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Apical membrane aminopeptidase appears at site of cell–cell contact in cultured kidney epithelial cells

(Na$^+$,K$^+$-ATPase/α-actinin/cell polarity/cell junctions/membrane biogenesis)

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ABSTRACT **A dog kidney epithelial cell line (MDCK), grown in monolayer, displayed *in vitro* an asymmetric localization of surface proteins. Aminopeptidase [α-aminoacylpeptide hydrolase (microsomal), EC 3.4.11.2] was found only in the apical face whereas Na$^+$,K$^+$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) was found in the basolateral faces. These two faces are delineated by the junctional complex at which close cell–cell contact occurs. α-Actinin, a protein associated with plasma membranes, was concentrated near the region of cell–cell contact. When membrane proteins in the apical surface were crosslinked and subsequently removed from the surface by endocytosis, crosslinked antigens reappeared in the apical face at the region of cell–cell contact. Antigens that were not crosslinked were also (re)inserted in the same region. This process was not affected by cycloheximide, presumably because a large pool of apical membrane proteins (observed in small cytoplasmic vesicles) was used to replace the endocytosed antigens. It is proposed that the region containing the junctional complex is involved in guiding apical membrane proteins to their final location.**

Epithelial cells display a striking structural and functional polarity. Inside the cell, the arrangement of different organelles is highly ordered, and on the cell surface one can distinguish at least two specialized domains: the apical surface and the basolateral surface. The apical surface, which is covered by microvilli, is involved in absorptive or secretory processes, whereas the basolateral faces are generally free of microvilli. These two surfaces are separated from one another by junctional complexes comprising the zonula adherens, the zonula occludens (tight junction), and the desmosomes, where neighboring cells attach to each other.

The functional polarity of differentiated epithelial cells reflects the existence of distinct surface domains. Each domain possesses a characteristic set of proteins (1), such as specific enzymes or antigens, that are concentrated in one domain or the other (2–4). The tight junction limits the penetration of macromolecules into the intercellular space. It is also thought to prevent lateral diffusion of proteins from one surface domain to the other (5), thereby maintaining their unique protein composition.

The existence of these highly specialized surface domains raises interesting questions concerning plasma membrane biogenesis. How is such specificity in membrane structure generated and, in particular, where during their intracellular transport are different pools of membrane proteins sorted out?

The MDCK (Madin–Darby canine kidney) cell line, established in 1958, has been shown (6, 7) to provide a useful model system for study of some aspects of the development and maintenance of specialized cell surfaces. The early observation by Misfeldt *et al.* (6) of the vectorial transport of solutes by MDCK cells, followed by the work of Cereijido *et al.* (7) on the development of their tight junctions, suggested that the cell surface had a structural polarity. Additional information on the structural and functional polarity of these cells has been provided by different groups (8, 9). Rodriguez-Boulan and Sabatini (10), for example, showed that the budding of virus particles occurs exclusively at one face or the other, depending on the virus used to infect the cells.

The observations presented in this paper provide evidence for the asymmetric distribution of two specific membrane proteins in MDCK cells grown in monolayers. The two proteins were selected for this study because they are considered to be reliable markers for the polarity of intestinal and kidney cells *in vivo:* aminopeptidase [α-aminoacyl-peptide hydrolase (microsomal), EC 3.4.11.2] is specifically localized in the apical membrane (brush border) (2) and the Na$^+$,K$^+$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is found predominantly in the basolateral membrane (3, 4). In addition, this study shows that the junctional complex described by others (7) for MDCK cells is in close proximity to α-actinin.

A substantial internal pool of aminopeptidase was also found in MDCK cells, and it is proposed that this pool of antigens can be inserted specifically into the apical membrane. Finally, evidence is presented supporting the idea that membrane proteins from the apical face can be internalized by antibody-stimulated endocytosis and later recycled to the cell surface (11, 12).

MATERIALS AND METHODS

Cell Culture. MDCK cells (obtained from M. Taub, University of California, San Diego) were grown in minimal Eagle's medium supplemented with 10% (vol/vol) fetal calf serum and antibiotics in a humid atmosphere of 95% air/5% CO_2 at 37°C. Cells were plated onto glass coverslips at $\approx 2 \times 10^5$ cells per 35-mm dish 2 days prior to the experiment and were used just before the cells were confluent.

Antibodies and Staining Reagents. Na$^+$,K$^+$-ATPase was purified from dog kidney medulla (13), a gift from J. Kyte (University of California, San Diego). Aminopeptidase was purified from dog kidney cortex (14), and α-actinin from chicken gizzard (15). Primary antibodies were raised in rabbits against each individual protein and were purified by affinity chromatography by using the appropriate antigen immobilized on Ultrogel Ac22 activated with glutaraldehyde (16).

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Abbreviations: GxR, goat antibody against rabbit IgG; RxG, rabbit antibody against goat IgG; Rh, rhodamine-conjugated; Fl, fluorescein-conjugated; Biot-RxAP, biotinyl-conjugated anti-aminopeptidase; MDCK cells, Madin–Darby canine kidney cells; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate.

The specificity of the purified antibodies was checked by immunodiffusion or immunoprecipitation techniques, as well as by immunolocalization by indirect immunofluorescence on thin frozen sections of intestinal mucosa (17). Immunoprecipitation performed with a crude extract obtained from the appropriate antigen source led to the conclusion that each antibody can precipitate its specific antigen (unpublished data). Immunolocalization was also used to demonstrate the specificity of the antibodies: anti-aminopeptidase stained only the brush border, anti-Na^+,K^+-ATPase reacted specifically with the basolateral faces (unpublished observations), and the anti-α-actinin was found associated only with the terminal web area as described (18, 19).

Secondary antibodies used for indirect immunofluorescence staining were goat antibodies against rabbit IgG (GxR) and rabbit antibodies against goat IgG (RxG). These antibodies were purified by affinity chromatography and conjugated to rhodamine (Rh-GxR) or fluorescein (Fl-GxR) by standard techniques (20, 21). Biotinyl-conjugated IgG (Biot-IgG) and fluorescein (Fl)- or rhodamine (Rh)-conjugated avidin, used for some experiments, were prepared as described (22).

Cell Staining. Surface staining was performed on formaldehyde-fixed cells grown on coverslips (23). Intracellular staining was done after permeabilization of cells by either mild treatment with Triton X-100 or a freeze-thaw procedure (22, 23). Cells were photographed with Kodak Tri-X film.

Endocytosis Experiments. Two layers of antibodies—e.g., anti-aminopeptidase (50 μg/ml) followed by Rh-GxR (30 μg/ml)—were bound at 4°C in serum-free medium for 15 min each. At the end of the incubation, controls for binding and initial organization of antigens at the cell surface were fixed. The other coverslips were transferred into new medium (serum free) at 37°C and fixed at different times in order to follow the fate of the antibodies. Fixed coverslips were subsequently stained with Fl-RxG (30 μg/ml).

Saturation of Antigens with Antibodies. Surface antigens can be saturated with specific antibodies. For aminopeptidase, anti-aminopeptidase was used (200–300 μg/ml), followed by GxR or Rh-GxR (200–300 μg/ml). Free binding sites available on GxR were blocked with nonimmune rabbit IgG (1 mg/ml). Cells saturated under these conditions were then processed with a new set of reagents—e.g., Biot-RxAP (50 μg/ml) and Fl- or Rh-avidin (10 μg/ml)—which could not then react with the saturated antigens but only with newly accessible binding sites.

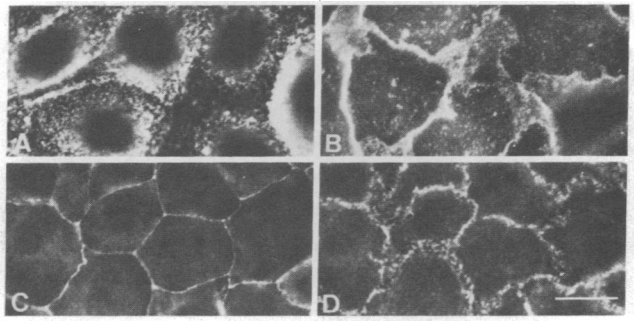

FIG. 1. MDCK cell stained with (*A*) anti-aminopeptidase; (*B*) anti-ATPase; and (*C* and *D*) anti-α-actinin. The primary antibody was at 50 μg/ml; the second antibody (Rh-GxR) was at 30 μg/ml. Controls were nonimmune rabbit IgG at 100 μg/ml. (*A*) Cells are stained on their apical face with the focus on top of the cells (dots are thought to represent projected microvilli). The center region of the cells seems to be unstained because apical faces are convex and appear out of focus. (*B–D*) Cells were fixed and opened with Triton X-100 prior to staining. (*B*) Lateral faces appear brightly stained in projection whereas basal membranes appear less intensely stained. The flat basal membrane is seen in one plane of focus. (*C*) Sharp belt-like appearance of fluorescence after staining with anti-α-actinin (focus is on top of the cell). Some diffuse staining is observed in the cytoplasm. (*D*) Same field as in *C* but plane focus is on bottom of the cell monolayer; observe the fluorescent broad diffuse belt (compare with *B*) resulting from projected interdigitated membranes. (Bar = 20 μm.)

RESULTS

Asymmetric Localization of Specific Membrane Proteins in MDCK Monolayer. When confluent monolayers of MDCK cells were fixed with formaldehyde and stained with anti-aminopeptidase, the apical faces (the only membranes accessible) fluoresced brightly. The staining was patchy because this surface is covered with microvilli (Fig. 1*A*). Cells labeled in the same way with anti-ATPase [found mostly on the basolateral face in normal dog kidney cells (4)] showed a negligible background staining of the cell surface similar to that obtained when nonimmune IgG was used (data not shown). These observations suggest that aminopeptidase molecules are readily accessible when the apical face is the only surface available, whereas ATPase molecules are inaccessible. If, however, fixed cells were first permeabilized with Triton X-100 or by freezing and thawing,* the basolateral surface could then be stained with anti-ATPase. Fluorescence appeared as an intense, broad, diffuse line corresponding to the lateral faces of the cells. This pattern could be observed at various focal planes, but it was most intense on the bottom of the cells, where the basal membrane also showed positive staining (Fig. 1*B*). The differences in intensity for the lateral and the basal faces do not necessarily reflect the presence of a greater number of antigenic sites in the lateral faces compared to the basal face because lateral faces are projected within the plane of focus. In contrast, permeabilized cells treated with anti-aminopeptidase showed no staining of the basolateral membranes (see Fig. 4).

Association of α-Actinin with Apical Edges of Cell. Fixed cells can only be stained with anti-α-actinin after permeabilization (Fig. 1 *C* and *D*). The pattern observed was remarkably different from that described by others using fibroblast-like cells in culture (24). Stress fibers were not seen, but the α-actinin in these epithelial cells was concentrated at the edges of the cells. A sharp belt encircled the top of the cell (Fig. 1*C*) whereas when the focal plane was on the bottom of the monolayer, it was observed as a broad diffuse line (Fig. 1*D*) similar to that seen in cells stained with anti-ATPase (Fig. 1*B*). The concentration of α-actinin in the top part of the cells supports the notion (18, 19) that the distribution of α-actinin is related to the zonula adherens, which defines the region of cell–cell contact. When the junctions were destroyed by brief treatment with EGTA, the sharp band of α-actinin also disappeared and, instead, a diffuse staining was seen in the cytoplasm. When EGTA was removed and the junctions were allowed to reform, with concomitant recovery of electrical resistance of the monolayer (7), the organized band of α-actinin reappeared at the same time (within 45 min) (not shown).

Antibody-Induced Endocytosis of Aminopeptidase. Confluent monolayers were incubated at 4°C with specific antibodies (anti-aminopeptidase followed by Rh-GxR). Control

* Another procedure that reveals ATPase sites includes a short treatment with ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate (EGTA) (4 mM, 4°C, 15 min) before fixation. This procedure gives similar results, but is not quantitative; only 10–20% of the cells are stained after this treatment. This is probably because the junctions are not fully opened after a 15-min treatment. A longer incubation induces rounding up of cells and, finally, disruption of the monolayer.

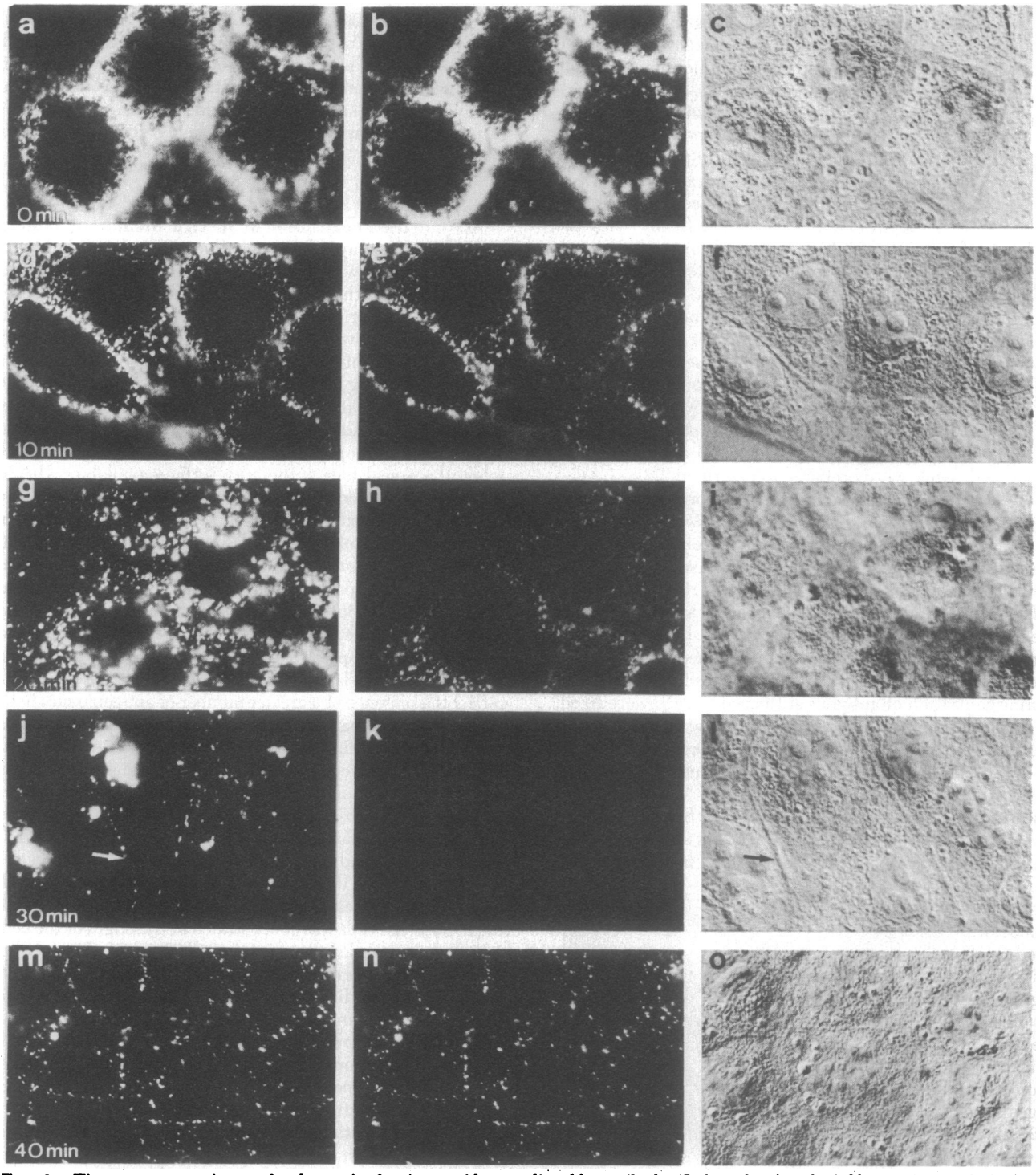

FIG. 2. Time-course experiment of endocytosis of aminopeptidase mediated by antibody. (*Left; a, d, g, j,* and *m*) Observations with rhodamine filters. (*Center; b, e, h, k,* and *n*) Observations with fluorescein filters. (*Right, c, f, i, l,* and *o*) Corresponding fields observed with Nomarsky optics with the focal plane on top of the cells identical to the fluorescent pictures; cell borders (arrow) are clearly seen (except in extreme top focus, *i*). (*a–c*) Control experiments showing surface staining at the end of incubation at 4°C. The surface is identical in *a* and *b* and similar to cells stained after fixation (Fig. 1*A*). (*d–f*) Cells were incubated for 10 min at 37°C. Notice the extremely patchy aspect of the cell surface; *d* and *e* are on the same focus level and are superimposable. (*g–i*) Cells were incubated for 20 min at 37°C. Globular structures are seen inside the cell (*g*) and mostly are above the nucleus (*i*). Surface labeling is shown in *h*. (*j–l*) Cells were incubated for 30 min at 37°C. Cell surface (*k*) is negative. Internalized patches (*j*) are seen along the cell edges (*l*) (arrow). (*m–o*) Cells were incubated for 40–90 min at 37°C. Top focal plane shows immunocomplexes at the cell surface (*n*). At lower focal plane, other exclusively internal structures are seen (not shown). Peripheral patches are clearly superimposable on cell borders (*o*). (×870.)

specimens fixed at 4°C prior to addition of Fl-RxG displayed a surface pattern identical to that seen in cells fixed prior to incubation with antibodies (compare Fig. 2 *a* and *b* with Fig. 1*A*). The remaining samples were then incubated at 37°C to allow redistribution and internalization of their surface antigens. Samples were fixed at 10-min intervals during 1 hr and incubated with Fl-RxG. Surface Rh-GxR still accessible to the Fl-GxR were then seen doubly stained, whereas internalized an-

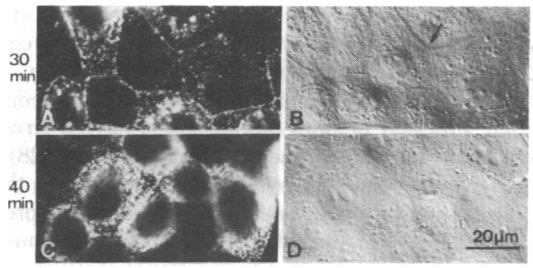

FIG. 3. Appearance of aminopeptidase on the cell surface. The cell surface was first saturated with unlabeled antibodies at 4°C and endocytosis of surface antigens was performed as in the legend of Fig. 2. The cell surface was stained, after antibody treatment in the presence of cycloheximide (20 μg/ml added 10 min before antibody treatment) and fixation (at times indicated), with biotinyl-conjugated anti-aminopeptidase (Biot-RxAP) (50 μg/ml) and Rh-avidin (10 μg/ml). (*A* and *B*) Same focus level 30 min after the beginning of endocytosis. Antigens appear concentrated in a continuous belt at the sites of cell–cell contact (arrow). (*C* and *D*) Same focus level 40 min after the beginning of endocytosis. Intense staining of cell surface is seen identical to that seen in Fig. 1*A* or Fig. 2 *a* and *b*. Control for saturations was performed in the following way. At the end of incubation with antibodies at 4°C, cells were fixed and stained as described above. Negligible background labeling was observed (not shown).

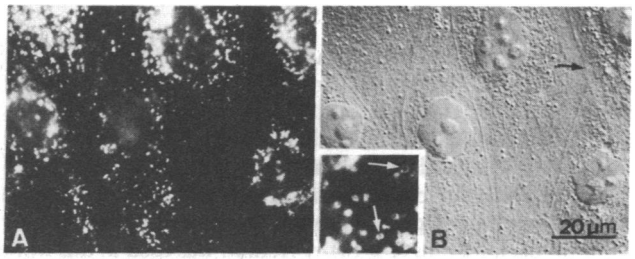

FIG. 4. Intracellular localization of aminopeptidase. (*A*) Subconfluent MDCK cells in monolayer, fixed and subsequently frozen (on dry ice) and thawed (at 20°C), once cell surface antigens had been saturated with unlabeled antibodies. Intracellular antigens were labeled with Biot-RxAP and Rh-avidin. Controls: incubation with anti-aminopeptidase (not biotinated) and Rh-avidin or with Biot-RxAP and Rh-avidin in the presence of 10 mM biotin (not shown). Notice that labeling of the lateral faces is very low compared with positive labeling of these faces (see Fig. 1 *B–D*). (*Inset*) Ring appearance of the labeling is shown (arrows). (*B*) Nomarsky optics: cell borders are clearly seen (arrow).

tibodies were labeled only with rhodamine. After a 10-min incubation at 37°C, the cell surface appeared more patchy (Fig. 2 *d* and *e*) than the control cells (Fig. 2 *a* and *b*). Nevertheless, the rhodamine pattern was identical to the fluorescein one. I conclude that clustering of surface proteins by their antibodies had occurred but little internalization had yet taken place. During the next 10 min a dramatic change was observed. Many large globular structures were seen inside the cell, located predominantly above the nucleus (Fig. 2*g*). The cell surface, on the other hand, became very weakly stained (Fig. 2*h*). After 30 min the cell was completely devoid of its surface markers. Two types of endocytosed material were observed: large globular structures without any precise location inside the cell and small patches randomly distributed around the nucleus and occasionally lined up along the edges of the cell at the apical surface (Fig. 2*j*). Later the reappearance of some of the immunocomplexes at the cell surface was observed. These were lined up along the edges of the apical membrane in a pattern strikingly similar to that demonstrated for α-actinin (compare Fig. 2 *m* and *n* and Fig. 1*C*). This pattern remained unchanged for a long period of time (40–60 min) (not shown). Moreover, the number of clusters on the cell surface did not increase significantly nor did they invade the cell apex. During the second hour the surface staining diminished and the labeling of the large internal globular structures was also reduced. Finally, after 2–2½ hr, the cells were completely unlabeled (not shown). These experiments clearly show that recycling of some endocytosed material occurs and that reappearance at the cell surface takes place primarily in the region of cell–cell contact.

Appearance of Aminopeptidase on Cell Surface. The observation that internalized aminopeptidase reappeared at the surface near the region of cell–cell contact led us to examine the possibility that this region also plays a role in the insertion of antigens into the plasma membrane. Cells were treated at 4°C with saturating amounts of unlabeled antibodies and were then transferred into medium at 37°C for 30 min or longer in order to induce complete internalization of the surface aminopeptidase (Fig. 2*k*). Cells were then fixed and stained *without permeabilization*. In this way, only apical surface antigens could be seen. Fig. 3 *A* and *B* shows (30 min after endocytosis began; focal plane on top of cells) that antigens are appearing on the cell surface. These appeared to be concentrated at the edges of the cells and formed a characteristic, almost continuous, belt around the cell.† In contrast, the rest of the cell surface showed a weak, dispersed, spotted labeling of the microvilli. However, this pattern was transient; 40 min after endocytosis had begun, the cell surface was intensely labeled with the usual pattern, identical to control cells‡ (compare Figs. 1*A* and 3*C*).

An identical experiment performed on cells treated with cycloheximide led to the same observations. These data suggest that newly inserted antigens are drawn from an intracellular pool of preexisting proteins.

Evidence for an Intracellular Pool of Aminopeptidase in MDCK Cells. Subconfluent MDCK cells, well-spread and relatively flat, were used for these experiments because their internal structures are easier to observe. Fixed cells were first saturated with unlabeled specific antibodies, then permeabilized by freezing and thawing, before being indirectly stained for internal labeling. Numerous dots, heterogeneous in size, were found scattered throughout the cell, although they were usually more concentrated around the nucleus (Fig. 4*A*). When observed at high magnification, some of these dots were seen as rounded profiles suggestive of membrane-bound antigens (Fig. 4 *Inset*). The freezing and thawing procedure was chosen instead of Triton treatment because it gives less cytoplasmic background and seems to be less damaging to these internal structures. The saturation of the cell surface with unlabeled reagents before permeabilization was not really necessary, but it facilitated observation of the vesicles, particularly those located just beneath the plasma membrane. Cells stained first with fluorescent antibodies, then frozen and examined immediately afterwards, showed apical staining with no vesicles inside. This excluded the possibility that surface-stained antigens in fixed cells were artifactually brought into the cell by freezing.

† Scanning electron microscopic studies have shown that microvilli are lined up along the cell borders. This explains the patchy aspect of the observed pattern.

‡ The 30-min time point was chosen for this study because the intensity of staining at the edges of the cells usually appeared to be maximal after 30 min. However, new antigens, weakly stained, were already observed after 10–15 min of endocytosis (not shown). This can be explained in two ways. Either the input of antigens is maximal after 30 min or the binding of the biotinyl-conjugated antibody is sterically inhibited by immunocomplexes still present during the first 20 min of endocytosis.

DISCUSSION

The results presented in this paper provide evidence that the cell line MDCK displays features usually observed *in vivo* in kidney epithelia. Aminopeptidase is concentrated in the microvilli on the apical surface, whereas Na^+,K^+-ATPase is predominantly localized in the basolateral faces. An additional feature of epithelial cells, also observed in MDCK cells, is the localization of a peripheral protein, α-actinin, in the vicinity of the zonula adherens (18, 19). α-Actinin was used in this work as a convenient marker for the cell–cell contact region which delineates two distinct domains, the apical surface and the basolateral membrane.

A substantial pool of aminopeptidase was found inside the cells. The internal antigens (destined for the cell apex) were seen in patches which may correspond to vesicular structures in which they are presumably membrane bound. The resolution of the light microscope, however, does not allow their morphological characterization. These vesicles (rather heterogeneous in size) may represent a biochemically heterogeneous fraction composed of newly synthesized antigens as well as material that is normally endocytosed by the cell. The origin and fate of this cytoplasmic pool of antigens were not clarified by this study, but presumably at least part of this pool can be transferred to the cell surface.

The antibody-mediated endocytosis of a surface marker antigen from the apical surface (aminopeptidase) led to two observations. The first, unexpected, finding was the reappearance of antibody, presumably bound to antigens at the cell surface. This observation suggests the existence of a cycle whereby antigens from the cell surface are transported via unidentified intracellular components back to the cell surface again (11, 12). The second striking observation was that the appearance of antibody occurred in a localized pattern at the apical cell borders. Even in the absence of protein synthesis (presence of cycloheximide), the cells responded to the induced endocytosis of specific membrane proteins by inserting antigens drawn from an internal pool that may or may not be identical to the one observed inside the cells (Fig. 4). Similar experiments were performed on antibody-mediated endocytosis and (re)-appearance of concanavalin A receptors, localized predominantly in apical membrane; these led to similar observations (unpublished data). The conclusion presented here seems to concern a group of apical membrane molecules.

In MDCK cells the (re)appearance of apical membrane markers occurred first at the site of cell–cell contact, as shown by the striking similarity between the pattern of recycled or newly inserted proteins and α-actinin (compare Figs. 1*C* and 3*A*). For a short time (between 30 and 40 min) it was possible to observe a belt (at the edges of the cells) of newly inserted antigens. This belt, it must be emphasized, can be labeled with biotinated antibodies and seems to be almost continuous. Because the biotinated antibody could still react specifically, the antigenic sites are not blocked and these molecules are probably not complexed with antibody used to induce endocytosis. In addition, if they were complexed, the belt would be more patchy (Fig. 2 *m* and *n*). Because protein input is continuous and lateral diffusion occurs, this picture rapidly faded and the cell apex reverted to its normal pattern.

Preferential appearance of membrane antigens at the cell borders was first demonstrated by Marcus (25), who followed the appearance of hemagglutinin molecules on giant HeLa cells infected with NVD viruses. In addition, two groups have studied the reappearance of fibronectin (LETS) on the surface of trypsinized fibroblasts during their spreading on coverslips (26, 27). They both observed that fibronectin first appears on the cell surface in patches, distributed like a belt. This belt is concentric with the outer rim of the spreading cell, and between it and the leading edge is an area of close contact between the cell membrane and the substratum (adhesion plaque). Such observations are relevant to this study because it has been proposed that adhesion plaques of fibroblasts have a structure similar to that of the zonula adherens of epithelial cells (28). This idea has recently been supported by observations on the attachment of α-actinin and vinculin (130,000-dalton protein) to adhesion plaques of fibroblasts (29) and to the zonula adherens of intestinal cells (30). The junctional complex and its equivalent, in fibroblast-like cells (the adhesion plaques) might contain receptors necessary for the appearance of some membrane proteins at the cell surface.

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