Immunofluorescent Localization of 100K Coated Vesicle Proteins

Margaret S. Robinson and Barbara M. F. Pearse

Medical Research Council (MRC) Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England

Abstract. A family of coated vesicle proteins, with molecular weights of ~100,000 and designated 100K, has been implicated in both coat assembly and the attachment of clathrin to the vesicle membrane. These proteins were purified from extracts of bovine brain coated vesicles by gel filtration, hydroxylapatite chromatography, and preparative SDS PAGE. Peptide mapping by limited proteolysis indicated that the polypeptides making up the three major 100K bands have distinct amino acid sequences. When four rats were immunized with total 100K protein, each rat responded differently to the different bands, although all four antisera cross-reacted with the 100K proteins of human placental coated vesicles. After affinity purification, two of the antisera were able to detect a 100K band on blots of whole 3T3 cell protein and were used for immunofluorescence, double labeling the cells with either rabbit anti-clathrin or with wheat germ lectin as a Golgi apparatus marker. Both antisera gave staining that was coincident with anti-clathrin, with punctate labeling of the plasma membrane and perinuclear Golgi apparatus labeling. Thus, the 100K proteins are present on endocytic as well as Golgi-derived coated pits and vesicles. The punctate patterns were nearly identical with anti-100K and anti-clathrin, indicating that when vesicles become uncoated, the 100K proteins are removed as well as clathrin. One of the two antisera gave stronger plasma membrane labeling than Golgi apparatus labeling when compared with the anti-clathrin antiserum. The other antiserum gave stronger Golgi apparatus labeling. Although we have as yet no evidence that these two antisera label different proteins on blots of 3T3 cells, they do show differences on blots of bovine brain 100K proteins. This result, although preliminary, raises the possibility that different 100K proteins may be associated with different pathways of membrane traffic.

COATED vesicles are found in nearly all eukaryotic cells and participate in many different pathways of membrane traffic. They are derived from coated pits, which occur both on the plasma membrane and on intracellular organelles, and which concentrate those proteins that are to be transported to a different membrane compartment (9). Their cytoplasmic coats are composed of clathrin heavy and light chains, a 50,000 dalton protein that has been designated 50K, and a family of proteins with molecular weights of ~100,000 that has been designated 100K. Studies on isolated coated vesicles have implicated the 100K proteins in coat assembly (10, 17) and, more tentatively, in the attachment of clathrin to the vesicle membrane (14).

A number of questions have been raised about the 100K proteins in vivo. For instance, are they only found on coated vesicles that bud from the Golgi apparatus, or are they present on endocytic coated vesicles as well (17)? Do they remain behind on vesicles whose clathrin coats have been removed (11)? Could different 100K proteins be associated with different pathways of membrane traffic (10)?

We have recently purified the 100K proteins of bovine brain coated vesicles and have begun to characterize some of their properties (10). Based on the behavior of the proteins on hydroxylapatite columns, we have divided them into two groups: a minor group of proteins, HA-I (or Hydroxyl-Apatite-I), which elute first from the column, and the major group of proteins, HA-II, which elute at higher phosphate concentrations, and which are complexed with the 50K protein. Both groups migrate on SDS gels as three closely spaced bands, and both groups promote the assembly of clathrin into coats.

In this paper, we further characterize the HA-II group of proteins. We have examined the heterogeneity of the proteins within this group both by peptide mapping and by labeling immunoblots with antibodies raised in rats. In addition, we have used the antibodies to label tissue culture cells and thus have addressed some of the questions about the 100K proteins in vivo.

Materials and Methods

Protein Purification

The HA-II 100K proteins were purified from extracts of bovine brain coated vesicles by a combination of gel filtration on Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) and hydroxylapatite chromatography, as previously described (10). The proteins were further purified by preparative SDS PAGE (6, 10), resulting in a final yield of ~1 mg of 100K protein from 12 brains (~4 kg). Partial separation of the three major 100K bands was

achieved by hydroxylapatite chromatography in the presence of SDS, either before or after running the preparative gel. Using the method of Moss and Rosenblum (7), the HA-II protein from 12 brains was dialyzed into 10 mM Na phosphate, pH 6.4, containing 0.1% SDS and 1 mM dithiothreitol, adsorbed to a 5-ml hydroxylapatite column (Bio-Rad Laboratories, Richmond, CA), and eluted with an 80-ml gradient of 0.2–0.5 M Na phosphate, pH 6.4.

The three major 100K bands were peptide mapped by the partial proteolysis method of Cleveland et al. (2). Minigels (3.5 × 8.5 cm) were run both for the initial protein separation and for the enzymatic digestion. *Staphylococcus aureus* protease was used at either 75 or 375 ng/well, and the gel was stained with silver (16).

Antibody Production

The 100K protein that was to be used as antigen was fluorescently labeled so that it could be easily monitored. Before the preparative gel electrophoresis step, the purified HA-II 100K proteins were labeled with the fluorescent dye 5-iodoacetamidofluorescein (Molecular Probes Inc., Junction City, OR), using 1 mg of dye per mg of protein, as described by Taylor and Wang (12). After dialysis to remove unbound dye, the protein was electrophoresed, eluted, and concentrated by acetone precipitation. It was then emulsified with Freund's adjuvant and portions were injected intraperitoneally into four Lou rats, using 100 μg of 100K protein per animal. The primary injection was given in complete Freund's adjuvant, and the animals were boosted on days 14 and 56 using incomplete Freund's adjuvant. Serum was collected from tail bleeds on days 70, 80, and 87.

For some experiments, the antisera were affinity purified. Unlabeled electrophoretically purified 100K protein was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) and ~0.5 mg of coupled protein was used to absorb 1 ml of serum. The columns were eluted with 0.2 M glycine, pH 2.3, followed by 3 M sodium thiocyanate/0.5 M NH_4OH, and neutralized with 3 M Tris-Cl, pH 7.6, or 4 M NaH_2PO_4. The activity of the various fractions was assayed on immunoblots (see below), and fractions containing anti-100K activity were pooled for each rat, dialyzed into phosphate-buffered saline (PBS), and concentrated by ultrafiltration to about half the volume of the original serum.

Immunoblots

Protein samples used in immunoblots included bovine brain HA-I and HA-II 100K proteins, human placental coated vesicles (8), and NIH 3T3 cell protein. The 3T3 cell protein was prepared by adding 50 μl of boiling sample buffer (6) to a 35-mm dish of confluent 3T3 cells, quickly scraping the cells off the dish, and incubating the sample in boiling water for 2 min. The DNA was broken up by sonication and the sample was centrifuged before electrophoresis. All proteins were electrophoresed on SDS minigels and electrophoretically transferred onto nitrocellulose, essentially as described by Towbin et al. (13), except that the elution was carried out overnight and the buffer contained only 5% methanol instead of 20%. After blocking (5), the nitrocellulose blots were dried and refrigerated until needed. Antibody labeling was carried out as described by Kilmartin and Adams (5), using 10^6 cpm/ml ^{125}I-labeled affinity-purified rabbit anti-rat IgG (from J. V. Kilmartin, MRC Laboratory of Molecular Biology).

Immunofluorescence

NIH 3T3 cells plated onto multiwell glass slides (Flow Laboratories, Inc., McLean, VA) were prepared for antibody labeling in one of two ways. They were either fixed for 10 min with 3.7% paraformaldehyde in PBS and permeabilized for 10 min with 0.1% Triton X-100 in PBS, or they were fixed and permeabilized in methanol for 5 min at −20°C. They were then incubated for 1 h at a time with a series of antibodies and washed with PBS between incubations. When only the 100K proteins were to be labeled, the cells were incubated with the undiluted affinity-purified rat antibody, followed by affinity-purified fluorescein isothiocyanate rabbit anti-rat IgG (Miles Laboratories Inc., Naperville, IL). In some cases, this step was followed by a third incubation in affinity-purified fluorescein isothiocyanate sheep anti-rabbit IgG (Miles Laboratories Inc.) to amplify the fluorescence. They were then mounted in 90% glycerol containing 1 mg/ml *p*-phenylenediamine (4) and examined on a Zeiss standard WL microscope, equipped with a 50W high pressure mercury light and selective filters (Zeiss 487710 and 487715) for fluorescein and rhodamine fluorescence.

Two types of double labeling experiments were also carried out. To visualize 100K and clathrin simultaneously, cells had to be fixed in paraformaldehyde, since methanol destroyed the antigenicity of the clathrin. They were then incubated with a 1:50 dilution of rabbit anti-clathrin (1), a generous gift from Dr. Saul Puszkin. This was followed by incubations with affinity-purified rhodamine-labeled sheep anti-rabbit IgG (Miles Laboratories Inc.), which had been absorbed with rat IgG coupled to Sepharose, then with rat anti-100K, and finally with affinity-purified fluorescein isothiocyanate sheep anti-rat IgG (Miles Laboratories Inc.), which had been absorbed with rabbit IgG coupled to Sepharose. Two controls were carried out to ensure that neither the rat anti-100K nor the fluorescein isothiocyanate sheep anti-rat was binding to the first pair of antibodies. First, when the rat anti-100K was omitted, no specific labeling was seen in the fluorescein channel. Second, when the anti-clathrin was replaced with rabbit anti-thymus myosin (from J. Scholey, MRC Laboratory of Molecular Biology), the fluorescein labeling looked completely different from the rhodamine labeling.

Cells were also double labeled with anti-100K and 20 μg/ml rhodamine-labeled wheat germ lectin (Pharmacia Fine Chemicals, Piscataway, NJ), a marker for the Golgi apparatus (15). The lectin had been labeled by dissolving it in 0.1 M $NaHCO_3$ and reacting it for 1 h on ice with tetramethylrhodamine isothiocyanate (Nordic Immunology, Maidenhead, England) at a ratio of 0.1 mg of dye per mg of protein. Unbound dye was removed on a column of Bio-Gel P-6 (Bio-Rad Laboratories, Whatford, England). Controls for the double labeling included preincubating the lectin with 0.2 M *N*-acetyl glucosamine, which blocked the specific rhodamine staining, and omitting the rat anti-100K, which resulted in no specific fluorescein staining.

Results

Protein Characterization

Fig. 1*A* shows an SDS polyacrylamide gel of a typical preparation of bovine brain 100K coat proteins. These correspond to the HA-II group of proteins (10), which elute from hydroxylapatite columns as complexes with the 50K coat protein, along with a trace of clathrin and some other minor contaminants. On very lightly loaded gels (Fig. 1*B*), three major 100K bands can be resolved which otherwise tend to run together, particularly the middle and lower bands. The 100K proteins were further purified by preparative SDS gel electrophoresis, and the three major bands were partially separated from each other by chromatography on a second hydroxylapatite column in the presence of 0.1% SDS. The upper and lower 100K bands began to elute at a lower phosphate concentration than the middle band (Fig. 1*C*).

Peptide mapping by limited proteolysis (2) (Fig. 2) showed some similarities between the upper and lower bands (compare *a* with *c*, *e* with *g*), but considerable differences between these bands and the middle band (*b* and *f*). These patterns suggest, first, that all three bands contain distinct polypeptides; and second, that the protein or proteins making up the middle

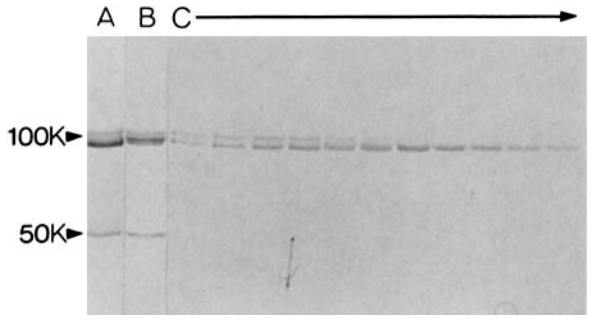

Figure 1. SDS PAGE of 100K proteins. Proteins were purified from bovine brain coated vesicles and electrophoresed on 7.5% acrylamide gels. (*A*) HA-II proteins. The 100K family and the 50K protein are indicated. (*B*) A lighter loading of a preparation similar to the one in *A*. Three bands can be resolved in the 100K region. (*C*) Sequential samples from a hydroxylapatite column of the electrophoretically purified 100K proteins, chromatographed in the presence of SDS. The upper and lower bands eluted first from the column; the middle band (which has a similar mobility to that of the lower band on this gel) began to elute at a higher phosphate concentration.

100K band differ markedly in amino acid sequence from the proteins of the other two bands, which may explain their different behavior on hydroxylapatite in SDS.

Antibody Production and Characterization

Four rats were immunized with electrophoretically purified HA-II total 100K proteins from bovine brain coated vesicles. Although all the rats were immunized with the same preparation, each rat responded somewhat differently. Fig. 3, *A* and *B*, shows immunoblots of the HA-II 100K proteins after hydroxylapatite chromatography in the presence of SDS, stained with the four antisera. Antiserum 1 stained the upper band most strongly, with weaker staining of the lower band and weakest staining of the middle band. Antiserum 2, although less intense than antiserum 1, preferentially stained the middle band. Antiserum 3 gave a pattern similar to that of antiserum 1, but the differences between the three bands were more pronounced. Antiserum 4 stained the upper and lower bands equally well, with much weaker staining of the middle band. These differences were maintained through serial dilutions ranging from 1:10 to 1:3,000.

When the four antisera were tested for their abilities to label a blot of the other HA-I group of 100K proteins from bovine brain (10), none of the antisera appeared to cross-react with any of the HA-I bands (data not shown). However, all four antisera cross-reacted strongly with one or more members of the 100K family of proteins from human placental coated vesicles (Fig. 3 *C*). Because the placental 100K bands are much more closely spaced on SDS gels than those of brain, it has not yet been possible to determine whether different antisera preferentially label different bands.

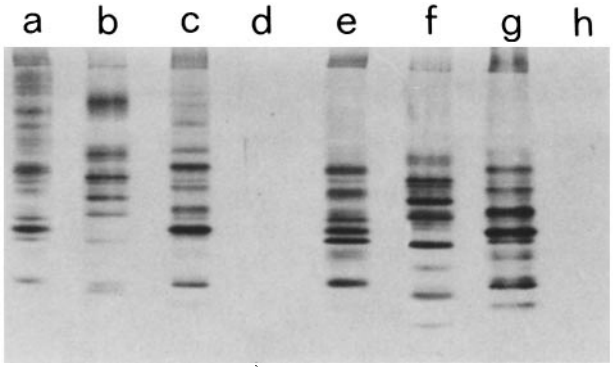

Figure 2. Peptide mapping by limited proteolysis of the three major 100K bands. Bands were cut from a gel similar to the one shown in Fig. 1 *C* and digested in a second 15% acrylamide gel with *S. aureus* protease, using 75 ng of protease per well in lanes *a–d* and 375 ng per well in lanes *e–h*. Lanes *a* and *e* are maps of the upper 100K band; lanes *b* and *f* are of the middle band; and lanes *c* and *g* are of the lower band. Lanes *d* and *h* are protease alone. The gel was silver stained.

Immunofluorescence

All four antisera were tested for their abilities to stain 3T3 cells. Those from rats 3 and 4 looked the most promising when the immune sera were compared with the preimmune sera: the cells looked brighter, and perinuclear labeling could be distinguished with antiserum 3. However, because of high background levels, all the antisera were affinity purified before further use. Of the four affinity-purified antisera, those from Rats 3 and 4 were able to detect a 100K band on blots of whole 3T3 cell protein (Fig. 3 *D*), and were also the only two that gave detectable immunofluorescent labeling. Serial dilutions of the two affinity-purified antisera resulted in fainter labeling, but the overall staining patterns were retained.

Cells stained with both antisera were labeled with a punctate pattern which was particularly bright in cells stained with antiserum 4 (Fig. 4 *A*). Focusing up and down on the cells indicated that most of the labeling was in the plane of the plasma membrane. In addition, perinuclear staining could be detected in most of the cells, and was particularly prominent in cells stained with antiserum 3 (Fig. 4 *B*). These results are

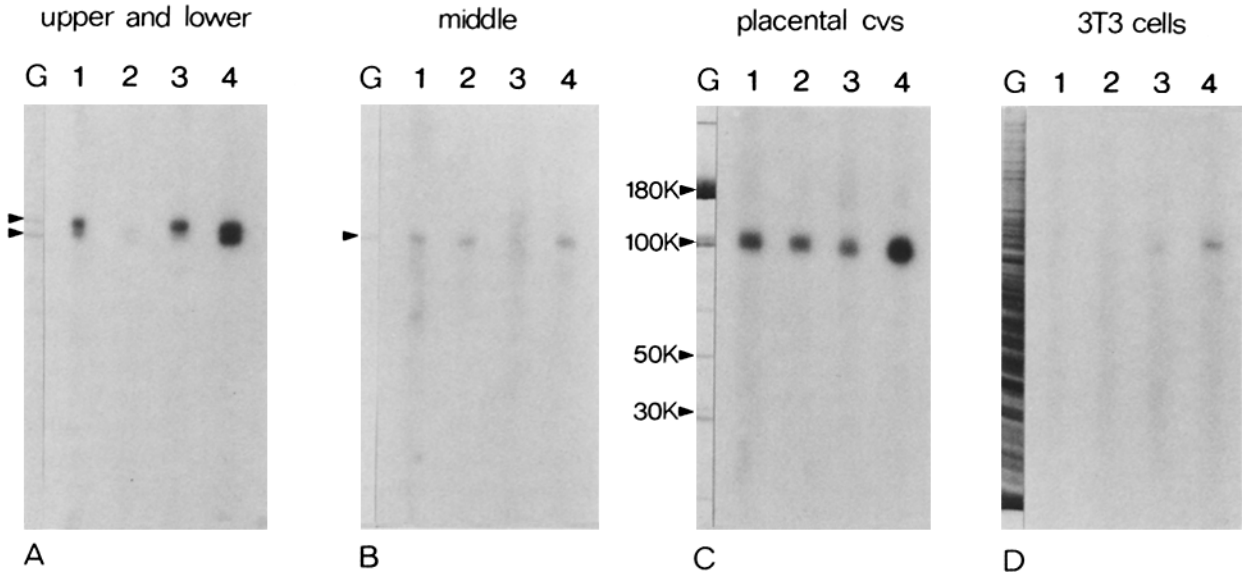

Figure 3. Immunoblots of proteins labeled with anti-100K antisera. Proteins were blotted and labeled with 1:50 dilutions of the four rat antisera (*A–C*) or with affinity-purified antisera (*D*) diluted 1:2 (*2* and *3*) or 1:10 (*1* and *4*). The number of the rat is indicated above each lane; lanes labeled *G* are the original gel, stained with PAGE blue 84. (*A*) Upper and lower 100K bands, separated from the middle band by hydroxylapatite chromatography in SDS (see Figure 1 *C*). (*B*) Middle band. (*C*) Human placental coated vesicle proteins (*CVS*). Clathrin heavy chain (*180K*), *100K*, *50K*, and light chains (*30K*) are indicated by their molecular masses. (*D*) Whole 3T3 cell proteins.

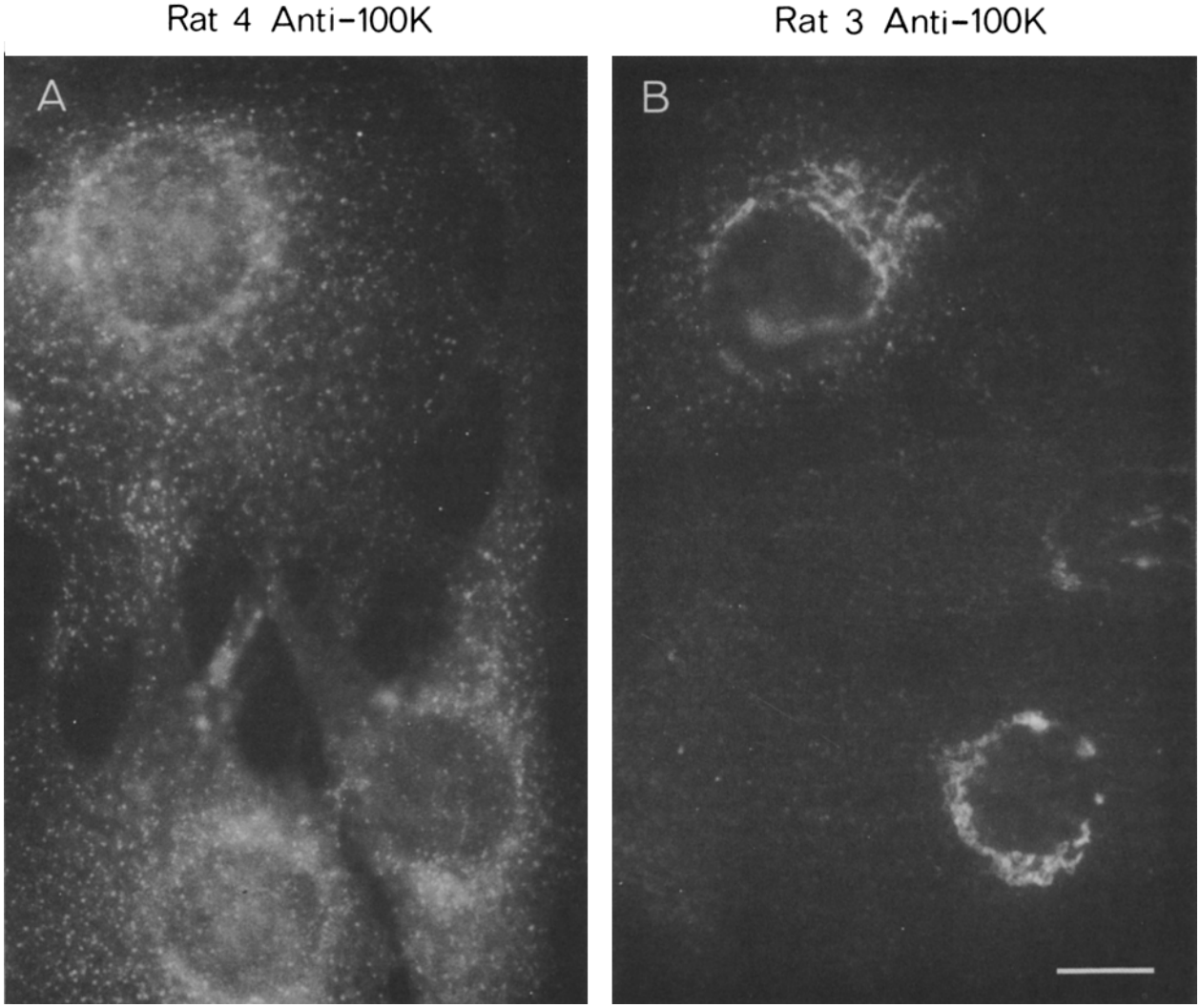

Figure 4. Indirect immunofluorescence micrographs of 3T3 cells stained with affinity-purified anti-100K. Cells were labeled with either antiserum 4 (*A*) or antiserum 3 (*B*). The photographs were taken focusing on the basal cell surface. Punctate labeling in the plane of the plasma membrane can be seen in cells stained with both antisera but is brighter in cells stained with antiserum 4 (*A*). Perinuclear labeling is also present, particularly in cells stained with antiserum 3 (*B*). Bar, 10 μm.

consistent with electron microscopic observations, which indicate that the majority of coated pits and vesicles in a cell are distributed in and around the plasma membrane or clustered in the Golgi region (3).

To confirm that the antisera were indeed labeling coated pits and vesicles, a rabbit antiserum against clathrin (1), which does not cross-react on blots with the 100K proteins (data not shown), was used to double label the cells. The anti-100K labeling was never as bright in these cells, since optimum labeling of the 100K proteins was achieved either by making a sandwich of fluorescent antibodies, which could not be done on double-labeled cells, or by fixing the cells with methanol, which destroyed the antigenicity of the clathrin. Nevertheless, the staining of the 100K proteins was bright enough to be compared with the clathrin staining. Fig. 5 shows cells double-labeled with antiserum 4 and anti-clathrin. The fluorescent dots in the spread margins of the cells are virtually superimposable with the two antisera. The main difference between the two labeling patterns is that the perinuclear region is stained more brightly with the anti-clathrin (*B* and *D*) than with the Rat 4 anti-100K (*A* and *C*). Double labeling with Rat 3 anti-100K (Fig. 6*A*) and rabbit anti-clathrin (Fig. 6*B*) also gave similar staining patterns, but now the perinuclear relative to punctate labeling appears brighter with the anti-100K antiserum. To test whether the perinuclear staining coincides with the Golgi region of the cell, cells were double labeled with Rat 3 anti-100K (Fig. 6*C*) and wheat germ lectin (Fig. 6*D*), which has been used as a marker for the Golgi apparatus (15). The regions of the cells that stained with the two labels are the same, although differences can be seen in the two staining patterns. The wheat germ lectin gave a certain amount of cell surface labeling, and although punctate labeling can be seen in some of the cells, it does not completely coincide with the punctate antibody labeling. In addition, the lectin labeling of the Golgi region is somewhat diffuse, while the pattern given by the Rat 3 anti-100K is much sharper, possibly because only discrete patches of Golgi membrane are stained (3).

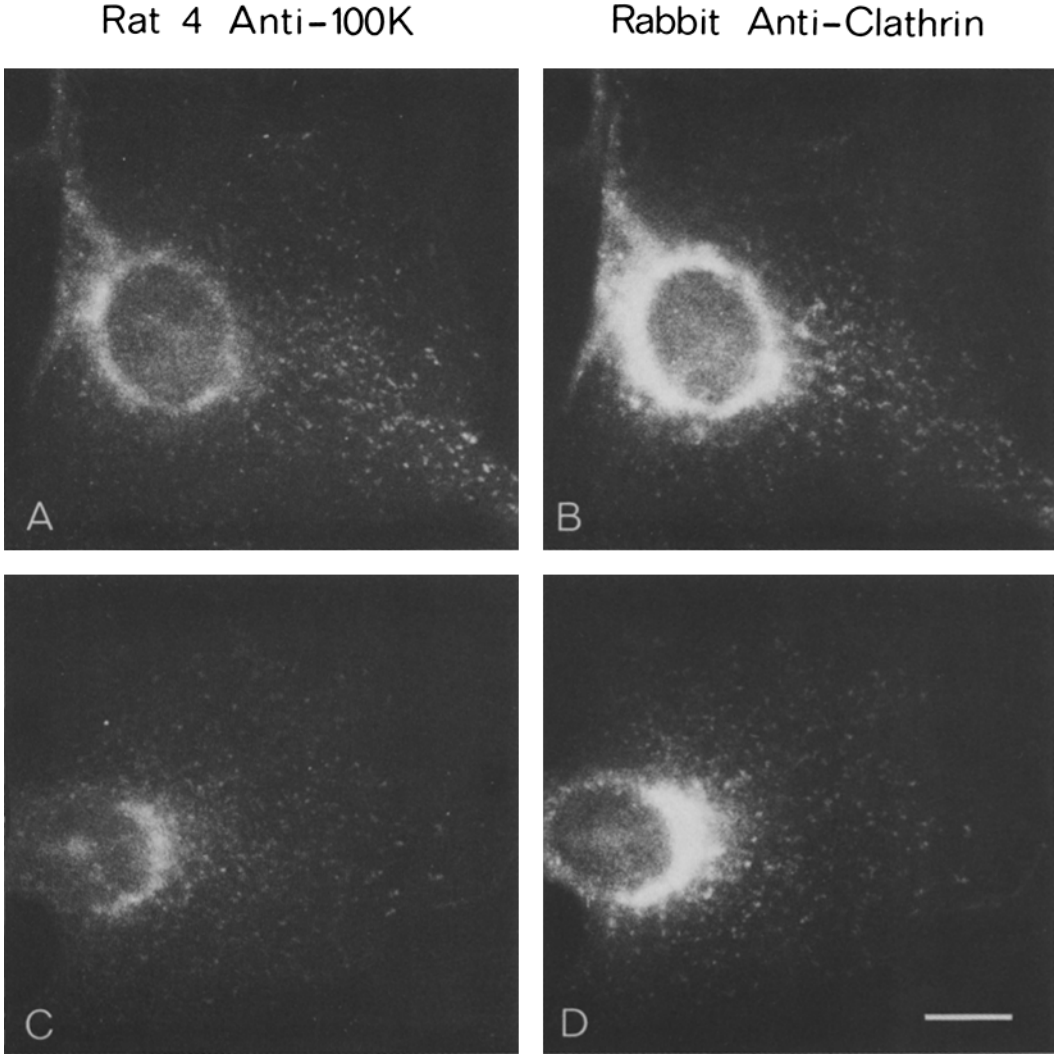

Figure 5. Cells double-labeled with Rat 4 anti-100K and rabbit anti-clathrin. One cell is shown in *A* and *B*; another cell is shown in *C* and *D*. *A* and *C* are labeled with anti-100K; *B* and *D* are labeled with anti-clathrin. The punctate labeling is essentially identical with the two antisera, although the perinuclear labeling is brighter with the anti-clathrin. Bar, 10 μm.

Discussion

We have raised four antisera against the 100K proteins of bovine brain coated vesicles and have used them both to help characterize the proteins biochemically and to localize the proteins in tissue culture cells.

Our results clearly indicate that the multiple bands seen on SDS gels of the 100K proteins represent distinct polypeptides rather than breakdown products or modifications of a single polypeptide. Two lines of evidence support this conclusion. First, peptide maps of the three major 100K bands in the HA-II group look markedly different from each other, although a certain amount of homology may be seen in the maps of the upper and lower bands. Second, antisera raised in four rats all reacted differently with the different bands. The antisera were also used to confirm that the HA-I group of proteins is different from the HA-II group: none of the antisera, which were raised against the HA-II proteins, cross-reacted with the HA-I group. Thus, there are probably at least six distinct 100K proteins in bovine brain coated vesicles. There may be even more, since each gel band is not necessarily made up of only one protein. However, all the proteins in both the HA-I and the HA-II groups co-assemble with clathrin into coats, and all the HA-II proteins form complexes with the 50K protein (10). Moreover, even though our results indicate that the different 100K proteins in bovine brain have distinct amino acid sequences, the ability of the antisera to cross-react with the 100K proteins of human placenta and mouse 3T3 cells indicates that a number of these proteins are conserved from one organism to another.

Two of our antisera (from Rats 3 and 4) were used to stain 3T3 cells and thus provide a picture of the intracellular distribution of the HA-II 100K proteins. Both antisera gave staining patterns similar to those seen with anti-clathrin; and when cells were double labeled with anti-100K and rabbit anti-clathrin, the two patterns of fluorescent dots were virtually superimposable. This result leads to several conclusions. First, it confirms that the 100K proteins are true components of coated pits and vesicles in vivo. Second, since our antisera gave punctate cell surface labeling as well as perinuclear labeling, it is clear that the 100K proteins are found in coated vesicles that bud from the plasma membrane and not just

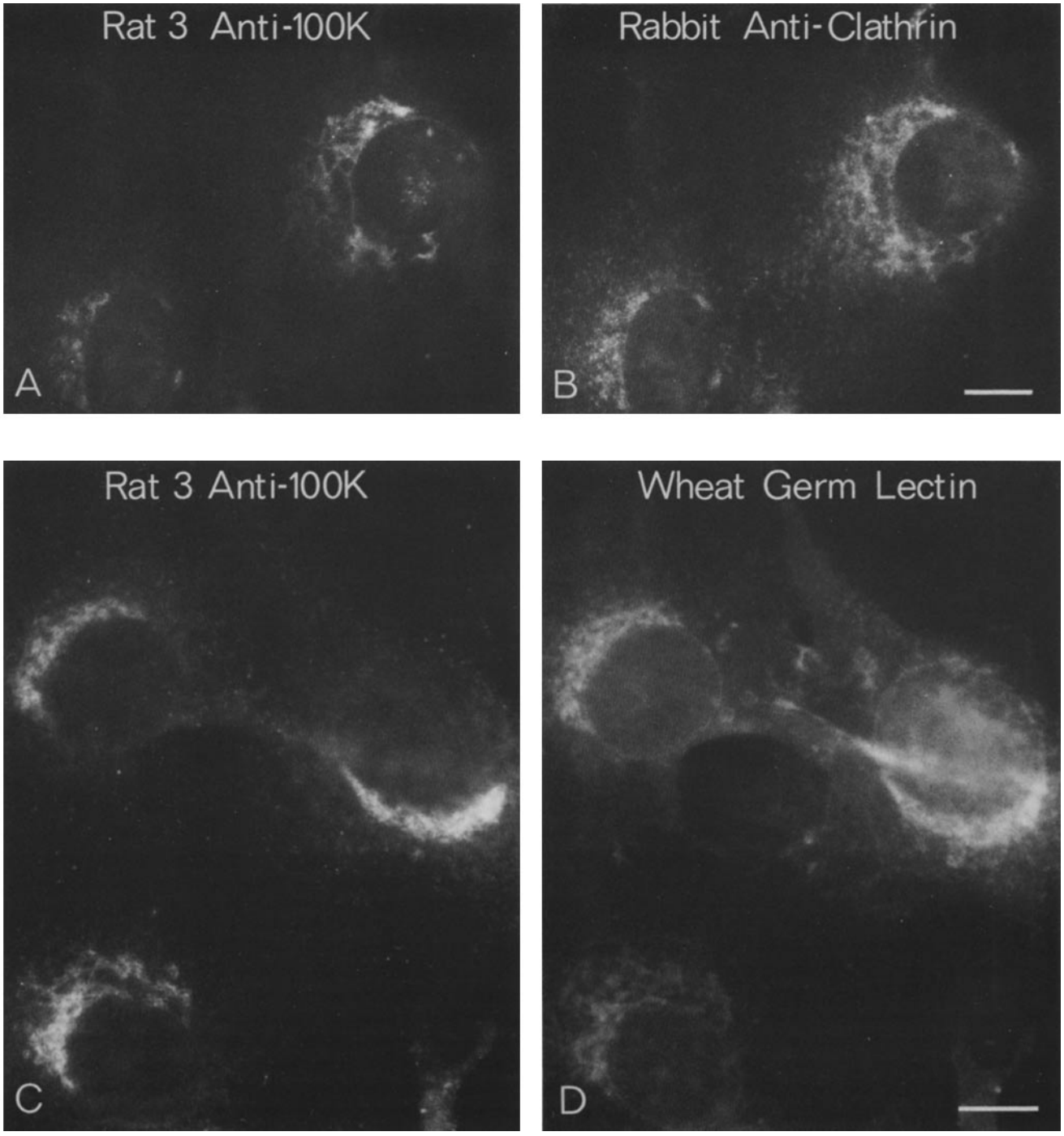

Figure 6. Double labeling using Rat 3 anti-100K. (*A* and *B*) Cells were double labeled with Rat 3 anti-100K (*A*) and rabbit anti-clathrin (*B*), and were photographed focusing on the perinuclear region. The staining is very similar with the two antisera. (*C* and *D*) Cells were double labeled with Rat 3 anti-100K (*C*) and wheat germ lectin (*D*), a marker for the Golgi apparatus. The perinuclear staining is again very similar, although it is more diffuse with the lectin than with the antibody. Bars, 10 μm.

those derived from the Golgi apparatus, as has been suggested (17). Third, since essentially all the fluorescent dots that were labeled with anti-100K were also labeled with anti-clathrin, it seems likely that when vesicles become uncoated, the 100K proteins come off as well as clathrin, and both are recycled to form new coated pits. An uncoating ATPase has recently been purified which removes clathrin from isolated coated vesicles but does not apparently solubilize the 100K proteins (11). It is possible, however, that there is a second uncoating factor in the cell which removes these proteins. In any case, our results indicate that the 100K proteins do not remain behind on uncoated vesicles, and therefore could not act as signals to direct the vesicles to their correct intracellular targets.

What, then, is the function of the 100K proteins in vivo? The work on isolated coated vesicles (14) suggests that they may promote the assembly of clathrin onto selected trans-

membrane proteins. Since one expects different membrane proteins to be found in coated vesicles that bud from different membrane compartments of the cell, then one might also expect that there would be different 100K proteins in these different compartments. To test this idea, it will be necessary to have antibodies that exclusively recognize individual members of the 100K family. Nevertheless, our results provide preliminary support for such an idea. Of our two affinity-purified anti-100K antisera, one (from Rat 4) gave stronger plasma membrane labeling than Golgi apparatus labeling when compared with the anti-clathrin antiserum; while the other (from Rat 3) preferentially labeled the Golgi region. These two antisera also showed differences on immunoblots of bovine brain 100K proteins. However, it is not yet possible to correlate these differences with the different staining patterns in intact 3T3 cells. We have as yet no evidence that the two antisera label different 100K proteins on blots of 3T3 cells; and in any case, with a polyclonal antiserum, we cannot be sure that the same antibodies that give the strongest staining on blots also give the strongest immunofluorescent staining.

For this reason, we are now in the process of raising monoclonal antibodies against the different 100K proteins. We hope to use these antibodies to clarify further how the different members of the 100K family may differ functionally.

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