid conserved region of the hinge [25]. Although previous work using elastase-treated plasma membrane sheets had suggested that the formation of coated pits could occur in the absence of the hinge/ear regions of the large chains [26], proteolytically 'shaved' AP-1, lacking the hinge/ear domains of the $\beta 1$ and γ chains, is unable to direct the recruitment of clathrin onto isolated Golgi membranes [27]. Moreover, AP-1 or AP-2 complexes, lacking all of their large-chain ears, are unable to drive the polymerization of clathrin into coats [28].

To determine whether the β hinge/ear is critical for clathrin recruitment, we examined the incorporation of AP complexes containing epitope-tagged truncated $\beta 1$ and $\beta 2$ chains into clathrin-coated vesicles in transfected CHO cells. Complexes containing the truncated chains, which lack the entire hinge/ear, are markedly deficient in incorporation into vesicles, whereas complexes containing epitope-tagged recombinant full-length chains are incorporated normally. We therefore propose that the β hinge/ear is indeed a principal determinant in APs for clathrin association in intact cells.

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Materials and methods

Full length cDNA clones for β1 and β2. The previously described full length cDNA clone referred to as βa [23] corresponds to the β1 chain of rat AP-1 complexes by virtue of sequence identity between segments of the predicted open reading frame and the amino-terminal amino acid sequence determined by automated Edman degradation of tryptic peptides from purified bovine brain β1 chains [23]. The DNA sequence of five independent cDNA clones revealed that two of them have an insertion of nucleotides GACTTGGAG following position 2814. This insertion introduces residues DLE at T_{925} located in the ear domain at the carboxyl-terminal of β1. Clone OK711, which contains the insertion, was used in this study.

The rat brain β2 cDNA, previously referred to as βb [23], lacks part of the open reading frame at its 5′-end. The missing portion was obtained from an additional screen of a rat brain cDNA library and a full length β2 cDNA created by appropriate ligation.

Generation of β1 and β2 epitope-tagged chains for expression in mammalian cells. Amino-terminal tagged β1 (HAβ1) and β2 (HAβ2) chains containing the sequence MGYPYDVPDYA instead of the initiator M were obtained using polymerase chain reactions and other standard molecular biology methods. The inserted sequence corresponds to the hemagglutinin epitope tag YPYDVPDYA recognized by the monoclonal antibody 12CA5 [29]. The truncated epitope-tagged forms of β1 and β2 (HAβ1-trunk and HAβ2-trunk), which contain the amino-terminal 591 residues and lack their carboxyl-terminal hinge and ear domains, were generated in a similar way. An amino-terminal HA-tagged rat brain clathrin light LCb3 chain (HALCb3) was used as a positive control for protein expression and for incorporation into the coat of clathrin coated vesicles. The DNA constructs were inserted into the vector pCMV for expression under control of the CMV promoter. The DNA sequences of the portions generated by polymerase chain reaction were verified by DNA sequencing.

Establishment of cell lines stably expressing the tagged chains. DNA constructs were purified by two successive CsCl gradients and used to transfect the Chinese hamster ovary CHO K1 cell line using Transfectam (IBF, Columbia, MD; Sepracor, Marlborough, MA; or Promega, Madison, WI) for HAβ1, HAβ1-trunk, HAβ2-trunk, HALCb3 and the vector pCMV. Calcium phosphate transfection was used to generate the cell line expressing HAβ2. Selection of expressor cells was achieved by cotransfection with the vector pSV2-neo [30] and growth in the presence of G418 (Geneticin; GIBCO-BRL, Gaithersburg, MD) for HAβ1 and HAβ1-trunk, or by cotransfection with the vector pBABE [31] and growth in puromycin (Calbiochem, La Jolla, CA) for HAβ2, HAβ2-trunk, HALCb3 and pCMV. Resulting cell lines expressing the tagged proteins were subcloned by serial dilution two or more times until all subclones were positive by Western blot analysis. Clonal cell lines expressing the highest amount of protein were frozen at earlier passages and used in the experiments.

The CHO cell lines were maintained at 37 °C with 5% CO_2 and 100% humidity in α-MEM (GIBCO-BRL, Gaithersburg, MD) supplemented with penicillin/streptomycin and glutamine (Sigma Co., St. Louis, MO) and 10% (vol./vol.) fetal bovine serum (GIBCO-BRL, Gaithersburg, MD) or 5% (vol./vol.) fetal bovine serum and 5% (vol./vol.) Serum Plus (JRH Biosciences, Lenexa, KS). For selection and growth of the transfected cells, the media contained either 1 mg/ml Geneticin or 2.7 μg/ml puromycin.

Subcellular fractionation. Samples containing AP complexes that are free in the cytosol or in association with clathrin-coated vesicles were obtained from cell lysates by differential centrifugation ending with a Ficoll/sucrose step gradient, essentially as previously described [32].

Briefly, expressor CHO cells were grown to 90–100% confluence in 4–6 150 mm petri dishes, washed twice in phosphate buffered saline (6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.65 mM KCl, pH 7.4) and scraped into 1 ml of buffer A (0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% (wt/vol.) NaN_3, pH 6.5) supplemented with the protease inhibitors 0.5 mM PMSF, 1 mM benzamidine, 1 mM 1,10-phenanthroline, 10 μg/ml leupeptin and 0.05 TIU/ml aprotinin. The scraped cells were subjected to sonication followed by centrifugation at 15,000 rpm in a TLA 100.4 rotor (Beckman Instruments, Fullerton, CA) for 10 minutes at 4 °C. The low speed supernatant was centrifuged at 85,000 rpm in the TLA 100.4 rotor for 20 minutes at 4 °C in order to generate a high speed supernatant and a high speed pellet. This pellet was resuspended in 0.2–0.4 ml of buffer A, diluted with one volume of buffer containing 12.5% (wt/vol.) sucrose and 12.5% (wt/vol.) Ficoll (Pharmacia, Piscataway, NJ), and centrifuged at 30,000 rpm in the TLA 100.4 rotor for 12 minutes at 4 °C. The supernatant was then collected, diluted with four volumes of buffer A and centrifuged at 85,000 rpm in the TLA 100.4 rotor for 20 minutes at 4 °C. About 50% of the vesicles in these samples correspond to clathrin-coated vesicles as determined by negative staining and electron microscopy (not shown). Aliquots of the resulting fractions were mixed with Laemmli sample buffer in the presence of β-mercaptoethanol, boiled for 2 minutes and processed for Western blot analysis [13].

Gel filtration chromatography. 0.2 ml of the high speed supernatants was loaded into a preparative grade Superose 6 gel filtration column (H10/30, Pharmacia),

pre-equilibrated with gel filtration buffer (0.5 M Tris, 1 mM EGTA, 0.02% NaN_3, 0.5 mM DTT, pH 7.4). The samples were eluted at a flow of 0.5 ml/minute and fractions of 0.5 ml were colledted for analysis. 0.2 ml of each fraction was concentrated by adding 0.25 ml 30% chilled TCA and 0.05 ml of lysozyme (0.6 mg/ml) used as carrier. After a 30 minute incubation on ice, samples were centrifuged at 4 °C for 10 minutes in an Eppendorf centrifuge at top speed. Supernatants were carefully removed, and acetone (−20 °C) added to the pellets. After another 10 minute spin, the supernatant was removed and the pellets were allowed to dry. The pellets were then dissolved with 20 µl of Laemmli sample buffer containing, 0.1 M Tris pH 10.0 and boiled for 2 minutes in the presence of β-mercaptoethanol. Aliquots of 20 µl were then processed for Western blot analysis.

Immunoprecipitation of AP complexes. Lysates from expressor CHO cells were obtained from confluent cultures grown in two 150 mm dishes. The cells were washed with phosphate-buffered saline and subsequently scraped off the dish in the presence of 1 ml of lysis buffer (0.5 M Tris-HCl, 0.5% (vol./vol.) Triton X-100, 0.5 mM PMSF, and 20 mM leupeptin, pH 7.4). The lysates were cleared at 4 °C by centrifugation at 15,000 rpm for 15 minutes. The supernatants were saved and the solution adjusted to immunoprecipitation buffer conditions (0·25 M Tris-HCl, 0.1 M NaCl, 0.5% (vol./vol.) Triton X-100, 0.5 mM PMSF, and 20 mM leupeptin, pH 7.4) in a final volume of 2 ml. 20 µl of anti-α or anti-β immunoprecipitating serum containing polyclonal rabbit antibodies [16] was added per ml of lysate, followed by incubation on an end-over-end rotator for 2 hours at 4 °C. 40 µl of Protein A Sepharose beads (50% slurry) was added to each sample and incubated for an additional hour. The beads were washed twice in immunoprecipitation buffer and suspended in Laemmli sample buffer.

Western blot analysis. After SDS-PAGE, proteins were electrotransferred onto nitrocellulose membranes, followed by overnight incubation at 4 °C with the appropriate primary antibody. This was followed by a two hour incubation at 4 °C with the secondary antibody. The signals were visualized by horseradish peroxidase (HRP) and enhanced chemiluminescence (Amersham, Arlington Heights, IL). Different exposures and sample loadings were used for quantitation. Alternatively, the blots were developed by a colorimetric alkaline phosphatase method (Promega, Madison, WI) reaction. The following antibodies were used: mouse monoclonal antibodies 12CA5, specific for the HA tag; AC1-M11, specific for the α chains; B1-M6 or 9A [19, 33], specific for the $\beta 1/\beta 2$ chains, and a mixture of rabbit polyclonal antibodies specific for the hinge and amino-terminal portion of the γ chain. Goat polyclonal horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Amersham, Arlington Heights, IL), HRP-conjugated anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN), and alkaline phosphatase-conjugated anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN) were used as secondary antibodies.

Immunogold electron microscopy. Cells grown in tissue culture dishes were released by treatment with PBS supplemented with 0.5 mM EDTA and fixed with 2% paraformaldehyde prior to processing of ultrathin frozen sections for immuno-electron microscopy exactly as previously described [33]. To detect the HA-tagged chains, the sections were incubated with 12CA5 followed by protein A-gold. The grids were examined in a JEOL 1200EX transmission electron microscope and images recorded at a primary magnification of 25,000.

Results

Epitope-tagged β chains are expressed normally. CHO cells were transfected with cDNAs encoding the full-length and truncated rat brain $\beta 1$ and $\beta 2$ chains tagged at the amino-terminal with the HA epitope. Epitope-tagging was required to differentiate the endogenous from the transfected β chains because the amino acid sequences of β chains are almost completely conserved among mammalian species, and because species-specific antibodies for the β chains are not readily available. Cell lines which expressed the highest levels of the proteins encoded by these constructs were cloned by three cycles of serial dilution and characterized by Western blot analysis. Figure 1 shows a Western blot of cell lysates probed with the antibody 12CA5 specific for the HA epitope, in which bands corresponding to the

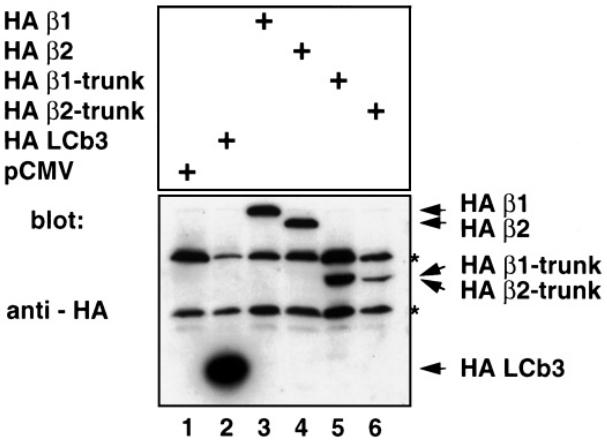

Figure 1. Stable expression of HA-tagged β chains in transfected CHO cells. SDS-10% PAGE and Western blot analysis of low speed supernatants obtained from lysates of CHO cell lines transfected with the expression pCMV vector alone (lane 1); the positive control HA-tagged LCb3 (lane 2); and the β chains HA-tagged $\beta 1$ (lane 3); HA-tagged $\beta 2$ (lane 4); HA-tagged $\beta 1$-trunk (lane 5); and HA-tagged $\beta 2$-trunk (lane 6). Presence of the HA-tag was determined with the monoclonal antibody 12CA5. The asterisks indicate nonspecific crossreacting bands.

expected size of the expressed full-length and truncated β-chain proteins, and not present in the parent cell line, can be readily seen. No additional specific bands are visible using the anti-HA antibody, suggesting that the epitope-tagged proteins are not subject to significant degradation. The tagged chains show similar levels of expression in the different cell extracts, although the $\beta 2$ trunk is expressed to a lower extent than the other chains. In the cases of cells expressing full length $\beta 1$, full length $\beta 2$ or truncated $\beta 1$, the level of expression was high enough for the extent of their incorporation into AP complexes to be estimated as similar to that of endogeneous $\beta 1$ and $\beta 2$ (see below and fig. 3).

Epitope-tagged β chains incorporate into AP-2 complexes. To examine whether the epitope-tagged β chains incorporate into AP complexes, we first performed gel filtration analysis on the cytosolic fraction (high speed supernatant) of cells expressing each of the tagged constructs and asked if they co-elute with endogeneous AP complexes. The gel filtration run was done in a Tris-containing buffer previously shown to prevent AP aggregation without dissociating the chains of the complexes [1, 2, 28, 34]. As shown in figure 2, the transfected chains all co-elute in the same range as the endogeneous α, $\beta 1/\beta 2$ and γ chains, with an approximate Stokes' radius of 60 Å which corresponds to the size of monomeric AP complexes (see for example refs. 24, 35). We did not detect any epitope-tagged β chains eluting at a position corresponding to the much smaller Stokes' radius of purified recombinant β chains or truncated β chains made in *E. coli* [24, 25]. These observations suggest that all of the transfected β chains, whether intact or truncated, incorporate equally well into large structures, presumably the AP complexes.

To confirm that the tagged β chains incorporate into AP complexes, we performed immunoprecipitations from lysates of transfected cells, using polyclonal rabbit antibodies that detect native AP complexes which are specific for the hinge/ear domains of either the α_c or the $\beta 1/\beta 2$ chains [16]. We first confirmed the specificity of the antibodies and demonstrated the absence of mixed aggregates of AP-1 and AP-2 in the cell lysate. As shown in figure 3, the immunoprecipitates obtained with the anti-α antibody that is specific for AP-2 (lanes 1–6) do not contain AP-1 (monitored by the absence of γ chain), whereas the immunoprecipitates generated with the anti-$\beta 1/\beta 2$ antibody specific for both AP-1 and AP-2 (lanes 7–12) contains both AP-1 (monitored by the presence of γ chain) and AP-2 (monitored by presence of the $\beta 1$ and $\beta 2$ chains).

Since all species of transfected HA-tagged β chains coprecipitate with the anti-α_c antibody (lanes 3–6) we conclude that both $\beta 1$ and $\beta 2$ variants incorporate into AP-2 complexes. Moreover, in the cases of cells expressing full length HA $\beta 1$ or $\beta 2$ or in cells expressing truncated $\beta 1$, we were able to compare in the anti-α_c precipitates of AP-2 the relative content of the tagged proteins with the amounts of endogenous $\beta 1$ and $\beta 2$ as an additional way to estimate the extent of incorporation of the modified β chains into AP complexes. As depicted in figure 3, the intensity of the bands elicited by Western blot using the $\beta 1/\beta 2$ antibody was similar for the tagged β proteins and for the endogenous $\beta 1$ and $\beta 2$, suggesting that about 50% of the endogeneous β chains in AP-2 were replaced by recombinant β chains. The promiscuity of the transfected β chains is consistent with the similar behaviour of the endogeneous β chains [16, 36, 37]. That this result is not an artifact of the transfection or of tagging is shown by probing the other half of the same immunoprecipitates with the monoclonal antibody B1-M6 specific for the amino-terminal domain of $\beta 1$ and $\beta 2$ chains. In the

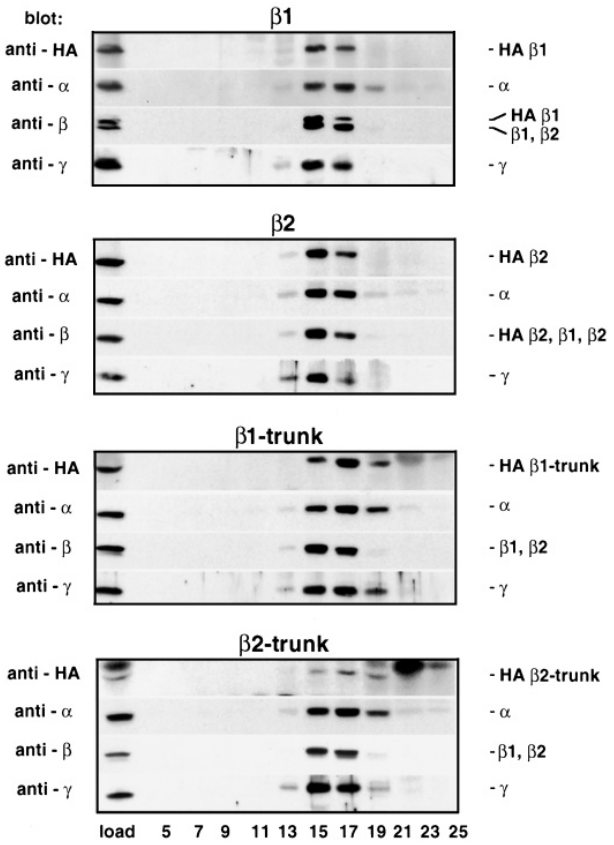

Figure 2. Gel filtration analysis of the HA-tagged β chains shows that they incorporate into complexes of size similar to intact AP complexes. Gel filtration analysis using a Superose 6 HR10/30 column of the high speed supernatants obtained from the transfected CHO cells expressing HA $\beta 1$, HA $\beta 2$, HA $\beta 1$-trunk and HA $\beta 2$-trunk. Alternate fractions were subjected to TCA precipitation followed by SDS-PAGE (8% gel) and Western blot analysis with several antibodies and developed with ECL. The HA-tagged proteins were probed with the antibody 12CA5; the same membranes were probed for $\alpha a/\alpha c$, $\beta 1/\beta 2$ and γ chains of AP-2 and AP-1 complexes with the monoclonal antibodies AC1-M11, 9A and the polyclonal antibodies against hinge/N-terminus of γ, respectively. The 'load' sample corresponds to 10% of the input into the column whereas the other lanes correspond to 40% of alternate fractions from the gel filtration run.

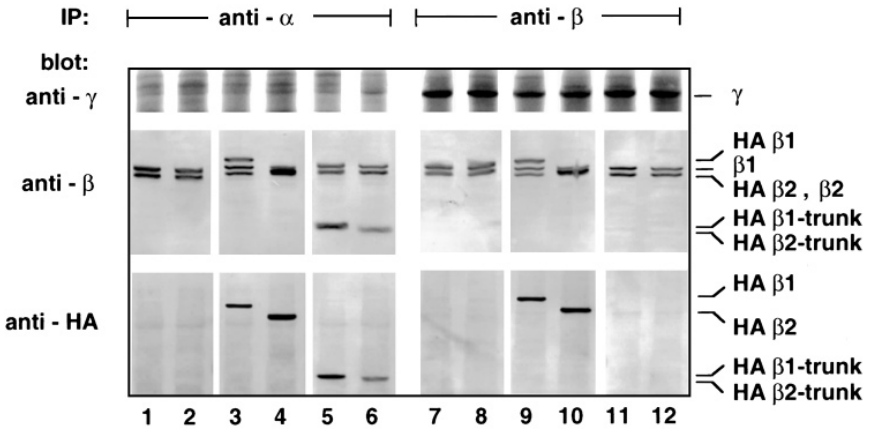

Figure 3. The HA-β chains assemble into AP complexes. To evaluate the incorporation of HA-β chains into AP complexes, low speed supernatants from CHO cells expressing the HA-β chains were generated by treating the cells with lysis buffer containing Tris and Triton followed by immunoprecipitation, SDS-PAGE (10% gel) and Western blot analysis. The samples correspond to control CHO cells transfected with the pCMV vector only (lanes 1 and 7), and to cells expressing the epitope-tagged clathrin light chain HA LCb3 (lanes 2 and 8), HA β1 (lanes 3 and 9), HA β2 (lanes 4 and 10), HA β1-trunk (lanes 5 and 11), and HA β2-trunk (lanes 6 and 12). Samples were first immunoprecipitated with anti-α_c or with anti-β1/β2 polyclonal antibodies, then split into two aliquots. One blot was sequentially probed with anti-β (B1-M6) followed with anti-γ antibodies; the second blot was probed with anti-HA (12CA5) antibody. The membranes were developed using the alkaline phosphatase colorimetric reaction.

parent cell line transfected with the expression vector pCMV (fig. 3, lane 1), or in the negative control cell line transfected with HA-tagged clathrin light chain LCb3 (fig. 3, lane 2), two bands are visible in the anti-α_c antibody immunopecipitate, at the appropriate positions for endogeneous β1 and β2. In cell lines expressing HAβ2 chain, the β2 band is noticeably denser than in controls (fig. 3, lane 4), while in lines expressing HAβ1, because of the size difference between rat brain β1 used in the transfection and endogeneous CHO β1, three β bands are visible (fig. 3, lane 3). In this Western blot (fig. 3, lanes 5 and 6), it is possible to see that the truncated β1 and β2 chains are also immunoprecipitated with the anti-α_c antibody, confirming that they too are incorporated into AP-2 complexes. We note that the anti-β1/β2 ear antibody brings down complexes containing the full length HA-β chains (fig. 3, lanes 9 and 10) but does not immunoprecipitate the truncated β1 and β2 chains (fig. 3, lanes 11 and 12), confirming that AP complexes carry only one β chain.

APs containing truncated β chains are deficient in incorporation into coated pits and coated vesicles. How important is the β chain hinge/ear domain for incorporation of the AP complex into clathrin-coated structures? To answer this question, we tested the ability of APs with truncated β chains to incorporate into clathrin coated structures by using two independent methods, one based on the biochemical isolation of coated vesicles and the other on direct visualization of coated structures by electron microscopy. In the first method, coated vesicles were prepared from lysates obtained from the transfected cells under conditions in which the coated vesicles are stable, and fractionated by SDS-PAGE followed by Western blot analysis to compare the extent of incorporation of APs that contain the full-length and the truncated epitope-tagged β chains. As shown in figure 4, lanes 3 and 4, the amount of tagged chain found in the high-speed supernatant (corresponding to free AP complexes) correlates with the level of expression (see fig. 1 for comparison). However, the levels of truncated epitope-tagged β chains found in the sample containing the coated vesicles (lanes 11, 12) are dramatically decreased compared to the levels of full-length epitope-tagged β chains (lanes 9, 10), even though the amounts of recovered coated vesicles, monitored by the content of endogeneous β1 and β2 chains, are about the same. Based on a comparison of the ECL-signal elicited by a serial dilution of the samples it was estimated that the level of incorporation of the truncated chains is $\sim$6–12% of that of the full-length chains.

In the second method, samples of cells were processed for immuno-electron microscopy to investigate the intracellular localization of the full-length or truncated HA-tagged β1 or β2 chains. The visualization was performed on ultrathin sections that had been incubated with the monoclonal antibody 12CA5 specific for the HA tag followed by labelling with protein A-gold (figs 5 and 6). The fields were inspected for presence of gold particles along the plasma membrane and the Golgi complex. As shown in the figure and summarized in table 1, gold particles corresponding to HA LCb3, used as a positive control, were found in profiles characteristic of clathrin coated pit and coated vesicles, both along the plasma membrane (fig. 5) and the trans-Golgi network (fig. 6). Negative control cells transfected with the pCMV expression vector did not show any gold particle decoration (not shown). Samples obtained from cells expressing HA β1 or HA β2 also showed the expected intracellular distribution; many gold particles

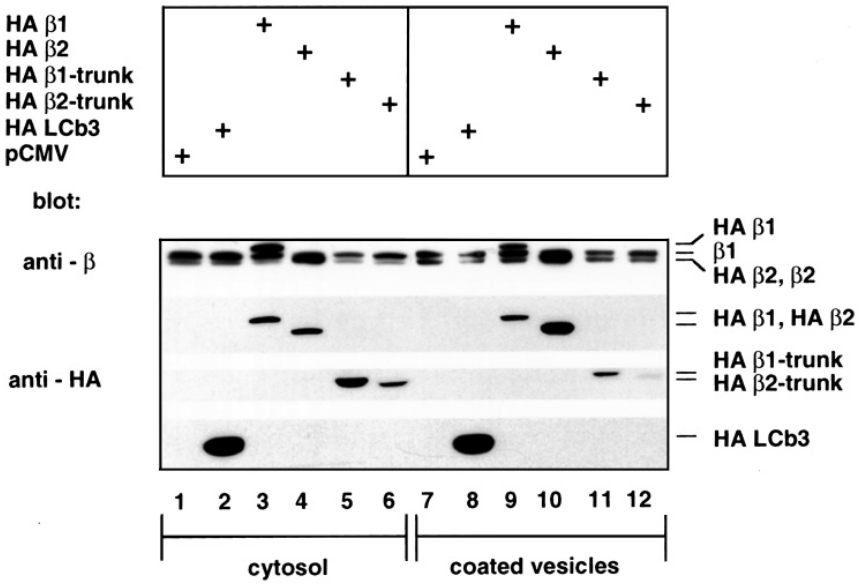

Figure 4. AP complexes with truncated β chains incorporate poorly into coated vesicles. Lysates of CHO cells, normalized to represent equivalent amounts of protein, were subjected to differential centrifugation to separate the cytosolic fraction containing free APs (high speed supernatant) from the fraction containing APs bound to coat vesicles (Ficoll/sucrose). Aliquots were subjected to SDS-PAGE (10%) and then characterized for the distribution of the tagged β chains or endogenous β1 and β2 chains by Western blot and ECL analysis using the antibodies 12CA5 and 9A, respectively.

corresponding to HA β1 were found at the trans-Golgi network (fig. 6) whereas fewer particles were found at the plasma membrane (fig. 5). Gold particles corresponding to HA β2 were found along profiles of coated pits and coated vesicles at the plasma membrane (fig. 5) whereas none were detected at the trans-Golgi network. In contrast, we were unable to detect gold particles corresponding to HA β1-trunk (figs 5 and 6) or HA β2-trunk (fig. 5) within coated profiles at either location. In summary, the morphological observations are in agreement with the biochemical results described above and they indicate that AP complexes containing truncated β chains seem to be hindered in their ability to incorporate into clathrin coated pits and coated vesicles. The morphological results allow us to rule out the possibility that the depletion of APs containing truncated β chains copurifying with coated vesicles described earlier is due to their artifactual release from the coats during the biochemical purification of the coats because of weak interactions with clathrin.

Discussion

The data in this report indicate that truncated β chains lacking their hinge/ear segment incorporate into AP complexes, but that the incorporation of such AP complexes containing truncated chains into clathrin coated pits and coated vesicles is hindered.

We have shown in previous work that intact β1 and β2 chains can drive the polymerization of clathrin coats in vitro [24]. In the case of β2, the hinge/ear region is essential for this activity. The similarity of β1 and β2 (93% sequence identity) [23] allows us to assume that this will also hold true for β1. We have further shown that the assembly activity resides in a conserved 50-amino acid portion of the β1 and β2 hinge/ear [25]. If, in vivo, this interaction likewise directs the formation of clathrin coated pits and coated vesicles, AP complexes containing truncated β chains will fail to participate in coat formation because they cannot recruit clathrin, and will be poorly represented in clathrin coated structures. The data presented here are consistent with this prediction. We have found that coated vesicles isolated from cells expressing truncated β chains have proportionally less APs with truncated β chains than APs with intact β chains. It is possible, however, that a weaker or less stable interaction of the truncated APs with the coats or with the membranes might result in their release during vesicle purification. We think that this is unlikely given the results obtained by direct electron microscopic visualization of coated profiles from cells expressing full-length and truncated β chains. It was found that the coated profiles from cells expressing full-length tagged-β chains were, as expected, decorated with gold particles indicated incorporation of the intact APs to the coats. In contrast, coated profiles from cells expressing the truncated β chains failed to be decorated. Another way in which deficient incorporation of APs into coats might occur is if APs with truncated β chains fail to be recruited to the appropriate membrane sites prior to coat assembly. Although we cannot rule out this possibility, binding to the plasma membrane of AP-2 containing truncated βs seems normal, since the amount of gold particle-labelling that we can detect in

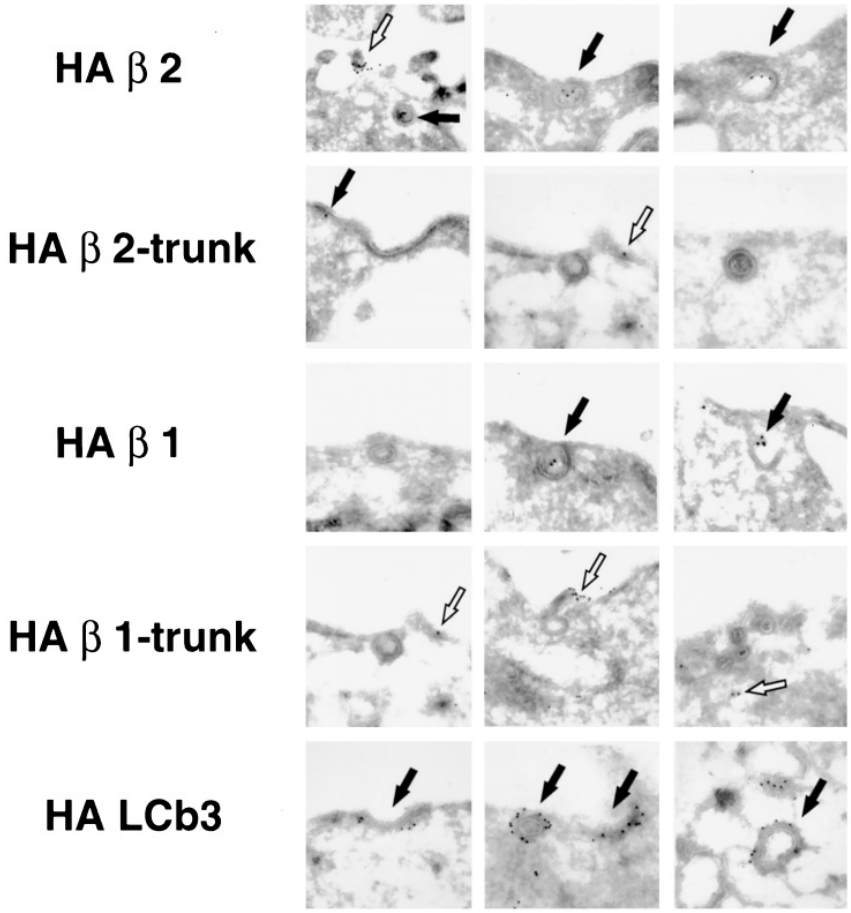

Figure 5. Immunoelectron microscopic localization of the tagged β chains to plasma membrane-coated pits and -coated vesicles. Sections of CHO cells expressing the HA β1, HA β2, HA β1-trunk, HA β2-trunk or HA LCb3 chains were stained with the anti-HA antibody followed by decoration with protein A-gold. The gallery of images represents views obtained for each cell type from at least 25 different fields. The black arrows point to membrane profiles corresponding to coated pits and coated vesicles; the outlined arrows point to noncoated membrane regions.

cells expressing full length or truncated HA βs is similar in regions of the plasma membrane devoid of coat. An additional indication that the β-hinge/ear portion is important for the interaction between APs and clathrin has been obtained in a recent study focused on the effect of phosphorylation on the interaction of APs with clathrin in vivo [38]. In this study it was found that there is a direct correlation between the extent of phosphorylation of the β-hinge and the ability of APs either to bind pre-formed clathrin cages or to partition with coated vesicles in cells.

When AP complexes are partially digested with proteases such as elastase or trypsin, a 'core' complex remains which contains the amino-terminal trunk domains of both of the large chains, together with the medium and small chains, μ and σ [2, 22, 23, 28, 39]. As these complexes appear stable in the absence of 'hinge/ear' domains, it is not unexpected that recombinant β trunk was efficiently incorporated into AP complexes. However, the AP complexes containing recombinant β trunk seem to incorporate poorly into clathrin coated pits, as shown here by immuno-electron microscopy, and into coated vesicles also shown here both biochemically and by electron microscopy. We therefore conclude that the β hinge/ear indeed links the AP complex to the clathrin lattice. Some truncated β-containing chains are incorporated into coated vesicles. Thus, there may be other elements within the AP complex that are able to interact with components of the clathrin-coated pit. The α chains are candidates since it has been reported recently that recombinant α chain can interact with clathrin, although it cannot drive clathrin coat formation [40].

Given our results, how can we explain the observation that AP complexes, treated on membranes with elastase to remove large chain hinge/ear domains, can initiate the formation of coated pits in plasma membrane sheets [26]? It is possible that the 'hinge/ear' of the β chain is not required for the *initiation* of pit formation, but that complexes containing truncated β chains are specifically excluded when the pit buds to become a coated vesicle. The mechanism for such exclusion would be difficult to

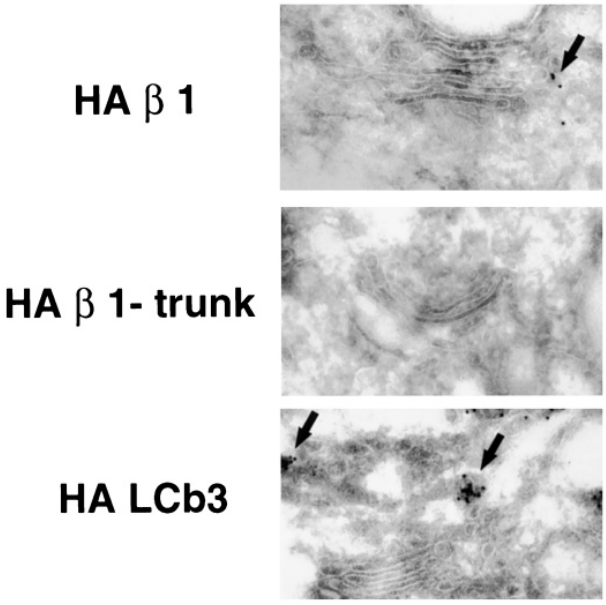

Figure 6. Immunoelectron microscopic localization of the tagged β chains to membranes at the trans-Golgi network. Sections of CHO cells expressing HA β1, HA β1-trunk and HA LCb3 were processed for electron microscopy as indicated in figure 5. The images correspond to areas close to the Golgi complex.

imagine, however. In the elastase-shaving experiments described above, only the proteolysis of the α chain was measured directly, as no antibody to β-chain hinge/ear was then available. Although elastase is capable of giving full cleavage of both large chain hinges in isolated AP-2 complexes [28], the conditions used in the membrane-sheet experiment were not tested for β chain cleavage. In particular, retention of AP complexes on membranes during the elastase digestion might have protected the β hinge from cleavage. We therefore think it likely that in this experiment some percentage of intact β chains remained, sufficient to drive some amount of coated pit formation.

In the course of our work, as noted above, we made the surprising observation that AP-2 complexes do not discriminate between β1 and β2 chains. The prevailing view in the literature, that AP-1 complexes contain only β1 chains, and AP-2 complexes contain only β2 chains, rests on the single observation that in bovine brain, where the level of β1 is extremely low, the β chains found in AP-2 complexes are almost all β2. A trace contaminant of another chain (referred to as b* [34]), which reacted with anti-β chain antibodies, was also found in AP-2 complexes but was never identified. This trace contaminant has the same electrophoretic mobility as the bovine brain β1 chain. The published data are not inconsistent with the idea that b* is indeed the bovine brain β1 chain, and the incorporation of β1 and β2 into AP-2 complexes is proportional to their expression. The promiscuity of the β chains has recently been observed by us and others [16, 36, 37]. This result has several implications for the assembly of AP-2 complexes. First, the α chain does not discriminate between β1 and β2, despite making direct contact with the amino-terminal domain of the β chain within the complex [1]. In contrast, the γ chain of AP-1 does seem to discriminate between β1 and β2 as suggested by the observation that in bovine brain, in which β2 is predominantly expressed, AP-1 complexes contain only β1 chains. Second, the selection of the medium and small chains, which do appear to show discrimination in the complexes, must be performed by the α (or by the γ) chain, as demonstrated by the analysis of AP complexes in cells transfected with chimeras of α and γ chains [36].

In conclusion, we present evidence that the hinge/ear domain of the β chains does not seem to be required for in vivo assembly of the AP complex, but appears to be essential for the normal incorporation of AP complexes into clathrin coats.

Table 1. Relative distribution of tagged β chains in coated pits located at the plasma membrane of the CHO cells.

Sample	Gold particles/μm of plasma membrane	Gold particles in coated pits (%)
HA β1	0.69 ± 0.13	15
HA β2	1.45 ± 0.027	73
HA β1-trunk	0.61 ± 0.15	3
HA β2-trunk	0.52 ± 0.08	8
HA LCb3	2.15 ± 0.41	70
Control	0.45 ± 0.11	6

Ultrathin sections produced from all samples were simultaneously incubated with the same concentration of HA antibody and, for each sample, 10 different plasma membrane profiles and coated pit fields were randomly selected and quantified.

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