

Endocytosis and Degradation of Interstitial Retinol-binding Protein: Differential Capabilities of Cells that Border the Interphotoreceptor Matrix

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ABSTRACT Between the pigment epithelium and the outer limiting membrane of the retina is an extracellular compartment filled with the interphotoreceptor matrix (IPM). A prominent component of the IPM is a glycoprotein known as interstitial retinol-binding protein (IRBP). Using *in vitro* techniques, we compared the ability of the cells that border this compartment to internalize colloidal gold (CG) coated with either IRBP or ovalbumin, a glycoprotein not found in the IPM. Neither IRBP-CG nor ovalbumin-CG was internalized by the Muller's cells. Both rod and cone photoreceptors take up IRBP-CG, which is observed in small vesicles and multivesicular bodies. Neither photoreceptor type takes up ovalbumin-CG. Acid phosphatase cytochemistry indicates that acid phosphatase reaction product in the multivesicular bodies co-localizes with IRBP-CG, which suggests that this molecule is degraded by rod and cone photoreceptors and is not recycled. The pigment epithelium internalizes IRBP-CG and ovalbumin-CG, both of which remain in small cytoplasmic vesicles near the apical plasma membrane. There is no indication that vesicles that contain either IRBP-CG or ovalbumin-CG fuse with the lysosomal system in the pigment epithelial cells during the incubation.

The interphotoreceptor matrix (IPM)¹ fills an extracellular compartment between the outer limiting membrane of the retina and the apical surface of the pigment epithelium. The inner and outer segments of the photoreceptors project from the outer retinal surface into the IPM. Because of the interdependence of the photoreceptors and the pigment epithelium for the continued function of the visual process, the unique location of the IPM implicates the components of this extracellular matrix in the mediation of interactions that occur between these tissues.

The IPM is composed of a mixture of glycoconjugates and non-glycosylated proteins (1, 3). *In vitro* studies of the retina and pigment epithelium have shown that these tissues actively synthesize and secrete components of the IPM (1, 4, 7). The ongoing addition of new constituents to the matrix must be accompanied by some mechanism for removal and degrada-

tion of the IPM as well, if steady state volume and concentration of the individual components are to be maintained (15). It is not known how any component of the IPM is removed, recycled, or degraded.

Recent studies identified an IPM glycoprotein of high molecular weight (M_r 144,000 in bovine, 135,000 in human) that is present in high concentration and binds vitamin A, which suggests a role in the transport of retinoids between the photoreceptors and pigment epithelium (5, 7, 13). Because of its localization and affinity for retinol, this IPM glycoprotein has been named interstitial retinol binding protein (IRBP). Although rod photoreceptors have been implicated in the synthesis and secretion of IRBP (10, 11), nothing is known about the interaction of this component with the tissues that border the IPM, nor is any information available about its retrieval and degradation. In this study we incubated isolated bovine retina and pigment epithelium with IRBP-coated colloidal gold (IRBP-CG) as a means of visualizing the interaction of IRBP with cells that border the IPM.

¹ *Abbreviations used in this paper:* CG, colloidal gold; IPM, interphotoreceptor matrix; IRBP, interstitial retinol-binding protein.

MATERIALS AND METHODS

Bovine IRBP was purified as described previously (8). Ovalbumin was purchased from Sigma Chemical Co. (St. Louis, MO). CG (40 nm) was prepared by reduction of 100 ml of 0.01% HAuCl₄ with 2 ml of 1% trisodium citrate dihydrate (19). The solutions were mixed on ice and then refluxed for 30 min to yield a red sol. After cooling on ice, the pH was adjusted to ~5.2 by addition of 0.25 ml of 0.2 M K₂CO₃. The amount of IRBP or ovalbumin required to protect the CG from precipitation by an equal volume of 10% NaCl was determined to be 13 µg IRBP/ml of sol and 180 µg ovalbumin/ml of sol. Protein equal to 110% of these values was added to 25 ml of CG solution. After 5 min of gentle agitation, we added 0.25 ml of 5% polyethylene glycol to each protein-CG sol to ensure stabilization of the suspension.

We established that the glycoproteins added to the CG suspension were indeed associated with the CG particles. At the end of the incubations described below, the medium was recovered; the colloidal gold particles were pelleted (12,000 rpm for 3 min), resuspended in sodium acetate buffer (20 mM, pH 5.0), and pelleted again; and the pellet was then resuspended in an SDS dissociation buffer (13) to remove associated protein. After the CG was pelleted, aliquots of the SDS solution were then electrophoresed on SDS-polyacrylamide gels (13) and the gels were stained with Coomassie Blue or immunoblotted onto nitrocellulose paper (10) by the use of anti-bovine IRBP IgG (7, 8). By following these procedures we could identify both IRBP (on the immunoblots) and ovalbumin (in the stained gel).

The retina and pigment epithelium used for studies of the association of CG tracers were isolated from bovine eyes, which had been enucleated and placed on ice within 10 min after death. Eyes reached the laboratory within 1 h after enucleation. The retina and pigment epithelium/choroid tissues were separated and circular discs of tissue were cut with a 3-mm trephine. Tissue discs were placed in 1-ml volumes of protein-CG suspended in Ringer's/bicarbonate/pyruvate incubation medium (6) and were incubated at 37°C with continuous gassing (95% O₂:5% CO₂) at a flow rate of 1 standard ft³/h. At incubation intervals of 15 min or 1 or 2 h, tissues were recovered and fixed for 30 min in cacodylate buffer (pH 7.4, 0.10 M) containing 2% formaldehyde and 2% glutaraldehyde. At the end of the primary fixation one-half of the tissue samples were postfixed in 1% OsO₄ for 30 min and processed for routine electron microscopy. We processed the remaining tissues for acid phosphatase cytochemistry using a modification (14) of the procedure of Gomori (9).

RESULTS

Retina

We observed single IRBP-CG particles adjacent to the extracellular surface of rod and cone outer segments and also within the fuzzy coat material surrounding the connecting cilium at the inner segment-outer segment junction. In the tissues recovered after 15 min of incubation in the presence of IRBP-CG, we observed single or clustered CG particles associated with the extracellular surface of the plasma membrane of both rod and cone inner segments. As shown in Fig. 1(A-C), small groups of IRBP-CG particles were associated with the surface of the outer plasma membrane, sometimes in small depressions in the plasma membrane surface in configurations that suggested an early stage of endocytosis.

IRBP-CG was frequently observed in the inner segment cytoplasm of both rods and cones within the lumen of lucent vesicles 100–500 nm in diameter in tissues from all recovery times (Figs. 1, D-G, and 2, A and B). Vesicles containing 1–5 CG particles were encountered in every third or fourth inner segment profile examined. The vesicles containing IRBP-CG could be found throughout the inner segment from the level of the connecting cilium to positions near the junctional contacts with Muller's cells, which form the so-called outer limiting membrane of the retina. We never observed IRBP-CG within the outer segment.

In retinas from the 1- and 2-h recovery times, 1–10 IRBP-CG particles were also observed within the luminal profiles of multivesicular bodies (0.5–1.0 µm diam) in both rod and cone inner segments (Fig. 2, C-E). In tissues prepared for acid phosphatase cytochemistry, co-localization of IRBP-CG

with acid phosphatase reaction product in the multivesicular body was observed (Fig. 2F). Acid phosphatase-positive material was not present in the smaller, electron lucent vesicles described above, which also contained IRBP-CG.

Examination of Muller's cell cytoplasmic contents failed to reveal the presence of any intracellular IRBP-CG, although the tracer was commonly observed in the extracellular space in close proximity to the villous processes.

In the retinal tissues incubated with ovalbumin-CG, this tracer was also observed in the extracellular space but was not found associated with the plasma membrane of photoreceptors as described above for IRBP-CG. In the four blocks of retinal tissue examined from each of the recovery times, only one rod was observed to contain ovalbumin-CG in the inner segment cytoplasm of more than 50 photoreceptor sections examined. In this rod from a retina recovered after 1 h of incubation, we observed a vesicle 150 nm in diameter that contained two ovalbumin-CG particles. Except for this single example, ovalbumin-CG was not present within the cytoplasm of any of the other photoreceptors or Muller's cells examined.

Pigment Epithelium

In samples recovered after 15 min of incubation, IRBP-CG was observed in close association with the apical surface and villous processes of the pigment epithelium. In the tissues recovered after 1 and 2 h of incubation, in addition to the extracellular IRBP-CG, the tracer was also observed within the luminal cavities of vesicles 200–300 nm in diameter, some of which appeared to be connected to tubular cisternae in the apical cytoplasm of the pigment epithelium just below the plasma membrane (Fig. 3, A and B). At all recovery times examined, the IRBP-CG remained in these membrane-bound compartments near the apical surface and were not observed in more basal regions of the cell. In the pigment epithelium/choroid samples incubated for acid phosphatase cytochemistry, reaction product was present in the Golgi apparatus, phagosomes, and residual bodies but was not observed in the apical vesicles containing IRBP-CG.

In the pigment epithelium/choroid tissues incubated with ovalbumin-CG, we observed CG particles in locations virtually identical to those of the IRBP-CG described above. They were present extracellularly and were also found in the lumen of small vesicles and tubular cisternae within the pigment epithelial cells subjacent to the apical plasma membrane (Fig. 3, C and D).

DISCUSSION

These observations show that the cells bordering the IPM varied in their ability to endocytose protein-coated CG and also that this response varied depending on whether IRBP or ovalbumin was the protein in question. IRBP-CG particles were observed in close apposition to the plasma membranes of rod and cone inner segments and were also seen associated with depressions in the plasma membrane of the inner segments, which suggested stages in endocytosis. Within the photoreceptors we observed from one to five IRBP-CG particles in small lucent vesicles located in positions from just below the plasma membrane to deep within the interior of the inner segment. IRBP-CG was also observed in multivesicular bodies in the 1- and 2-h recovery samples. The absence of acid phosphatase reaction product in the smaller lucent

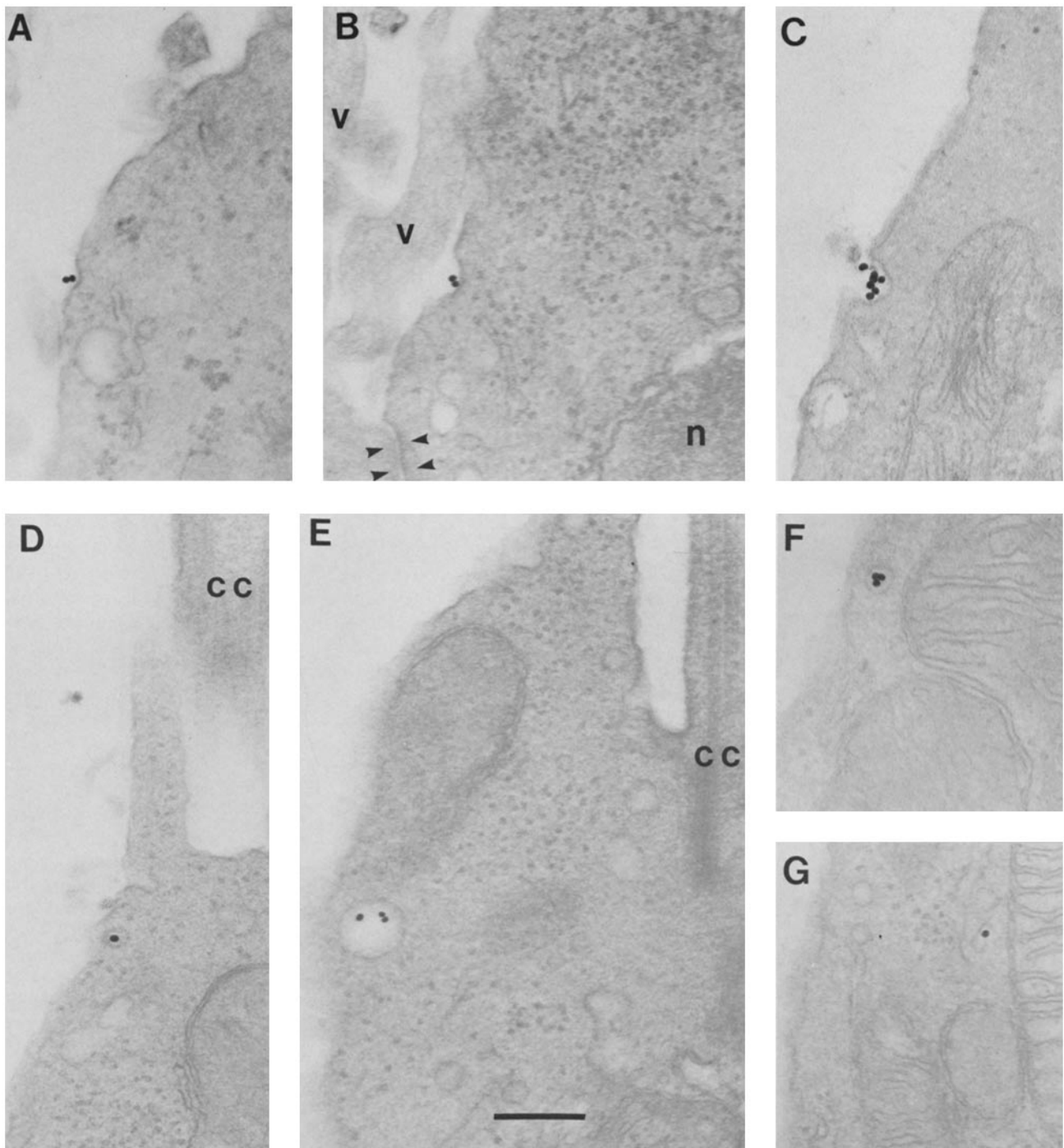


FIGURE 1 IRBP-CG localization in bovine photoreceptors. (A and C) Colloidal gold particles clustered in depressions in the plasma membrane of the inner segment of rod photoreceptors. (D and G) IRBP-CG in small lucent vesicles inside the inner segment of rod photoreceptors (B, E, and G) and a cone photoreceptor inner segment (F). cc, connecting cilium; n, nucleus; v, villous processes of Muller's cell. Double arrows in B indicate junctional complex at the outer limiting membrane. All micrographs printed at same magnification. Bar, 0.25 μ m.

vesicles suggests that these components represent primary endocytotic vesicles that are involved in intracellular transport of the IRBP-CG to the multivesicular bodies. The presence within these multivesicular bodies of many IRBP-CG particles that co-localize with the acid phosphatase reaction product, a marker for lysosomal enzymes, suggests that IRBP, once interiorized by the photoreceptor cells, is shunted into a degradative pathway and is not reused or recycled. This interpretation must be tempered somewhat because of the

nature of the tracer used. The solid phase IRBP-CG particles are somewhat large and may not precisely follow the intracellular pathway taken by native IRBP when interiorized. Additional studies with radiolabeled or biotinylated IRBP will be required to delineate further this pathway.

The paucity of ovalbumin-CG within the cytoplasm of the photoreceptor cells suggests that the endocytosis of IRBP-CG may involve a specific recognition process rather than bulk uptake of the tracer from the extracellular space. Receptor-

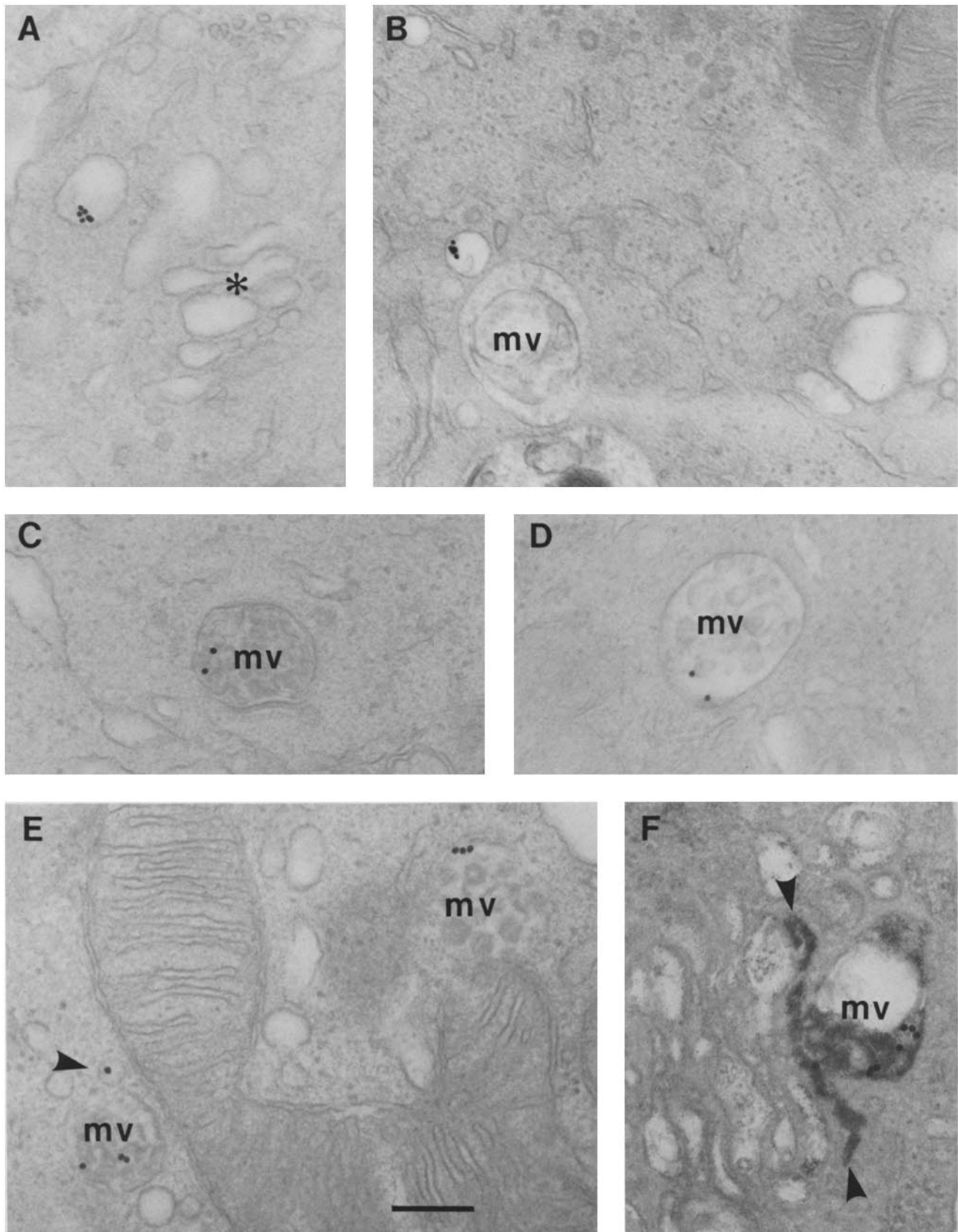


FIGURE 2 IRBP-CG in the inner segment of cone (A, B, and E) and rod photoreceptors (C, D, and F). In A a large lucent vesicle containing five CG particles is visible near the Golgi (*) of a cone. (B) Small lucent vesicle containing 4 CG profiles near a multivesicular body that contains what appears to be a partially degraded mitochondrion. (C and D) Two CG particles within the lumina of multivesicular bodies. In E, two multivesicular bodies, each containing three CG particles are evident, as is a small lucent vesicle (arrowhead) near a multivesicular body, suggesting a stage just before fusion with the multivesicular body. (F) Acid phosphatase reaction product in the trans-face of a Golgi body (arrowheads) and also within a multivesicular body that contains eight IRBP-CG gold particles. All micrographs printed at same magnification. mv, multivesicular body. Bar, 0.25 μ m.

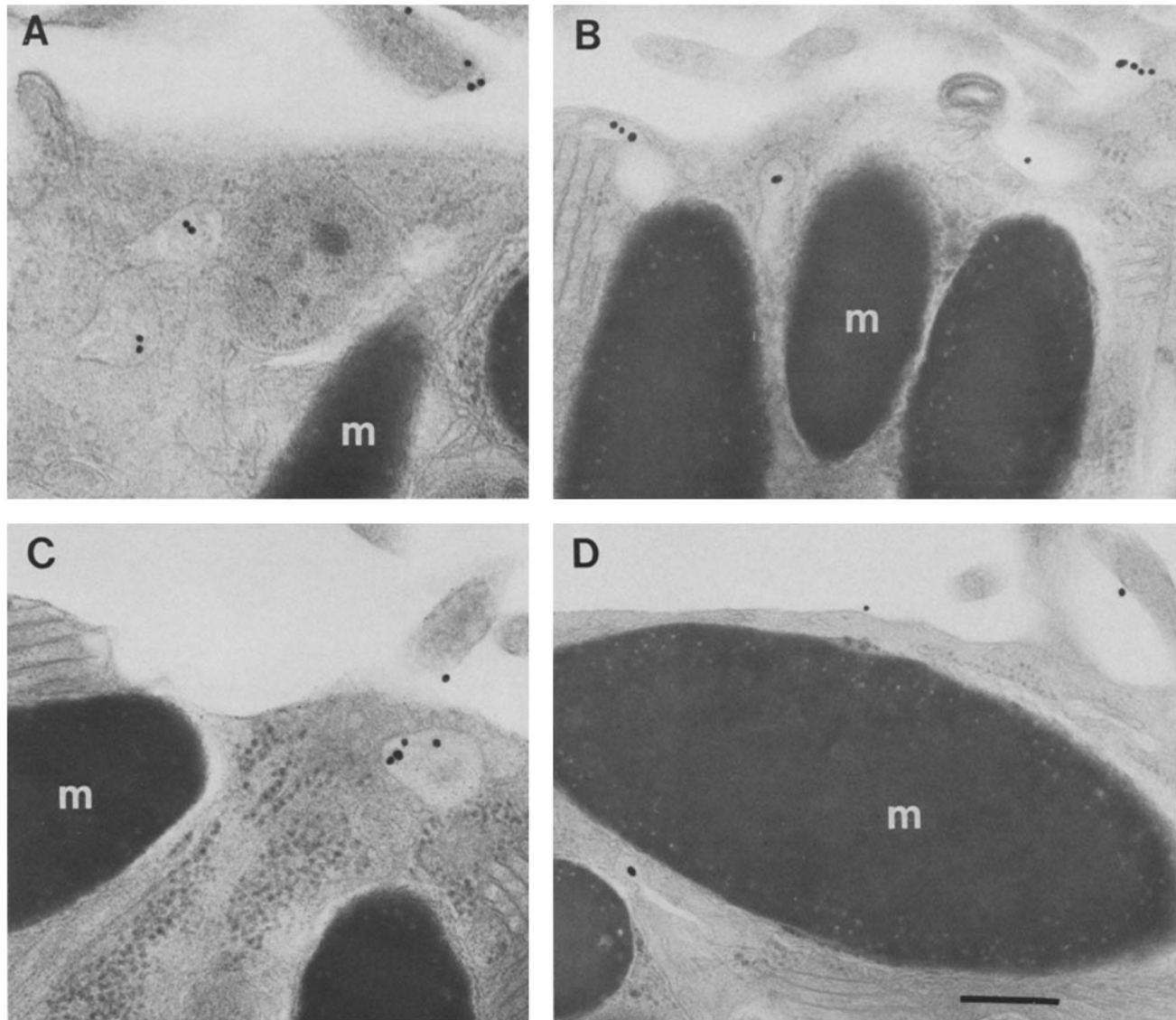


FIGURE 3 IRBP-CG (A and B) and ovalbumin-CG (C and D) contained in small vesicles and cisternae near the apical surface of the pigment epithelium. A and C are from tissues incubated with the tracer for 1 h; B and D are from tissues incubated for 2 h. All micrographs printed at same magnification. *m*, melanosome. Bar, 0.25 μ m.

mediated endocytosis in other systems is thought to occur in conjunction with specific receptors that cluster in clathrin-coated pits (2, 16, 18). Although coated vesicles have been observed in the inner segments of amphibian photoreceptors (12), we should point out that we did not observe IRBP-CG in coated vesicles in these conventionally fixed and stained preparations. The uptake of IRBP-CG by uncoated vesicles suggests that this response may not be receptor mediated. Additional studies using methods which more clearly reveal clathrin will be required to substantiate this observation.

We did not observe the differential response of the photoreceptors to IRBP-CG and ovalbumin-CG in the pigment epithelium. In this tissue, both tracers were taken up into small endocytotic vesicles that remained near the apical surface of the pigment epithelium. Note also that whereas in the photoreceptors the IRBP-CG moved rapidly into the multivesicular bodies, in the pigment epithelium both IRBP-CG and ovalbumin-CG remained in the apical cytoplasm and were not observed to fuse with lysosomes during the incubation. Because both tracers were engulfed by the pigment

epithelium, it is likely that this response reflects nonspecific uptake of small components from the IPM by this tissue. Since IRBP has been implicated as a carrier molecule for transport of retinoids across the IPM (13, 17), the small vesicles observed under the apical surface of the pigment epithelium may be the sites at which IRBP delivers or acquires its ligand. Additional studies with longer incubation times will be required to determine whether the IRBP-CG is degraded or recycled by the pigment epithelium.

Although this report deals with the response of the cells that surround the IPM to only one of its major established constituents, other soluble proteins and glycoconjugates are also present (1, 3). Future studies that focus on other matrix components may reveal entirely different patterns of cellular response. Because of the interdependence of the retina and pigment epithelium for the turnover and biosynthesis of the IPM and for the sustained function of the vision process, an understanding of the interactions of components of the matrix with the surrounding tissues is an important area for future study.

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