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# Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane

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**We have established by peptide mapping and immunochemical analysis of purified clathrin assembly protein preparations from bovine brain, that the cluster of components of mol. wt 100-120 kd fall into four classes, which we term $\alpha$, $\beta$, $\beta'$ and $\gamma$. The $\beta$ and $\beta'$ proteins are immunologically related and generate a series of common tryptic peptides. The same criteria reveal no such homologies between the $\alpha$, $\beta(\beta')$ and $\gamma$ polypeptides. The so-called HA-II assembly protein group contains equimolar amounts of $\alpha$ and $\beta$ class polypeptides, which are shown to interact with each other. In the HA-I group assembly protein complex $\gamma$ and $\beta'$ class polypeptides form a stoichiometric complex. Immunofluorescence microscopy reveals that the HA-I complex is specifically associated with clathrin-coated membranes in the Golgi region of cultured cells, whereas the HA-II complex appears to be restricted to coated pits on the plasma membrane. The data lead to the tentative conclusion that the clathrin assembly proteins are involved in the recognition of the intracellular targets by uncoated vesicles.**

*Key words:* clathrin-coated vesicles/100 kd assembly proteins/Golgi complex/protein transport

## Introduction

Clathrin-coated membranes are involved in specific intracellular transport processes such as receptor-mediated endocytosis and the transfer of newly-synthesized proteins from the Golgi to lysosomes and secretory storage vesicles (Pearse and Bretscher, 1981; Goldstein *et al.*, 1985; Pfeffer and Rothman, 1987). Correspondingly, the major coat component clathrin was shown by immunocytochemical techniques to be abundant in the *trans*-Golgi region and in coated pits that are distributed throughout the cytoplasmic face of the plasma membrane (Anderson *et al.*, 1978; Orci *et al.*, 1985). From these observations it has been conjectured that there are at least two subpopulations of coated membranes within each cell, which differ not only in the identity of their cargo molecules but also in the composition of their structural proteins. The major proteins of clathrin-coated vesicles from bovine brain tissue are resolved by SDS-PAGE into polypeptides with apparent mol wts of 180 kd (clathrin and the protein AP 180), several more between 100 and 115 kd and another group between 50 and 30 kd (Pearse, 1978; Schook *et al.*, 1979; Keen *et al.*, 1979; Ungewickell and Branton, 1981; Ahle and Ungewickell, 1986). The 100-115 kd polypeptides are seen as the most likely determinants of specificity on the grounds that they (i) mediate the binding of clathrin to the membrane of coated vesicles (Unanue *et al.*, 1981), (ii) interact with a transmembrane receptor (Pearse, 1985); (iii) interact with clathrin and thereby promote its assembly *in vitro* into cage-like structures (Keen *et al.*, 1979) and (iv) are located between the clathrin coat and the membrane (Vigers *et al.*, 1986).

The 100-115 kd polypeptides are peripheral membrane proteins, which *in vitro* are released together with clathrin from the coated vesicle membrane at alkaline pH (Woodward and Roth, 1978) or on addition of protonated amines (Keen *et al.*, 1979). The native 100-115 kd polypeptides are fractionated by hydroxyapatite adsorption chromatography into two groups, referred to by Pearse and Robinson (1984) as the HA-I and HA-II 100 kd polypeptides. Both contain several 100-115 kd polypeptides and probably two smaller species. The components of the HA-II group can be chemically cross-linked to form a covalent complex of some 300 kd (Pearse and Robinson, 1984). Comparable values for the native molar masses of the HA-I and HA-II complexes were recently obtained by gel filtration (Manfredi and Bazari, 1987). Subsequent studies by Robinson and Pearse (1986) indicated that the HA-II complex contains at least three distinct polypeptides with mol. wts between 100 and 115 kd. Two of these appear to be structurally related by immunological and biochemical criteria. Polyclonal antisera against the 100-115 kd polypeptides of the HA-II complex stained in cultured cells the Golgi region and the plasma membrane. It was inferred that the 100-115 kd polypeptides partition between clathrin-coated membranes in the Golgi and at the plasma membrane. More recently the results of immunofluorescence studies with monoclonal antibodies specific for the two related 100 kd proteins of the HA-II complex suggested that they are confined to the plasma membrane (Robinson, 1987).

The purpose of the work described here was to clarify the structural relationship between the polypeptides making up the HA-I and HA-II groups by means of a combination of biochemical and immunological approaches. Monoclonal and polyclonal antibodies to 100-115 kd polypeptides from both groups were employed to study their intracellular distribution. The results suggest that the HA-I group of assembly proteins are confined to the Golgi region of cultured cells while those of the HA-II group are probably associated only with coated pits at the plasma membrane. On the basis of the results, we develop a simple model for the structural organization of the HA-I and HA-II group polypeptides and suggest a possible function for them in the cell.

## Results

### *Purification of 100-115 kd polypeptides from coated vesicles*

The 100-115 kd polypeptides and clathrin were extracted with 0.5 M Tris from the membrane of purified bovine brain

coated vesicles. To avoid the danger of proteolytic damage during the preparation we eliminated the time consuming gel filtration step used in previous procedures. Instead, the HA-I and HA-II complexes were purified by a combination of ion-exchange and hydroxyapatite adsorption chromatography. First clathrin, the most abundant protein in the extract, was removed by anion-exchange chromatography on a preparative Mono Q column (Figure 1). Fractions containing AP 180 and the 100–115 kd polypeptides were briefly dialysed against or diluted into low salt buffer and then further fractionated on an analytical Mono Q column, which removed protein AP 180 and also partially separated the polypeptides belonging to the HA-I group from those of the HA-II group (Figure 2). Fractions containing these groups of proteins were separately applied to a hydroxyapatite column. This final chromatographic step removed all remaining traces of cross-contamination as judged by immunoblotting with monoclonal antibodies (see below).

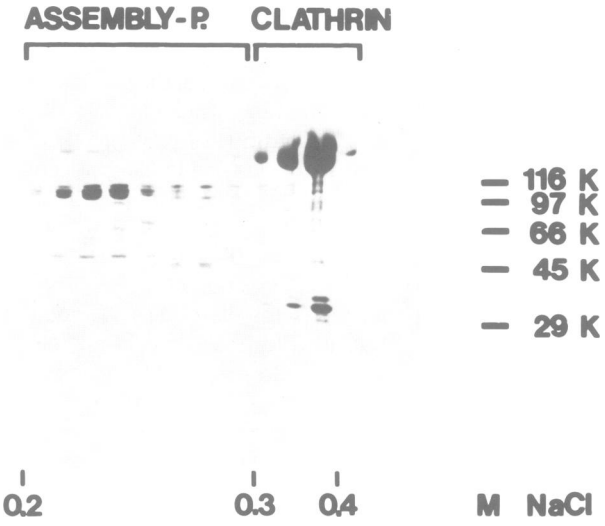


**Fig. 1.** Purification of assembly proteins and clathrin by anion-exchange chromatography. Clathrin and the assembly proteins were extracted with 0.5 M Tris–HCl, pH 7.0, from coated vesicles and after dialysis against 20 mM ethanolamine, 2 mM EDTA, pH 8.9, applied to a semi-preparative Mono Q column. The column was eluted with a linear 0–0.5 M NaCl gradient. Fractions were analysed by SDS–PAGE. The approximate salt concentration of the fractions is indicated by the numbers below the electrophoretogram and the fractions that were pooled are marked by the brackets.

As already noted by others (Zaremba and Keen, 1985) the HA-II complex consists of at least five polypeptides, which migrate on 7.5–20% polyacrylamide gradient gels in the presence of SDS with apparent mol. wts of 112, 106, 104, 50 and 17 kd (Figures 2 and 3A). The purified HA-I complex resolves into five polypeptides with apparent mol. wts of 115, 110 (a minor species), 105, 40 and 20 kd (Figures 2 and 4A).

Before investigating the structural relationship between the polypeptides in the region between 100–115 kd we attempted to improve on their separation. The standard two-dimensional procedure with isoelectric focusing in 8 M urea, followed by SDS-electrophoresis failed, because the proteins either precipitated at the origin of the isoelectric focusing gel or they smeared over a broad pH range (data not shown). Addition of zwitterionic detergents gave no improvement. While exploring other electrophoretic systems we observed that addition of 6–8 M urea to the separation gel of a Laemmli-type SDS polyacrylamide gel significantly altered the mobility of at least one of the 100–112 kd polypeptides of the HA-II group (Figure 3b). The apparent mol. wts in this system were then 115, 112 and 106 kd. By combining the urea–SDS gel electrophoresis in the first dimension with SDS electrophoresis according to Laemmli in the second, it was shown that the 106 kd polypeptide of the HA-II group migrated in the first dimension with an apparent mol. wt of 115 kd (zone b in Figure 3c). The two-dimensional gel also reveals that the 112 and the 104 kd bands resolve into two components, which we designate a1, a2, c1 and c2.

The HA-I group polypeptides were similarly examined in the two-dimensional system but none of its components gave evidence of anomalous migration in the urea–SDS gel system, nor were any additional bands observed (Figure 4b, c).

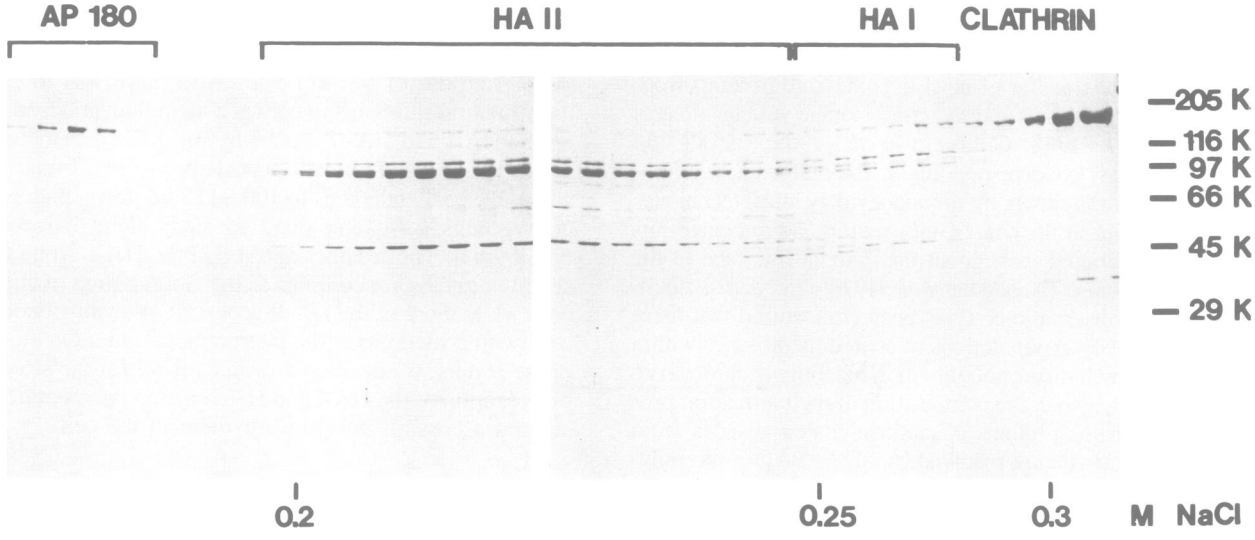


**Fig. 2.** High resolution anion-exchange chromatography of clathrin assembly proteins. Assembly protein containing fractions from the first chromatographic step (see Figure 1) were applied to an analytical Mono Q column and eluted with an extremely shallow salt gradient (see Materials and methods for details). HA-I and HA-II assembly proteins were pooled as indicated by the brackets on top of the electrophoretogram.

### *Structural relationship between the HA-I and HA-II polypeptides*

*Tryptic peptide maps of HA-II polypeptides.* To determine to what extent the polypeptides making up the 100–115 kd group are structurally related, we examined the two-dimensional fingerprints of their radioiodinated tryptic peptides. This method is more informative than the one-dimensional fingerprinting procedure, previously applied to some members of the same group of proteins (Robinson and Pearse, 1986). For iodination the 100–115 kd polypeptides from the HA-II group were excised from two-dimensional gels while all other polypeptides were obtained from standard SDS–PAGE gradient gels. A comparison of the fingerprints of the components a1, a2 (both ~112 kd in the Laemmli gel), c1 and c2 (both ~105 kd in the Laemmli gel) indicates that these polypeptides are very similar in structure (Figure 5). In contrast, the tryptic peptide map generated from zone b (106 kd) appears to be completely unrelated to those of either the a-type or c-type polypeptides. This result was corroborated by co-maps in which mixtures of a- or c-type tryptic peptides with those from zone b were sujected to fingerprinting (data not shown). The peptide maps of the 50 and 17 kd polypeptides also appeared unrelated to those of the larger polypeptides, and it seems therefore unlikely that the former are proteolysis products of any of the latter (Figure 5).

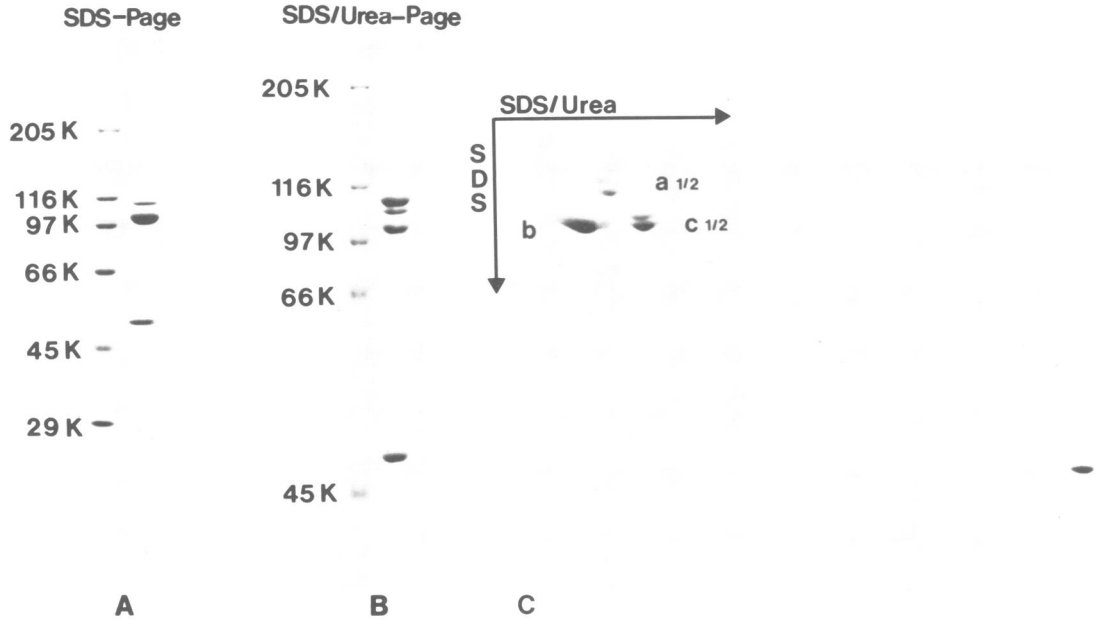


**Fig. 3.** Analysis of HA-II assembly proteins by SDS–PAGE (**A**); urea–SDS–PAGE (**B**); and two-dimensional electrophoresis (**C**). Left lanes in A and B contain protein standards. The 106 and 104 kd HA-II polypeptides (right track in A) were not resolved because of too high loadings.

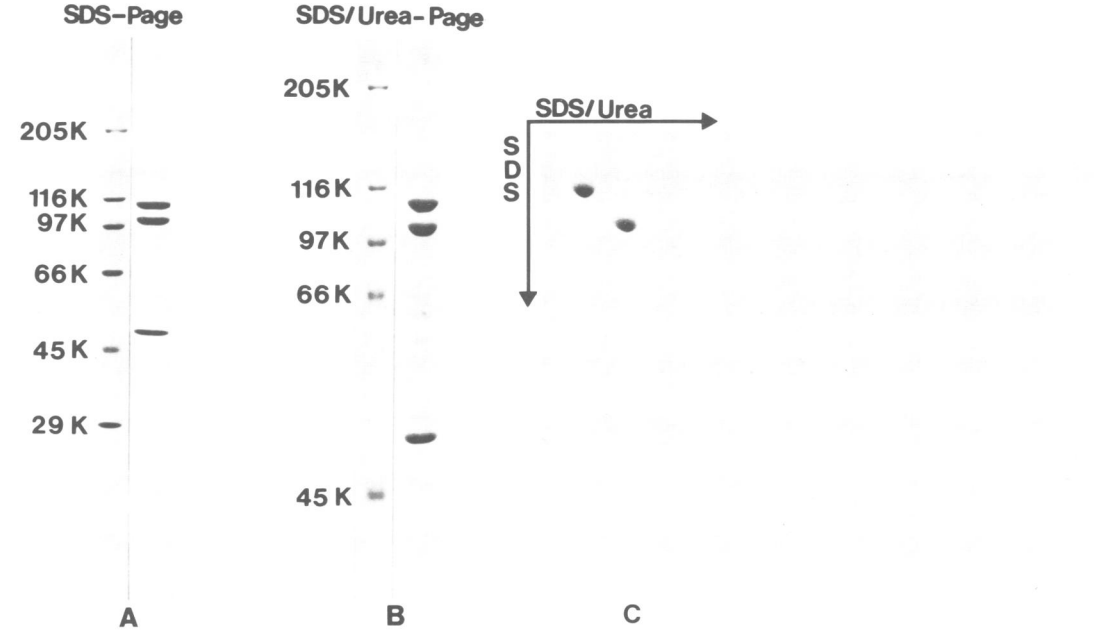


**Fig. 4.** Analysis of HA-I polypeptides by SDS–PAGE (A); urea–SDS–PAGE (B); and two-dimensional electrophoresis (C). Note that none of the polypeptides in the 110 kd region migrates anomalously in the presence of urea.

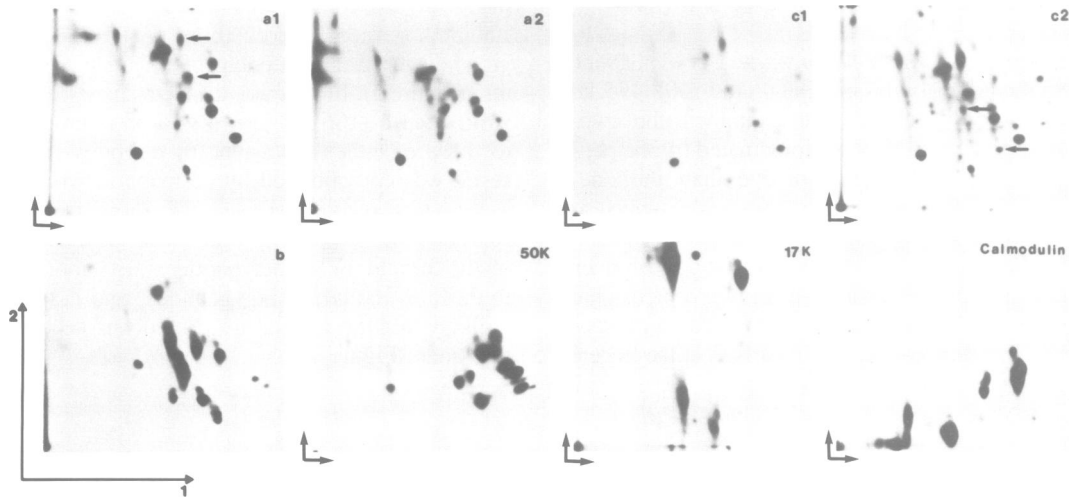


**Fig. 5.** Autoradiographs of two-dimensional peptide maps of $^{125}$I-labelled tryptic peptides from HA-II assembly polypeptides and calmodulin. Tryptic peptides were separated on cellulose thin layer plates by electrophoresis at pH 3.5 in the first dimension and ascending chromatography in the second. Arrows in a1 point at major peptides not found in a2. One of them (the upper one) is, however, present in c1 and c2. Arrows in c2 label spots missing from c1. Only the upper one is also found in a1.

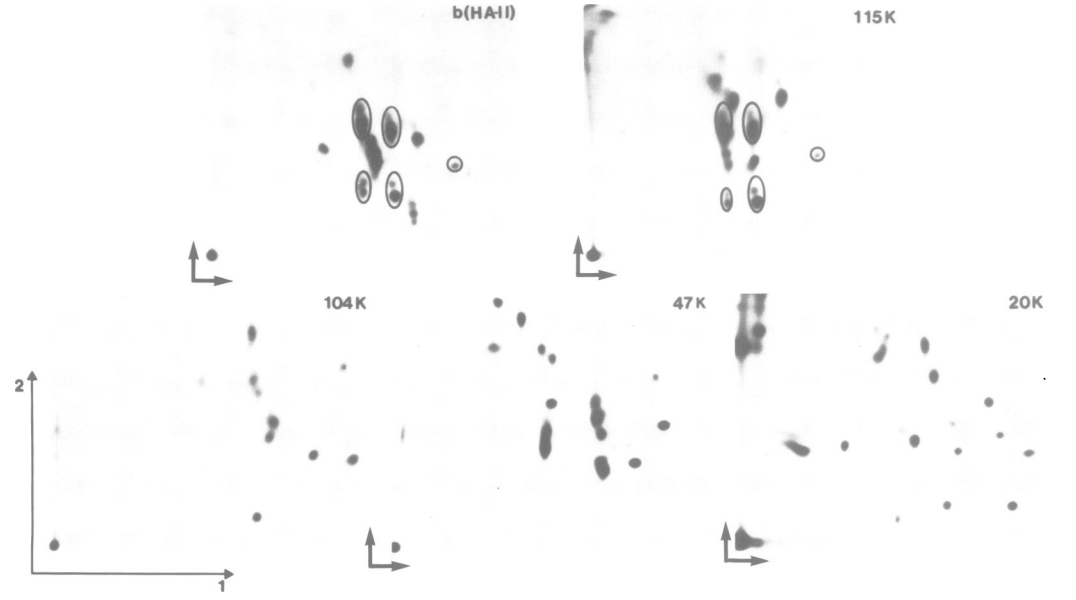


**Fig. 6.** Two-dimensional tryptic peptide analysis of HA-I assembly polypeptides. The map of the b-type polypeptide is included for comparison. Encircled peptides are common to both the 115 kd HA-I polypeptide and the b-type polypeptide from the HA-II assembly protein complex.

The 17 kd polypeptide resembles the regulatory protein calmodulin in its migration. Therefore we have also generated a peptide map from calmodulin and compared it with that of the 17 kd polypeptide. Figure 5 shows that the two polypeptides are unrelated. This result is in agreement with the observations of Zaremba and Keen (1985) who showed that unlike calmodulin the 17 kd polypeptide does not change its electrophoretic mobility on addition of $Ca^{2+}$.

### *Tryptic peptide maps of the HA-I polypeptides*

The HA-I group polypeptides, which show less electrophoretic heterogeneity than the HA-II polypeptides, were purified for peptide mapping by SDS–PAGE. Tryptic peptide maps were prepared from the 115, 110, 105, 40 and 20 kd components. The results obtained indicated that the first two are related (not shown), but there were no obvious similarities between any other members of the group (Figure 6). When peptide maps of the members of the HA-I and HA-II group were compared, the only clear resemblance was between the 106 kd polypeptide of the HA-II group and the 115 kd polypeptide of HA-I; these maps have at least eight out of 16 major peptide spots in common (Figure 6).

### *Monoclonal antibodies to 100–115 kd polypeptides*

To support the inferences from the peptide maps, and to study the distribution of the assembly proteins in the cell, we raised monoclonal antibodies against the 100–115 kd polypeptides from both the HA-I and HA-II group (Table I). mAb 100/1 was elicited in a mouse after immunization with highly purified HA-II protein. This antibody reacts on immunoblots of one-dimensional SDS gels with the 104 or 106 and 112 kd polypeptides of the HA-II group (Figure 7A,

**Table I.** Properties of monoclonal antibodies to clathrin assembly proteins

| Antibody | Isotype | Specificity | Intracellular target |
|---|---|---|---|
| mAb 100/1 | $IgG_1$ | HA-IIb and HA-I (115 and 110 kd) | Plasma membrane and Golgi |
| mAb 100/2 | $IgG_1$ | HA-II a/c | No data available |
| mAb 100/3 | $IgG_{2b}$ | HA-I (105 kd) | Golgi |

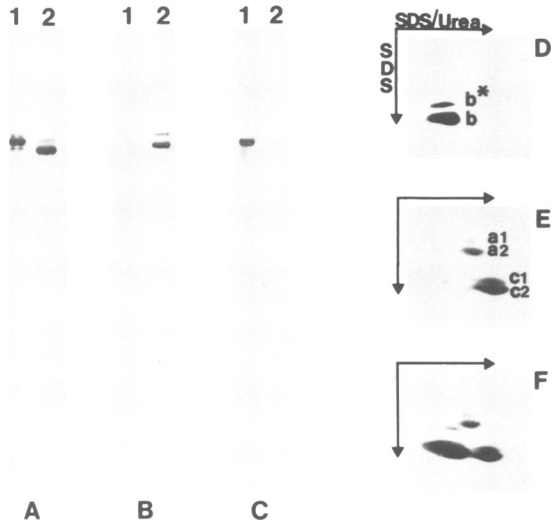


**Fig. 7.** Characterization of monoclonal antibodies by immunoblotting. Purified HA-I protein (track 1 in **A**–**C**) and HA-II protein (track 2 in A–C) were electrophoresed in SDS–PAGE and blotted onto nitrocellulose. Strip A was reacted with mAb 100/1, strip B with mAb 100/2 and strip C with mAb 100/3. Immunoblots containing HA-II polypeptides, which had been separated on two-dimensional gels, show that mAb 100/1 recognizes component b and a polypeptide which is referred to as b* (**D**), while mAb 100/2 binds to the a- and c-type polypeptides from HA-II (**E**). The immunoblot shown in (E) was rehydrated and reacted with mAb 100/1 to visualize the b-type polypeptides on the same blot to allow unequivocal identification of the antigens (**F**).

track 2) and with the 115 and 110 kd polypeptides from the HA-I group (Figure 7A, track 1). This suggested that the antibody recognizes component b of the HA-II group, since this is the only one to have tryptic peptides in common with the 115 and 110 kd proteins of HA-I. This inference was confirmed by immunoblotting of total HA-II polypeptides after separation on two-dimensional gels (Figure 7D, F). The antibody reacted strongly with zone b and in addition with a minor polypeptide above it which we refer to as b*. The latter is barely visible in gels stained with Coomassie brilliant blue and has therefore hitherto escaped detection.

A second monoclonal antibody (mAb 100/2) originated from a mouse immunized with 104 and 106 kd HA-II polypeptides that had been purified by SDS–PAGE. mAb 100/2 reacts on immunoblots of one-dimensional SDS gels with two HA-II polypeptides of about 105 and 110 kd (Figure 7B). However, in contrast to mAb 100/1 it does not react with polypeptides from the HA-I group. On two-dimensional gel blots of HA-II it reacts with a1, a2, c1 and c2 (Figure 7E). To establish the identities of the labelled components unequivocally, we incubated a sheet of nitrocellulose paper that had been stained with mAb 100/2 (Figure 7E) with mAb 100/1 to expose all the polypeptides in the

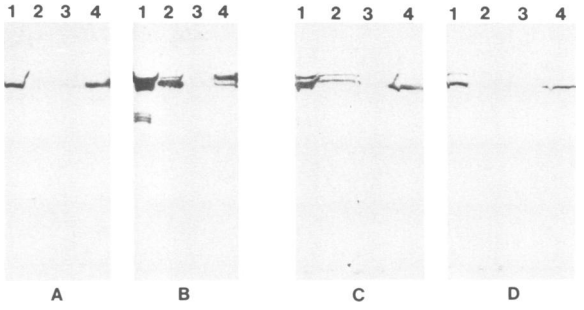


**Fig. 8.** Interactions between the 100–120 kd polypeptides of the HA-I and HA-II assembly proteins. A 'Tris-extract' from coated vesicles was incubated with mAb 100/3 which binds specifically to the 105 kd polypeptide of HA-I. Antigen–antibody complexes were precipitated with *S.aureus* cells and analysed by SDS–PAGE and immunoblotting. The immunoblot in (A) was reacted with mAb 100/3 in order to control for the efficiency of the precipitation reaction. The immunoblot shown in (B) was reacted with mAb 100/1 which is specific for the 115 and 110 kd components of HA-I, but also for the b-type polypeptides of HA-II. Note the presence of the 115 and 110 kd polypeptides in the precipitate (B, track 4). The immunoblots C and D show the results of an immunoprecipitation experiment with a monospecific polyclonal antiserum to HA-IIb. Blot C was reacted with mAb 100/1 and blot D with mAb 100/2 to visualize the HA-II specific polypeptides a and c in the precipitate. **Gel tracks. 1**: 'Tris-extract' before immunoprecipitation; **2**: supernatant after immunoprecipitation; **3**: high-salt wash of the immunoprecipitate; **4**: immunoprecipitate.

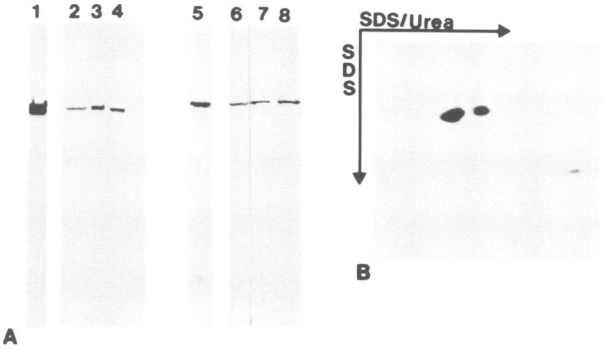


**Fig. 9.** HA-I and HA-II assembly polypeptides in bovine liver and cultured cell lines. Crude liver coated vesicles and total cell extracts from MDBK cells and human heart fibroblasts were subjected to SDS–PAGE and immunoblotting. Tracks **1**–**4** were reacted with mAb 100/1 and tracks 5–8 with mAb 100/3. Tracks **1** and **5**: bovine brain HA-II; **2** and **6**: liver coated vesicles; **3** and **7**: MDBK cell extract; **4** and **8**: human heart fibroblast extract. An immunoblot of liver coated vesicles separated by two-dimensional electrophoresis is shown in (B). The blot was reacted simultaneously with mAB 100/1 and mAb 100/2. Note that liver contains also a b-type component which migrates anomalously in the first dimension.

region between 100 and 112 kd on the same immunoblot (Figure 7F).

The third monoclonal antibody that we have characterized (mAb 100/3) reacts exclusively with a 105 kd polypeptide of the HA-I group (Figure 7C). Judged by two-dimensional peptide maps this species has no homologies with any of the other 100–115 kd polypeptides.

### *Interactions between the 105–115 kd HA-I polypeptides*

As judged by the intensities of stained protein bands the proportion of the combined (structurally related) 110 and 115 kd bands in HA-I matches that of the 105 kd polypeptide. By

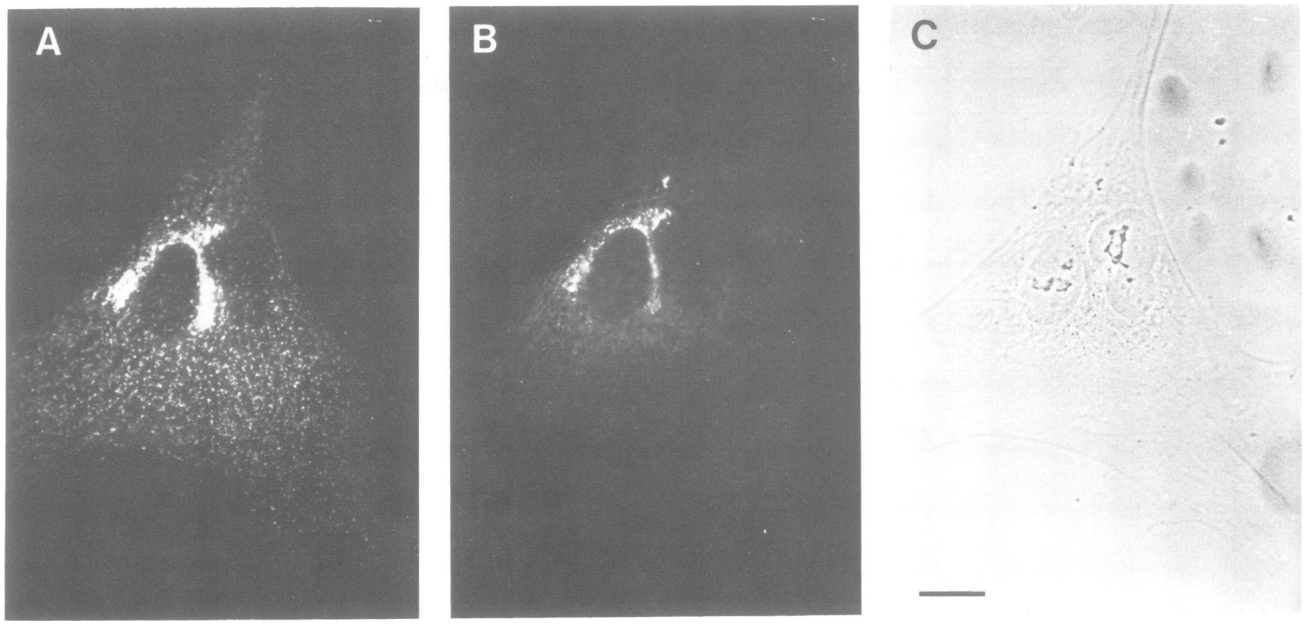


**Fig. 10.** Distribution of clathrin and HA-I assembly proteins in human heart fibroblasts. The cells were simultaneously labelled with a polyclonal antibody to clathrin light chains and with a monoclonal antibody to HA-I (mab 100/3), followed by fluorescein-conjugated goat anti-rabbit and rhodamine-conjugated goat anti-mouse antibodies. (**A**) Distribution of clathrin light chains; (**B**) distribution of HA-I; (**C**) phase-contrast image. Bar: 20 $\mu$m.

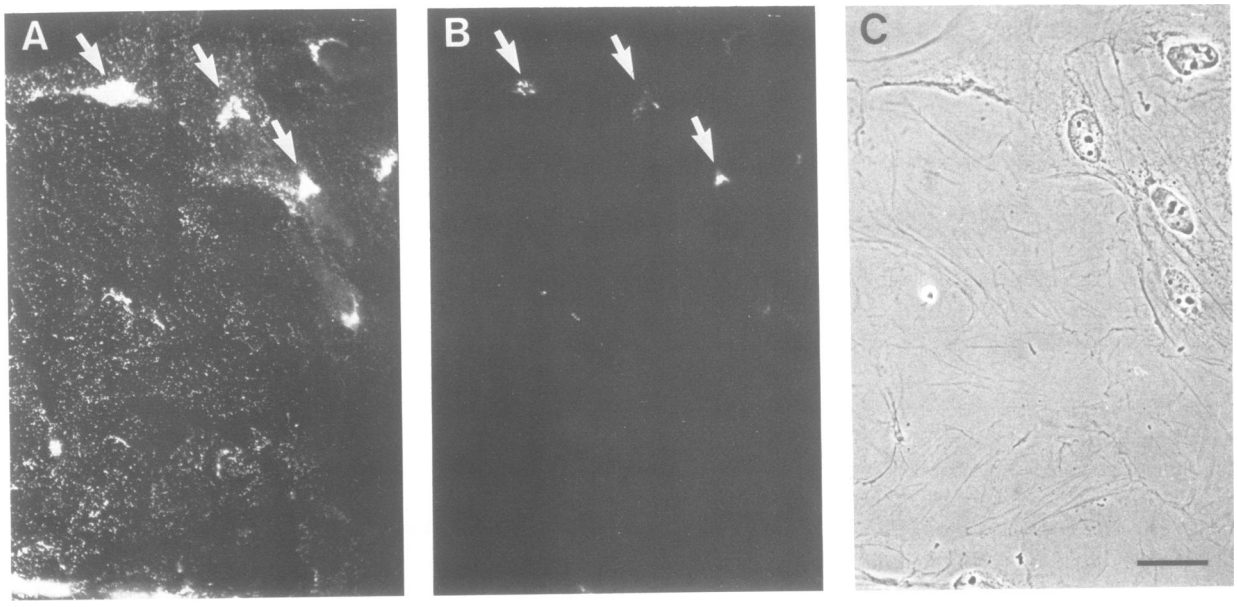


**Fig. 11.** Labelling of MDBK-cells after lysis squirting with antibodies to clathrin light chains and to HA-I assembly protein. Plasma membranes were prepared from a confluent layer of MDBK-cells by lysis squirting. (**A**) Antibodies to light chains decorate coated membranes on the plasma membrane and remaining Golgi structures (arrows), while the antibody to HA-I labels only the Golgi-region of intact cells (arrows in **B**); (**C**) phase-contrast image. Bar: 20 $\mu$m.

the same criterion the related a- and c-type species in HA-II are jointly present in equimolar proportions to the b-type. These results raise the possibility of a stoichiometric interaction between polypeptides from the two unrelated populations within each assembly protein fraction. We tried to validate this conjecture by quantitative immunoprecipitation. To examine the interactions in the HA-I complex, a crude 'Tris-extract' from coated vesicles was incubated with mAb 100/3, which was known to bind only to the 105 kd component of HA-I. The antibody was then precipitated with fixed *Staphylococcus aureus* cells. The precipitate was analysed by SDS−PAGE and immunoblotting using mAb 100/3 to estimate the extent of binding, and mAb 100/1 to test for the presence of the 115 and 110 kd polypeptides in the preciptate. The results showed clearly that both were indeed brought down, strongly suggesting that they interact with the 105 kd polypeptide (Figure 8A, B). The reaction of mAb 100/1 with a 106 kd polypeptide in the supernatant (Figure 8B, track 2) serves as an internal control, because it is HA-II b which is also recognized by mAb 100/1 but not precipitated by the HA-I specific monoclonal antibody mAb 100/3.

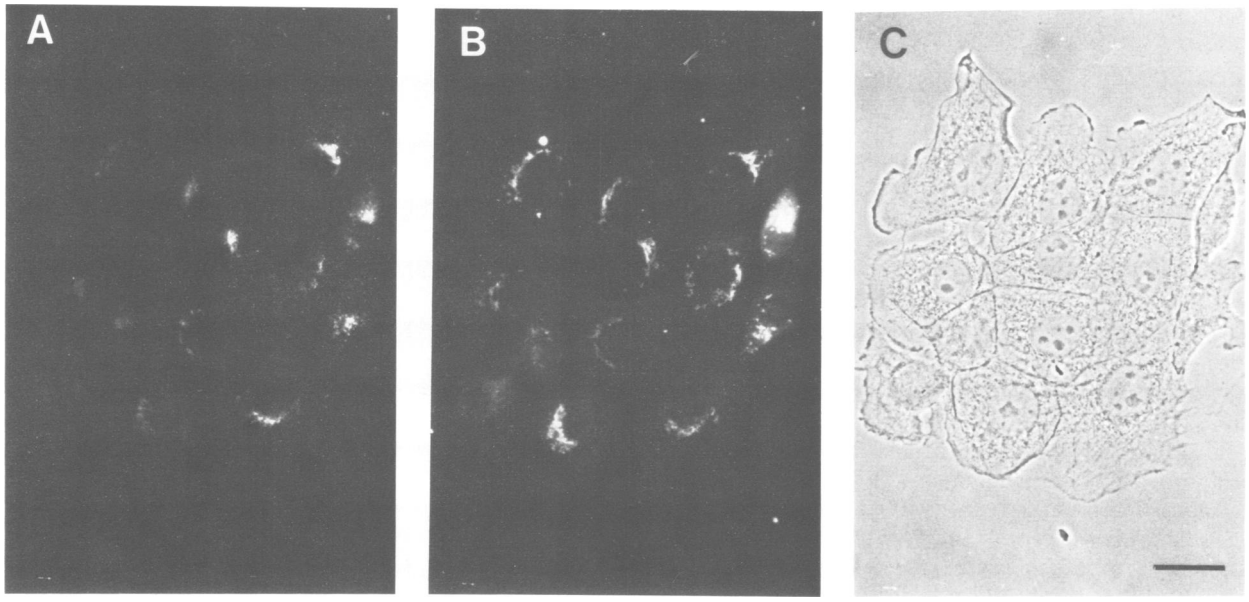


**Fig. 12.** Co-localization of the Golgi enzyme galactosyl-transferase and HA-I assembly protein in MDBK-cells. The monoclonal antibody against HA-I (mAb100/3) was visualized with rhodamine-conjugated goat anti-mouse antibodies (**A**) and the polyclonal antibody to galactosyl-transferase with fluorescein-conjugated goat anti-rabbit antibodies (**B**); (**C**) phase-contrast image; bar: 20 μm.

An analogous experiment for detecting interactions between the polypeptides of the HA-II complex could not readily be performed, because neither mAb 100/1 nor mAb 100/2 reacted with solubilized HA-II polypeptides under non-denaturing conditions. A polyclonal rabbit antiserum against the total assembly protein fraction was therefore purified by affinity chromatography on nitrocellulose strips carrying electrophoretically pure b-type polypeptide from HA-II. This polypeptide could be obtained in a highly purified form from nitrocellulose replicas of two-dimensional gels where it migrates off the diagonal (see Figure 3). Analysis of the immunoprecipitate by immunoblotting showed that the monospecific anti b-type antibody also precipitated c- and a-type polypeptides (Figure 8D; due to the low amount of a-type polypeptides they are difficult to discern). This result strongly suggests an interaction of the c- and a- with the b-type polypeptides within the HA-II complex.

### *Immunoreactive forms of assembly polypeptides in liver and cultured cell lines*

We next tested various cell and tissue extracts for their content of 100−115 kd polypeptides by immunoblotting. All three antibodies reacted with polypeptides of ~100 kd in liver, human heart fibroblasts and Madin−Darby bovine kidney (MDBK) cells but not with any components in the 110−115 kd range from these sources (Figure 9A, B) or from PC12 cells, neuroblastoma and astrocytes (data not shown). These results suggest that the 110 to 115 kd polypeptides may be specific variants that occur only in some cell types of brain. Robinson and Pearse (1986) arrived at a similar conclusion in regard to the 112 kd components of the HA-II complex. To test whether HA-II b from other sources than brain displays the same anomalous migration in urea−SDS compound gels we fractionated a crude coated vesicle extract from bovine liver on our two-dimensional gel system, transferred it to nitrocellulose and reacted it with mAb 100/1 and mAb100/2. The component was indeed seen to lie off the diagonal whereas the zone corresponding to HA-II-c migrated normally in the first dimension (Figure 9B).

### *Intracellular distribution of HA-I and HA-II polypeptides*

We employed both monoclonal and polyclonal antibodies to study the intracellular distribution of the 104−115 kd polypeptides from the HA-I and HA-II groups in a human heart fibroblast line and in MDBK cells. Cells from rodents were not studied in detail because mAb 100/3 does not recognize their HA-I assembly polypeptide. Figure 10 shows a human heart fibroblast that was simultaneously labelled with polyclonal rabbit antibodies to clathrin light chains and with mAb 100/3. The anti-light chain antibodies decorate clathrin-coated pits on the plasma membrane and most conspicuously clathrin-coated structures in the Golgi region (Figure 10A). Binding of mAb 100/3 appears to be largely confined to the Golgi region of the cell, where it evidently stains the same structures as the polyclonal anti-light chain antibody (Figure 10B). We also observed weak fluorescent speckles in other parts of the cell. To determine whether these represented vesicular structures or plasma membrane associated pits we applied the lysis squirting technique of Nermut (1982) to MDBK cells. Figure 11 shows a confluent cell layer from which most of the internal membranes had been removed by a buffer jet from a hypodermic needle. Only the plasma membranes which adhere tenaciously to the glass cover slip remain (Figure 11C). These were then double-labelled with antibodies to clathrin light chains and mAb 100/3. The light chain antibody gave rise to a punctate pattern over areas that were completely devoid of internal membrane structures and to strong labelling wherever intact cells remained (Figure 11A). In contrast, mAb 100/3 stained only intact cells and remaining Golgi structures, while the plasma membrane was not labelled at all (Figure 11B). We conclude that the 105 kd polypeptide of the HA-I group,

which is recognized by mAb 100/3, associates specifically with coated membranes in the Golgi and to a lesser extent possibly with post-Golgi vesicles but does not bind to the plasma membrane. The same result was obtained when the plasma membranes of human heart fibroblasts were stained with mAb 100/3 after lysis squirting (data not shown). Next we compared in double-labelling experiments on MDBK cells the distribution of the 104 kd polypeptide of the HA-I group with that of galactosyl-transferase, an enzyme that resides in the lumen of the *trans*-Golgi. Both antibodies gave rise to similar fluorescent juxtanuclear patches (Figure 12).

mAb 100/1, applied to MDBK-cells, strongly stained clathrin-coated membranes at the plasma membrane and in the Golgi region (Figure 13). This result was not unexpected because mAB 100/1 recognizes an epitope common to the HA-I and HA-II complexes (see above). To localize the components HA-IIa and c in cultured cells we had to revert to a polyclonal antiserum since mAb 100/2 would not decorate these antigens in intact cells. The rabbit polyclonal serum was affinity-purified on nitrocellulose strips bearing electrophoretically pure HA-IIc polypeptide. The antibodies against component c, which also reacted with the a-type polypeptides, decorated only coated pits on the plasma membrane (Figure 14). This result confirms the observations by Robinson (1987) who studied the distribution of a- and c-type polypeptides in fibroblasts with monoclonal antibodies.

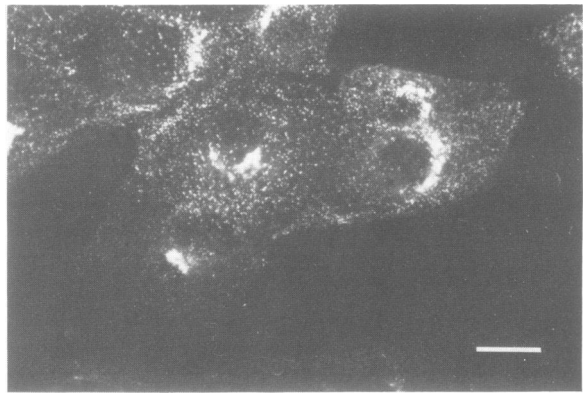

**Fig. 13**. Distribution of HA-I and HA-IIb assembly polypeptides in MDBK cells. Methanol fixed MDBK-cells were incubated with mAb 100/1 followed by rhodamine-conjugated goat anti-mouse antibody. Bar: 20 $\mu$M.

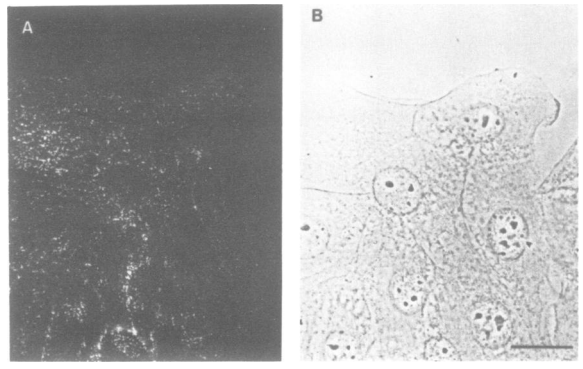


**Fig. 14.** Distributon of a- and c-type assembly polypeptides in MDBK-cells. The cells were incubated with affinity-purified antibodies to HA-IIc and subsequently with fluorescein-conjugated goat anti-rabbit antibodies. Note that only coated pits on the plasma membrane are labelled (**A**). (**B**) Phase-contrast image. Bar: 20 $\mu$m.

## Discussion

### *Structural relationship between the 100 – 115 kd polypeptides*

The data presented above show that the HA-I and HA-II groups of clathrin assembly proteins from bovine brain tissue together contain nine polypeptides with apparent mol. wts ranging from 105 to 115 kd. The results of peptide mapping and immunoblotting suggest the subdivision of these polypeptides into four classes henceforth referred to as $\alpha$, $\beta$, $\beta'$ and $\gamma$. The first two are part of the HA-II assembly protein complex, while the $\beta'$ and $\gamma$ species occur only in HA-I. The $\alpha$ class comprises the 112 kd polypeptides a1 and a2, and the 105 kd polypeptides c1 and c2, polypeptides b (106 kd) and b* (110 kd) belong to the $\beta$ class. Because of the extensive homologies between the $\beta$ class polypeptides and the 115 kd and the 110 kd polypeptides of the HA-I complex, the latter are referred to as the $\beta'$ class polypeptides. The 104 kd polypeptide is classified as the $\gamma$ chain of the HA-I complex. The major observable difference between the polypeptides within each class is electrophoretic mobility; this corresponds to an apparent mol. wt difference of up to 10 kd. The underlying cause of the differences in mobility has not yet been investigated, but it could equally well result from variations in polypeptide chain length, as from post-transcriptional modifications, such as different degrees of phosphorylation. It has recently been observed that the 105 – 115 kd polypeptides are substrates of kinases *in vitro* and *in vivo* (Keen and Black, 1986; Bar-Zvi and Branton, 1986; Schook *et al.*, 1987; Cantournet *et al.*, 1987). We also do not know why the $\beta$-type polypeptides are uniquely retarded in urea – SDS compound gels. This unusual behaviour, which has also been observed in the tropomyosins (Bretscher and Weber, 1978) provides a more convenient tool for separating the $\beta$ and $\beta'$ species from the $\alpha$ class polypeptides than the previously described method by Robinson and Pearse (1986).

As regards the HA-II group, Robinson and Pearse (1986) recognized three polypeptides in the 100 – 120 kd range, of which two (referred to as the 'upper' and 'lower' bands) were related. The 'middle' band was unrelated and behaved in a different manner from the others when chromatographed on hydroxyapatite in the presence of SDS. This band corresponds to our $\beta$-type polypeptides, which migrate anomalously in the urea – SDS gels and perhaps for the same (unknown) reasons they also elute anomalously from hydroxyapatite. Ketis (1987) resolved six polypeptides, migrating between 100 and 116 kd by SDS – PAGE of preparations of purified coated vesicles. All were found to give rise to a series of common tryptic or chymotryptic peptides. These data are difficult to reconcile with our results but we feel that the less rigorous methods used by Ketis (1987) for purifying the individual components may account for the differences from our results.

### *Structural implications for the HA-I and HA-II complexes*

The native mol. wts of the HA-I and HA-II complexes were estimated by chemical cross-linking (Pearse and Robinson, 1984) and by gel filtration experiments (Manfredi and Bazari,

1987) to be close to 300 kd. Based on this value and an apparent equimolar ratio of the 100–115 kd to the 50 kd constituent it was suggested (Pearse and Robinson, 1984) that the HA-II complex is made up from two 100–115 kd polypeptides and two copies of the 50 kd polypeptide, disregarding the 17 kd polypeptide, which forms part of the HA-II complex (Zaremba and Keen, 1986). Our data allow a refinement of this model. The α- and the β-type polypeptides of the HA-II complex and the β′ class and γ polypeptide of the HA-I complex occur in roughly equimolar amounts. Moreover, antibodies directed against the β polypeptides also quantitatively precipitated the α type polypeptides, and a monoclonal antibody against the γ polypeptide resulted in coprecipitation of the β′ component. Taken together these results suggest that the HA-II complex is composed of one α type and one β type polypeptide, and correspondingly the HA-I complex consists of a γ type and a β′ type polypeptide. A heterodimeric arrangement of the 100–115 kd polypeptides is also rational in functional terms: both assembly protein complexes are capable of binding to clathrin which implies that they should have at least one domain in common. Such a common domain is found in the β and β′ class polypeptides. The α- and γ-class polypeptides, which are specific for the respective assembly protein complexes, may determine the different distribution of the two assembly protein complexes in cells. Morover, the α-class polypeptides are likely to interact with the HA-II-specific 50 kd phosphoprotein and with the 17 kd polypeptide, while the γ polypeptide from the HA-I complex probably attaches to the 40 and 20 kd polypeptides. Our estimates of the molar ratio between the 100–115 kd and the 50 kd (40 kd) polypeptides, which are based on relative intensities of the stained protein bands in electrophoretic gels, vary between 0.6 and 0.7. Given the uncertainties of this method the proposed model of the assembly protein complexes should be regarded as tentative. More precise values for the mol. wts of the assembly protein complexes and of the molecular proportions of the polypeptides in both complexes will be needed to strengthen the model.

### *Intracellular distribution of 100–115 kd polypeptides*

The results of immunofluorescence studies on cultured cells, taken together with the immunological and biochemical characterization of the two assembly protein complexes lead to the conclusion that the HA-II complex is confined to coated pits on the plasma membrane, while the HA-I complex is located in coated pits in the *trans*-golgi network. How can this conclusion be reconciled with earlier studies of Robinson and Pearse (1986) which showed that a polyclonal antiserum against the 100 kd polypeptides from HA-II stains coated pits in both the Golgi and on the plasma membrane of fibroblasts? The most likely explanation is that the polyclonal antiserum reacted, like our monoclonal antibody mAb 100/1, with epitopes common to the β and β′ class polypeptides.

### *Function of assembly proteins*

Three types of clathrin-associated proteins with assembly promoting functions (HA-I, HA-II and AP 180) have now been described in coated vesicles from bovine brain. In spite of the differences between the HA-I and HA-II complexes they nevertheless appear to be more closely related to each other than to the brain-specific assembly protein AP 180. The functions of AP 180 in the cell may therefore differ from those of the structurally more complex HA-assembly proteins. But what precisely is the function of the assembly proteins? They are certainly not expected to function in the cell in exactly the same way as *in vitro*, because in that case the cell would quickly accumulate redundant empty clathrin cages. It is more likely that the promotion of clathrin assembly is a secondary phenomenon. Previously we speculated that the primary function of assembly proteins might be to link the apparently structurally diverse constituents of coated membranes to the principal coat protein clathrin (Ahle and Ungewickell, 1986). For such an adaptor function the assembly proteins would have to be as diverse in structure as the cargos of coated membranes. The results described here suggest that this is not the case. Alternatively the recognition of certain receptors by the coat proteins may either be mediated by still unknown factors or, perhaps more likely by a recognition signal encoded with the conformation of the cytoplasmic domains of the receptors, as suggested by Pfeffer and Rothman (1987).

In cells such as fibroblasts, which do not possess a regulated exocytotic pathway, clathrin-coated membranes were shown to be involved in receptor-mediated endocytosis and in the transport of newly synthesized lysosomal proteins. If the assembly proteins are only involved in connecting the cargo to the clathrin and thereby also inducing the assembly of clathrin, then one species of assembly protein would suffice. However, in fibroblasts both the HA-I and HA-II assembly protein complexes are present. If one assumes that the assembly proteins are also involved in targeting the transport vesicles to their correct destination (lysosome *versus* endosome) than the requirement for two types in fibroblasts is readily explained. Moreover, in cells which in addition possess a regulated exocytotic pathway, one might expect to find a third 'HA-III' assembly protein complex. If the assembly proteins are indeed involved in recognition of the target membrane it would be obligatory that only the clathrin is lost when the coated vesicle uncoats. This has been observed by Schlossman *et al.* (1984), who showed that the uncoating ATPase selectively dissociates clathrin from the membranes of coated vesicles, and leaves the assembly proteins behind. Immunofluorescence microscopy has so far failed to demonstrate the existence of assembly protein-containing transport vesicles that are free of clathrin (Robinson and Pearse, 1986 and our data). However, immunofluorescence, being an essentially qualitative rather than quantitative method may in this case not be very conclusive, since a transport vesicle with residual clathrin could be easily mistaken for a coated vesicle.

## Materials and methods

### *Materials*

Fresh bovine brains were obtained from a local abattoir and processed within 1 h of slaughter. Mono Q HR 5/5 and HR 10/10 anion-exchange columns, protein standards for SDS–PAGE, CNBr-activated Sepharose 4B, Ficoll 400, protein-A Sepharose CL-4B and PD-10 gel filtration columns were from Deutsche Pharmacia, Freiburg (FRG); t.l.c. cellulose plastic sheets without fluorescence indicator were obtained from Merck, Darmstad (FRG) and ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA) were from Serva, Heidelberg (FRG); reagents for SDS–PAGE were from LKB Instrument GmbH, Gräfelfing (FRG); 2-(N-morpho-

lino)ethanesulphonic acid (MES), dithiothreitol (DTT), galactosyl-transferase (EC 2.4.1.22); phenylmethylsulphonyl fluoride (PMSF) and unconjugated rabbit IgGs were from Sigma Chemie GmbH, Deisenhofen (FRG); carrier-free $^{125}I$ (3.7 GBq/ml) were from Amersham Buchler GmbH, Braunschweig (FRG); Fuji RX X-ray film was from Fuji Film Co., Ltd (Japan); peroxidase-conjugated IgGs to mouse and rabbit antibodies were from Dakopatts GmbH, Hamburg (FRG); fluorescein- and rhodamine-conjugated anti-rabbit and anti-mouse IgGs (both from goat) were from Dianova, Hamburg (FRG); nitrocellulose transfer membranes (BA 83, 0.2 μm) were from Schleicher and Schüll, Dassel (FRG); media and most ingredients for cell cultures were from Gibco, Eggenstein (FRG); foetal calf serum from Boehringer, Mannheim (FRG); disposable plastics from Nunc GmbH, Weisbaden (FRG). *Staphylococcus aureus* cells (Pansorbin) were obtained from Calbiochem. T-max 400 photographic film was from Kodak and Ultrafin developer from Tetenal Photowerk GmbH, D-2000 Norderstedt. MDBK cells were obtained from Prof. K.Weber (Max-Planck-Institute for Biophysical Chemistry, Göttingen) and a human heart fibroblast line from Dr Kandolf (Max-Planck Institute for Biochemistry, Martinsried).

### Methods

*Purification of HA-I and HA-II assembly proteins.* Coated vesicles from bovine brain tissue were prepared according to Campbell *et al.* (1984). For a typical assembly protein preparation ~50 mg coated vesicles were extracted in the presence of 0.1 mM PMSF with 0.5 M Tris according to Keen *et al.* (1979). The extract was clarified by ultracentrifugation at 100 000 *g* for 45 min and then extensively dialysed against 20 mM ethanolamine or diethanolamine, pH 8.9, 2 mM EDTA and 1 mM DTT. To separate the assembly proteins from clathrin the extract was chromatographed on a semi-preparative Mono Q column (8 ml resin). The column was eluted at room temperature with a linear 0–0.5 M NaCl gradient. The gradient volume was 240 ml and the flow rate 3 ml/min. Fractions containing the assembly proteins which eluted between 0.2 M and 0.3 M NaCl were pooled, once more dialysed against low salt at pH 8.9 and then further purified by ion-exchange chromatography on an analytical Mono Q column, which was eluted first with a steep linear gradient from 0–0.15 M NaCl (5 ml volume) followed by a 15 ml gradient from 0.15–0.25 M NaCl. Fractions containing the HA-I and HA-II assembly proteins were pooled separately and then applied to a hydroxyapatite column (1 ml volume), and chromatographed as described previously (Ahle and Ungewickell, 1986). HA-I proteins were eluted with 0.18 M phosphate and HA-II proteins with 0.39 M phosphate. A typical preparation yielded ~0.15 mg HA-I and 0.5 mg HA-II assembly protein which were either stored frozen at −20°C in 50% glycerol or rapidly frozen in liquid nitrogen and stored at −70°C.

*Electrophoretic methods.* SDS–PAGE was performed according to Laemmli (1970). For two-dimensional analysis the samples were heated for 5 min in standard SDS-sample buffer (Laemmli, 1970) and then electrophoresed in 7.5% polyacrylamide slab gels (10.5 × 11.5 × 1 mm) that contained in the separation gel 6 M urea and 2 mM EDTA in addition to 0.1% SDS. Gel strips from the first dimension were equilibrated for 45 min with 0.125 M Tris–HCl, 0.1% SDS, 1% β-mercaptoethanol, pH 6.8 (three changes) and then mounted on top of the stacking gel of a 5–10% SDS–polyacrylamide gradient gel. Gels were either stained for protein or electroblotted onto nitrocellulose paper (for details see Ahle and Ungewickell, 1986). The amount of protein in stained polyacrylamide gels was quantified by eluting the Coomassie brilliant blue stain with 25% pyridine and by determining its concentration spectrophotometrically as described by Fenner *et al.* (1975).

*Peptide mapping.* Two-dimensional peptide analysis of $^{125}I$-labelled tryptic peptides was performed essentially according to the procedure of Elder *et al.* (1977) as modified by Ahle and Ungewickell (1986). Polypeptides were labelled in the polyacrylamide gel with at least 18.5 Mbq. The reaction was terminated by addition of DTT (final conc. 0.4 M) instead of sodium bisulphite.

*Antibody production.* The three monoclonal antibodies used in this paper were obtained from two cell fusion experiments. mAb 100/1 and mAb 100/3 were obtained from 6–8 weeks old BALB/c mice that had been immunized at intervals of two weeks with unfractionated HA-I and HA-II assembly proteins. Sera were tested by immunofluorescence microscopy. Spleen cells from the mouse giving the strongest reaction were fused with the myeloma line PA1 following the procedure of Debus *et al.* (1983). Fused cells were distributed between thirty 24-well plates using HAT-medium. Supernatants were tested by immunofluorescence microscopy for their ability to stain coated pits in MDBK cells. Colonies of interest were subcloned twice by limited dilution. For large-scale production of monoclonal antibodies hybridomas were cultured in roller bottles and harvested after 1 week. The antibodies were concentrated by precipitation with 50% ammonium sulphate. The precipitate was redissolved in saline and then dialysed against 1.5 M glycine, 3 M NaCl, pH 8.9. IgGs were purified by affinity chromatography on protein-A Sepharose according to the manufacturer's instructions. Typical yields of antibodies from 600 ml of hybridoma supernatants varied between 15–25 mg. mAb 100/2 was obtained from a mouse that had been immunized with the 104 and 106 kd HA-II polypeptides. Both polypeptides were purified by preparative SDS–PAGE. Supernatants were tested by immunoblotting (for details of the procedure see Ahle and Ungewickell, 1986).

Polyclonal antibodies against HA-I and HA-II assembly proteins were obtained from two rabbits that had been immunized by repeated injections (4 × subcutaneously and intramuscularly) with the total assembly protein fraction over a period of 12 weeks. The polyclonal rabbit antisera against bovine galactosyl-transferase was a gift from Prof. K.Weber (Hiller and Weber, 1982). Antibodies to clathrin light chains from bovine brain tissue were elicited in a rabbit that had been immunized with purified clathrin light chains. The light chains were prepared by heat-denaturing highly purified clathrin triskelia (Lisanti *et al.*, 1982).

*Purification of polyclonal antisera.* Polyclonal antisera against the assembly proteins were incubated with nitrocellulose strips from a 2-D gel replica bearing HA-IIb or HA-IIc. Up to 20 of them were used to purify sufficient monospecific antibodies to these polypeptides. (For details of this method see Olmsted, 1981 and Ungewickell, 1985.) Antisera to galactosyl-transferase were affinity-purified using commercially available bovine galactosyl-transferase from bovine milk. The enzyme was further purified by SDS–PAGE and electroblotted onto nitrocellulose paper. A band with an apparent mol. wt of 46 kd was excised and used for the purification of monospecific antibodies. Monospecific antibodies to clathrin light chains were prepared by affinity chromatography on light chains coupled to CNBr-activated Sepharose (for details see Ungewickell, 1985).

*Immunoprecipitation experiments.* HA-I and HA-II assembly proteins were extracted with 0.5 M Tris–HCl, pH 7.0, from coated vesicles. The extract was clarified by ultracentrifugation and then transferred by gel filtration on a PD-10 column into a buffer containing 50 mM Tris–HCl, 0.15 M NaCl, 2 mM EDTA, pH 8.2 (TNE-buffer). 60 μl of the extract which corresponded to 30 μg protein was incubated for 45 min at room temperature with 50 μg mAb 100/3. Antibody–antigen complexes were precipitated with 0.1 mg *S.aureus* cells (Pansorbin). Before use, the cells were incubated with 3% BSA and then washed three times with TNE-buffer. The immunoprecipitates were washed five times with TNE-buffer and once with 50 mM Tris, 0.5 M NaCl, 0.5% Nonidet P-40, pH 8.2. The final pellet was resuspended in a final volume of 70 μl SDS-sample buffer and briefly boiled. Insoluble particles were removed by centrifugation and the supernatant was subjected to SDS–PAGE and immunoblotting. In order to block the binding of peroxidase-conjugated rabbit anti-mouse antibody to solubilized protein A, the BSA-quenched blot was incubated for 30 min at room temperature with rabbit IgGs at a concentration of 1 mg/ml. HA-II and HA-I complexes were immunoprecipitated from a 'Tris-extract' with affinity-purified polyclonal antibodies against HA-IIb.

*Immunofluorescence microscopy.* MDBK-cells and human heart fibroblasts were grown on round 1.2 cm glass coverslips in DME-medium, supplemented with fetal calf serum (10% for MDBK cells and 20% for the fibroblasts). The cells were either fixed with 3.7% formaldehyde in PBS and then permeabilized with 0.5% Triton X-100 for 5 min at room temperature or fixed and permeabilized for 6 min with methanol at −20°C. Methanol fixation gave the best results for mAb 100/1. For preparing plasma membrane monolayers from MDBK-cells the cells were grown on coverslips until confluent, washed in PBS and then allowed to swell for 5 min at 4°C in 20 mM MES, 2.5 mM $MgCl_2$, 2.5 mM EGTA, 1 mM benzamidine, 1 mM 1,10-phenanthroline, 1 mM leupeptin (MES-buffer). The upper plasma membrane and internal organelles were then removed by a stream of MES-buffer from a 5 ml syringe fitted with a 0.6 × 25 mm hypodermic needle. The monolayers were immediately fixed with 3.7% formaldehyde and subsequently incubated with 0.5% Triton X-100. Fixed cells and monolayers were incubated for 60 min at 37°C with 10–20 μl of purified monoclonal antibodies at a concentration of 1 μg/ml in the presence of 3% albumin. The cells were further processed for indirect immunofluorescence according to Osborn and Weber (1982), using fluorescein-conjugated goat anti-rabbit and/or rhodamine-conjugated goat anti-mouse antibodies. Before use the anti-rabbit antibody was passed through a column with immobilized mouse IgGs and the anti-mouse antibody was passed through a column with rabbit IgGs to remove any cross-reacting antibodies. Coverslips were mounted in Moviol 4-88 which contained 1 mg/ml *p*-phenylenediamine

to minimize the bleaching of the fluorescein conjugate. The specimens were viewed with a Zeiss IM 35 inverted microscope. Photographs were taken on Kodak Tmax 400 film and developed with Ultrafin for 8 min.

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## References

Ahle,S. and Ungewickell,E. (1986) *EMBO J.*, **5**, 3143–3149
Anderson,R.G.W., Vasile,E., Mello,R.J., Brown,M.S. and Goldstein,J.L. (1978) *Cell*, **15**, 919–933.
Bar-Zvi,D. and Branton,D. (1986) *J. Biol. Chem.*, **261**, 9614–9621.
Bretscher,A. and Weber,K. (1978) *FEBS Lett.*, **85**, 145–148.
Campbell,C., Squicciarini,J., Shia,M., Pilch,P.F. and Fine,R.E. (1984) *Biochemistry*, **23**, 4420–4426.
Cantournet,B., Creuzet,C., Komano,O. and Loeb,J. (1987) *FEBS Lett.*, **220**, 143–148.
Debus,E., Weber,K. and Osborn,M. (1983) *EMBO J.*, **2**, 2305–2312.
Elder,J.H., Pickett,R.A., Hampton,J. and Lerner,R.A. (1977) *J. Biol. Chem.*, **252**, 6510–6516.
Fenner,C., Traut,R.R., Mason,D.R. and Wikman-Coffelt,J. (1975) *Anal. Biochem.*, **63**, 595–602.
Goldstein,J.L., Brown,M.S., Anderson,R.G.W., Russell,D.W. and Schneider,W.J. (1985) *Annu. Rev. Cell Biol.*, **1**, 1–19.
Hiller,G. and Weber,K. (1982) *Exp. Cell Res.*, **142**, 85–94.
Keen,J.H. and Black,M.M. (1986) *J. Cell. Biol.*, **102**, 1325–1333.
Keen,J.H., Willingham,M.C. and Pastan,I.H. (1979) *Cell*, **16**, 303–312.
Ketis,N.V. (1987) *Biochim. Biophys. Acta*, **924**, 303–311.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685
Lisanti,M.P., Shapiro,L.S., Moskowitz,N., Hua,E.L., Puszkin,S. and Schook,W. (1982) *Eur. J. Biochem.*, **125**, 463–470.
Manfredi,J.J. and Bazari,W.L. (1987) *J. Bio. Chem.*, **262**, 12182–12188.
Nermut,M.V. (1982) *Eur. J. Cell Biol.*, **28**, 160–172.
Olmsted,J.B. (1981) *J. Biol. Chem.*, **256**, 11955–11957.
Orci,L., Ravazzola,M., Amherdt,M., Louvard,D. and Perrelet,A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5385–5389.
Osborn,M. and Weber,K. (1982) *Methods Cell Biol.*, **24**, 97–132.
Pearse,B.M.F. (1978) *J. Mol. Biol.*, **126**, 803–812.
Pearse,B.M.F. (1985) *EMBO J.*, **4**, 2457–2460.
Pearse,B.M.F. and Bretscher,M.S. (1981) *Annu. Rev. Biochem.*, **50**, 85–101.
Pearse,B.M.F. and Robinson,M.S. (1984) *EMBO J.*, **3**, 1951–1957.
Pfeffer,S.R. and Rothman,J.E. (1987) *Annu. Rev. Biochem.*, **56**, 829–852.
Robinson,M.S. (1987) *J. Cell Biol.*, **104**, 887–895.
Robinson,M.S. and Pearse,B.M.F. (1986) *J. Cell Biol.*, **102**, 48–54.
Schlossman,D.M., Schmid,S.L., Braell,W.A. and Rothman,J.E. (1984) *J. Cell Biol.*, **99**, 723–733.
Schook,W.J., Parker,C., Silva,W.I., Kohtz,S. and Puszkin,S. (1987) *J. Neurochem.*, **49**, 434–441.
Schook,W., Puszkin,S., Bloom,W., Ores,C. and Kochwa,S. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 116–120.
Unanue,E.R., Ungewickell,E. and Branton,D. (1981) *Cell*, **26**, 439–446.
Ungewickell,E. (1985) *EMBO J.*, **4**, 3385–3391.
Ungewickell,E. and Branton,D. (1981) *Nature*, **289**, 420–422.
Vigers,G.P.A., Crowther,R.A. and Pearse,B.M.F. (1986) *EMBO J.*, **5**, 2079–2085.
Woodward,M.P. and Roth,T.F. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4394–4398.
Zaremba,S. and Keen,J.H. (1985) *J. Cell. Biochem.*, **28**, 47–58.

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