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Internalisation of desmosomes and their entry into the endocytic pathway via late endosomes in MDCK cells

Possible mechanisms for the modulation of cell adhesion by desmosomes during development

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SUMMARY

MDCK cells grown in media with normal levels of Ca^{2+} (~2 mM) contain internalised desmosomes, referred to as desmosome-associated vacuoles (DAVs). The DAVs consist of one to three plaques retained in the plane of a surrounding vacuolar membrane, and their entry into the endocytic pathway has been investigated using HRP, cationised ferritin and BSA/gold in combination with electron microscopy and immunogold labelling of frozen sections.

Endocytic tracers supplied from the apical and basolateral surfaces to filter-grown MDCK cells met in a common perinuclear compartment but DAVs were not labelled during short (5-30 minutes) pulses of marker, whether applied apically or basolaterally. Only when the tracers were taken up from the basolateral surface and then chased for periods of 2-18 hours, were DAVs labelled. It is proposed that entry of an endocytic tracer to DAVs occurs by the association of the desmosomal vacuole with late endosomes. Immunolabelling studies with antibodies to desmosomal components (to Dsg, DPI/II), to HRP and to the cation-independent mannose 6-phosphate receptor (MPR), confirmed that Dsg and DPI/II are located within DAVs and late endosomes, but not in early endosomes. Passage of Dsg, but to a lesser extent DPI/II, was detected in MPR^- structures (lysosomes). DAV-like structures have also been observed in developing tissues such as mouse kidney. Such engulfment may provide a general mechanism for handling insoluble junctional proteins, particularly where rapid morphogenetic changes are occurring in the pattern of cell-cell adhesion.

Key words: MDCK cells, desmosome-associated vacuole, desmosome internalisation

INTRODUCTION

The maintenance of tissue architecture and communication between neighbouring cells in epithelia occurs through elements of the junctional complex (Farquhar and Palade, 1963). Desmosomes form a major adhesive component of this complex and are assembled, in response to cell-cell contact, as well-defined plaques situated on the plasma membranes of adjacent cells. The desmosome itself consists of a number of proteins organized into three structurally and functionally defined domains, an extracellular portion, a membrane core, and a cytoplasmically located plaque to which are attached bundles of intermediate filaments (for reviews, see Steinberg et al., 1987; Garrod et al., 1990; Schwarz et al., 1990). The major desmosomal proteins have been localised to distinct regions of the plaque and intercellular space by immunoelectron microscopy (Miller et al., 1987).

The plaque domain contains non-glycosylated proteins such as desmoplakin (DP)I (M_r 250,000), desmoplakin (DP)II (M_r 215,000) and desmoplakin (DP)III (plakoglobin; M_r 83,000). The protein sequence and structure of DPI/II have been reported (Green et al., 1990; O'Keefe et al., 1989) and also the possible nature of the association between DPI/II and intermediate filaments (Stappenbeck and Green, 1992). Glycosylated proteins possessing a transmembrane region and an extracellular portion domain include desmoglein (Dsg1) (M_r 150,000) and the desmocollins, Dsc3a/b (M_r 117,000/97,000); the nomenclature of the desmosomal glycoproteins used here follows the conventions proposed by Buxton et al.(1993). Analysis of the protein sequence has established homology of the amino terminus of desmoglein and the desmocollins with that of the cadherins, a group of Ca^{2+}-binding transmembrane glycoproteins involved in cell-cell adhesion (Takeichi, 1988; Koch et al., 1991; Parker et al., 1991; Wheeler et al., 1991;

Collins et al., 1991; for reviews, see Schwarz et al., 1990; Magee and Buxton, 1991; Buxton and Magee, 1992). Use of Fab′ fragments from polyclonal antibodies to desmocollins were shown to inhibit desmosome formation in bovine kidney cells (Cowin et al., 1984), strongly suggesting that desmocollins were directly involved in desmosomal adhesion. Assembly and formation of desmosomes are responsive to the extracellular Ca^{2+} concentration (Jones and Goldman, 1985; Mattey and Garrod, 1986a,b; Penn et al., 1987,a,b; Duden and Franke, 1988; Pasdar and Nelson, 1988a,b, 1989; Mattey et al., 1990). The development of desmosomal and other adhesive contacts between cells, in response to an increase in Ca^{2+} concentration, may however depend on the presence of molecules other than the desmocollins. For example, uvomorulin (or E-cadherin), a Ca^{2+}-dependent glycoprotein found in mammalian epithelia, and which is not believed to be a significant component of desmosomes, appears to mediate an early event in cell-cell adhesion, because antibodies to uvomorulin can inhibit formation of the entire junctional complex when Ca^{2+} is added to MDCK cells grown initially in low-Ca^{2+} media (Gumbiner et al., 1988). Both Dsg1 and Dsc3a/b are reported to bind Ca^{2+} (Mattey et al., 1987; Steinberg et al., 1987), and putative Ca^{2+}-binding sites have been identified in both Dsg1 and Dsc3a/b. The mechanism of action of Ca^{2+} on junction formation is more complex than that of a bridge between cells, and may be part of a signal transduction pathway (Citi, 1992). Single cell studies confirm that changes of intracellular, as well as extracellular Ca^{2+} concentration may be important in regulating junction assembly (Nigam et al., 1992).

Biochemical studies of desmosome assembly have shown that in low Ca^{2+} media (<0.1 mM), and in the absence of cell contact, desmosomal components, notably the glycoproteins, are rapidly degraded (Penn et al., 1987a,b, 1989; Pasdar and Nelson, 1988a,b, 1989). Addition of Ca^{2+} (to ~2 mM) results in desmosome assembly through the transfer of components such as DPI/II from a soluble pool to an insoluble, detergent-resistant, state (Penn et al., 1987a,b, 1989; Pasdar and Nelson, 1988a,b, 1989), although Dsg1 and Dsc3a/b appear to become unextractable in non-ionic detergent in low as well as high Ca^{2+} (Penn et al., 1989), despite the lack of morphologically recognisable desmosomes.

At the morphological level, a number of uncertainties exist concerning the correlation of these biochemical studies of assembly with the events observed by immunofluorescence (Duden and Franke, 1988). Some of these difficulties arise, in part, from doubts concerning the structure of the presumed desmosomal ‘precursors’ or with structures with which they might be associated. Part of this problem arises because of the persistence, and possible contribution, of old, endocytosed desmosomes to the image, thereby offering scope for potential confusion with structures on the assembly route (see Duden and Franke, 1988). Treatments that result in the separation of cells, such as exposure to low Ca^{2+} concentrations or trypsinisation, promote the internalisation of desmosomes and other junctional elements. Internalised desmosomes have a very characteristic structure, composed of a plaque attached to a vesicle and to associated tonofilaments (Overton, 1968; Kartenbeck et al., 1982, 1991). Some of the earlier events of desmosome internalisation have been studied by Kartenbeck et al. (1982, 1991) and by Mattey and Garrod (1986b), but the subsequent stages, presumably leading to degradation in the terminal end-stations of the endocytic pathway, have not been described. This pathway is complex and terminates in lysosomes (for reviews, see Gruenberg and Howell, 1989; Griffiths and Gruenberg, 1991; Murphy, 1991; Smythe and Warren, 1991; Dunn and Maxfield, 1992). Intimately related to the pathways of endocytosis are those involved in membrane trafficking along the exocytic route (for reviews, see Simons and Fuller, 1985; Simons and Wandinger-Ness, 1990; Hopkins, 1992; Wollner and Nelson, 1992). In the context of desmosome assembly, therefore, the relative contributions of these different pathways to morphological studies are at present difficult to assess, particularly where the details of the endocytic route, perhaps involving fragmentation of internalised desmosomes, remain uncertain.

In this paper I describe the fate of internalised desmosomes in filter-grown MDCK cells, utilising a number of endocytic markers in combination with immunolabelling of desmosomal proteins, preparatory to attempting to reconstruct the assembly pathway. In such polarised cells, the separate routes of uptake of apically and basolaterally administered tracers can be assessed independently and the general features of endocytic and transcytotic routes are known in some detail (von Bonsdorf et al., 1985; Bomsel et al., 1989; Parton et al., 1989). To assess the possible relevance of the proposed mechanism of desmosomal degradation to situations encountered during normal development, embryonic material has been examined for the presence of internalised desmosomes. For this purpose mouse kidney, at a number of stages of development, has served as a representative tissue.

MATERIALS AND METHODS

Cell culture

MDCK strain II cells were grown to confluence in MEM (Penn et al., 1987a,b) on 35 mm diameter plastic Petri dishes (Nunc, Denmark). A cloned cell line (N8) of MDCK cells, obtained through the kindness of Dr W.J. Nelson, Stanford University, California, USA, was used for endocytic and immunolabelling studies, and grown in DME on 12 mm diameter polycarbonate Transwell filters (Costar, UK). The properties of this clone have been described by Nelson and Veshnock (1986). The Ca^{2+} content of the media (both MEM and DME) was ~2 mM; where necessary, a low Ca^{2+} medium (<0.1 mM) was prepared by Chelex treatment of the foetal calf serum, as described by Penn et al. (1987, 1988). The Ca^{2+} content was determined by atomic absorption spectrometry. Transfer of cells to a low-Ca^{2+} medium was preceded by washing the dish, or filter, with six changes of Ca^{2+}-free PBS.

Endocytic tracers

Experiments were performed on MDCK (N8) cells grown in DME on Transwell filters. Cells were cultured for six days, with three changes of medium, to obtain confluent, fully polarized monolayers.

Horseradish peroxidase (HRP) type II (Sigma Chemical Co.)

was used at a concentration of 10 mg/ml in DME and added to either the apical or basal surface of the filter for periods of 5-30 minutes, or chased with unlabelled medium for 2-18 hours. Cationised ferritin (Sigma) or BSA-coated gold particles (20 nm diameter, from Biocell Laboratories, Cardiff, UK) were added at concentrations of 0.1 mg/ml and A_{525} ~7, respectively, to DME buffered with 10 mM Hepes, pH 7.5. Filter inserts, wrapped in Parafilm, were placed on ice for 10 minutes to facilitate binding, then transferred to a water bath maintained at 37°C.

Electron microscopy

Following a brief wash in ice-cold PBS, cells were fixed in a mixture of 1% glutaraldehyde and 2% formaldehyde (prepared from para-formaldehyde) in 100 mM cacodylate buffer, pH 7.2, for 1 hour at room temperature. All samples were post-fixed in 1% OsO_4 in cacodylate buffer for 1 hour at room temperature before staining in 1% aqueous uranyl acetate, dehydrated in an ethanol series and embedded in Araldite. Cells grown on plastic Petri dishes were removed as sheets following exposure to propylene oxide, sedimented in a centrifuge as a series of layers, and embedded in Araldite (Griffiths et al., 1984b). In this way, several layers of cells could be sectioned simultaneously in any desired plane. Sections of cells exposed to HRP were lightly stained with uranyl acetate and lead citrate.

Mouse embryos (Parkes random bred, NIMR) at 12.5 and 13.5 days of gestation were dissected by Dr Blanche Capel (NIMR), and the kidneys placed into the fixative described above. The kidneys were fixed for 1 hour on ice then for a further hour at room temperature. Subsequent stages of OsO_4 postfixation, dehydration and embedding were as outlined above for MDCK cells. Samples for cryomicrotomy were prepared in parallel (see below).

Cryomicotomy and immunolabelling

MDCK clone N8 cells on Transwell filters were washed briefly with PBS, following uptake of endocytic markers such as HRP, and fixed immediately in 1% acrolein, 2% paraformaldehyde in 200 mM Hepes buffer, pH 7.5, for 1 hour at room temperature. Two filters were placed with the cell monolayers facing each other and maintained in 10% gelatin (in PBS) for 10 minutes at 37°C before cooling on ice and subsequent fixation for an additional 30 minutes. Small strips of the sandwiched cell monolayers were then infused with 2.3 M sucrose in PBS for a minimum period of 2 hours before freezing in liquid nitrogen. It was also possible to peel the layer of fixed cells from the filter, carefully roll or fold it whilst in 10% gelatin and infuse small pieces in sucrose before freezing. Cryosections were cut on a Reichert FC4 microtome with cryoattachment at a temperature of −100°C, and sections retrieved in a drop of 2.3 M sucrose in a wire loop, following the methods described by Tokuyasu (1986) and Griffiths et al. (1984b). The thawed sections were transferred to drops of 0.5% fish skin gelatin in PBS, on ice, and left for periods of 2-18 hours. Subsequent stages of the immunolabelling procedure were performed at room temperature. Grids were washed on droplets of 0.1 M NH_4Cl in PBS for 10 minutes, washed briefly in 0.5% fish skin gelatin (Birrell et al., 1987; Lucocq et al., 1989) before incubation on 10 μl drops of primary antibody for 1 hour. Sections were washed with six changes of PBS over 15 minutes and then labelled with Protein A-gold for 1 hour, washed for a further 30 minutes in PBS (six changes) followed by a 5 minute wash in four changes of distilled water before staining and embedding in 0.2% uranyl acetate/methyl cellulose (Griffiths et al., 1984a).

For double-, or triple-labelling, grids were first incubated with the smaller gold probe (5 nm) before applying the larger conjugate (10 nm or 15 nm). Where two or three rabbit primary antibodies were used for multiple labelling, the recommendations of Geuze et al. (1981) were followed, except that glutaraldehyde (0.25-0.5%) was used to block unreacted sites before applying successive rabbit antibodies (see Slot et al., 1991). The distributions of multiply-labelled samples were compared with those involving a single antibody. Immunocytochemical controls included the omission of the primary antibody, the use of non-immune serum and, in the case of multiple labelling, a reversal of the order of primary antibodies to establish the pattern of labelling.

Mouse embryo kidneys (at 12.5 and 13.5 days of gestation) were fixed in acrolein/paraformaldehyde (see above) for 1 hour on ice, washed in PBS, then embedded in 10% gelatin by orientating individual kidneys under a dissecting microscope. After a further 1 hour in fixative, on ice, gelatin blocks were trimmed and placed in 2.3 M sucrose in PBS for 2 hours on ice before freezing in liquid nitrogen. Frozen samples were cut and labelled as described above.

All sections were examined at 80 kV on a JEOL 1200EX electron microscope.

Antibodies

Antibodies to DP I/II (Pasdar and Nelson, 1988a,b) and to Dsg1 (Pasdar and Nelson, 1989), prepared as rabbit antisera to proteins purified against bovine muzzle, were obtained as a generous gift from Dr W. J. Nelson, Stanford University, California, USA. However, desmosomal cadherins are known to exist as a number of isoforms (Buxton et al., 1993), and the particular isoform recognised by this antibody to desmoglein has not been determined. In this paper it will be therefore referred to by the neutral connotation Dsg. Dr B. Hoflack, European Molecular Biology Laboratory, Heidelberg, Germany, kindly donated a rabbit antibody against the bovine large cation-independent mannose 6-phosphate receptor (MPR) (Griffiths et al., 1988). Antibodies to the cytoplasmic domain of Dsg1 (Wheeler et al., 1990) and to base-pairs 4247-5228 (Green et al., 1990) of DPI/II (Arnemann et al., 1993), were produced in this laboratory. Where necessary, these will be referred to as $Dsg1^r$ and DPI/II^r, respectively. No distinction could be usefully drawn in terms of specificity between the antibodies to muzzle proteins and recombinant proteins as far as assembled desmosomes, internalised desmosomes and endocytic structures were concerned, and corresponding pairs could be used interchangeably. Anti-HRP antibodies were obtained from Sigma Chemical Co. Protein A-gold conjugates were purchased from Biocell Research Laboratories, Cardiff, UK and were examined by electron microscopy before use to confirm that the suspensions were free of aggregates.

RESULTS

Initial stages of desmosome internalisation

Well-defined desmosomal plaques were seen in filter-grown MDCK cells (Fig. 1A,B). Groups of three to six desmosomes, each about 0.1-0.25 μm in length, were present along the lateral borders of adjoining cells. The intercellular space, some 30 nm wide, was bridged by fine filaments (Fig. 1B), although a central dense line was seen only in favourably sectioned cells. The plaque itself was some 16 nm wide and of homogeneous density, but occasionally appeared to be composed of two zones parallel to the membrane, the outer zone being slightly more dense than the inner. On the cytoplasmic edges of the plaque were bundles of tonofilaments (Fig. 1B). Desmosomes of cells grown on plastic dishes were of similar structure.

Internalised desmosomes were seen commonly in cul-

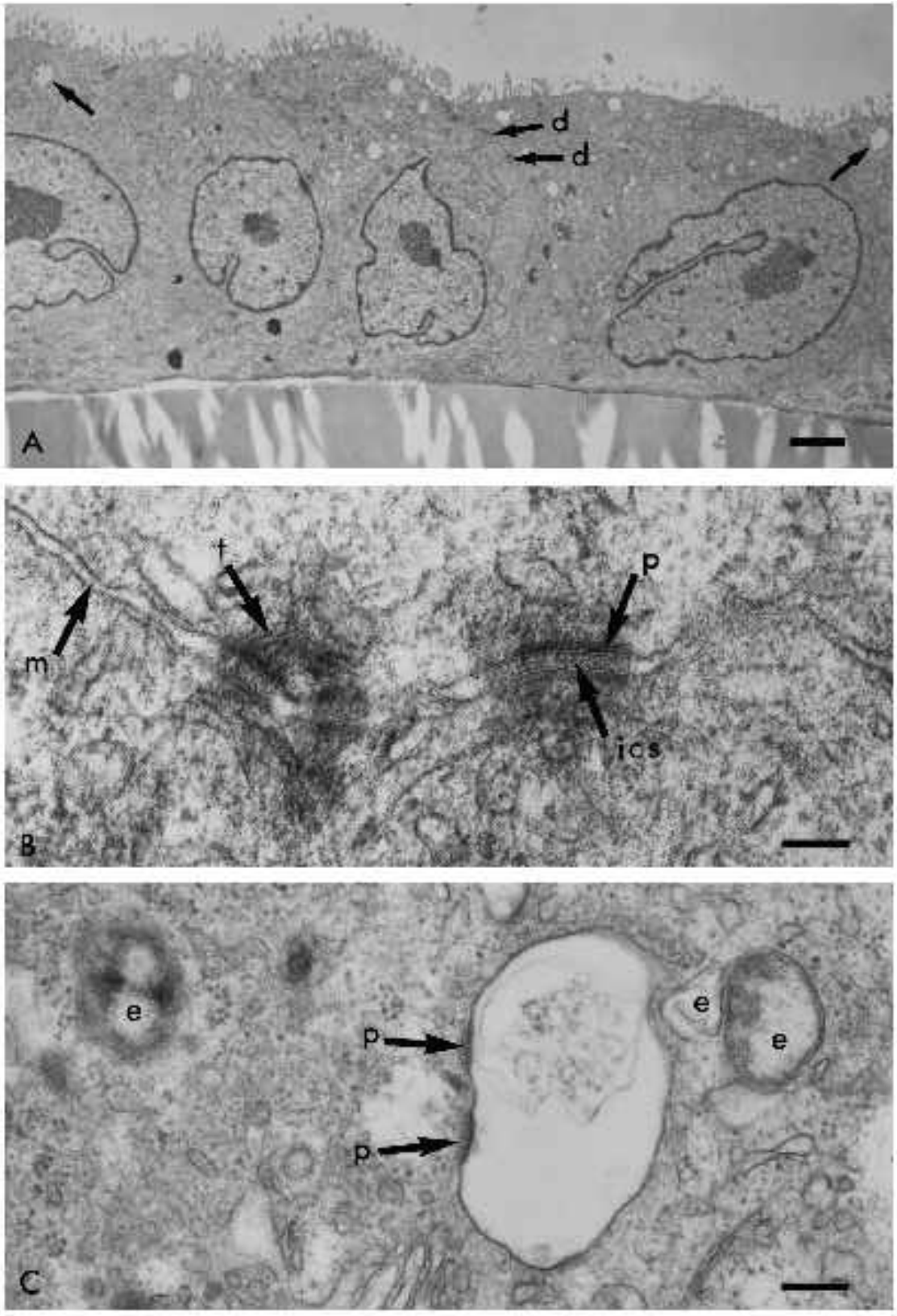


Fig. 1. Vertical thin sections of MDCK cells grown on filters. (A) Low magnification, showing desmosomes (d) on lateral plasma membranes and internalised desmosomes (arrows); (B) desmosome structure at higher magnification, showing plaque (p), with associated tonofilaments (t), lateral plasma membrane (m), intercellular space (ics) with fine fibrils; (C) internalised desmosome in filter-grown cells, showing plaques (p) and putative endosomal structures (e) in the cytoplasm. Bars: (A) 2 μm; (B) 0.1 μm; (C) 0.2 μm.

tures of MDCK cells grown in DME or MEM with physiologically normal levels of Ca^{2+} (~2 mM) and without further manipulation of the medium to reduce Ca^{2+} concentrations (see also Duden and Franke, 1988). Such internalised desmosomes will be referred to as desmosome-associated vacuoles (DAVs).

The essential structure of the DAVs was that of a vacuole in which one to three plaque profiles were visible in the plane of the vacuolar membrane. The presence of the plaques conferred an obvious angularity to the profile of the vacuole, perhaps due to an increased rigidity of the plaque with respect to the remaining portion of the membrane. On the cytoplasmic edge of the plaque fine spikes, with a spacing of ~13 nm, were visible. Tonofilaments were associated with the desmosomes in the initial stages of the process of internalisation (see Kartenbeck et al., 1982; Mattey and Garrod, 1986b), but were much less conspicuous among internalised desmosomes of cells not exposed to low-Ca^{2+} or EGTA treatment. Vesicular structures resembling endosomes or lysosomes were frequently observed in the vicinity of DAVs (Fig. 1C).

Uptake of endocytic tracers

Experiments were performed with MDCK cells grown on filters in order to determine the point of entry of DAVs into the endocytic pathway. As noted above, these cells already contained internalised desmosomes, perhaps originating wholly or partly in the course of trypsinisation during subculture. Their presence has been noted in cells maintained in low-Ca^{2+} medium for several weeks (Duden and Franke, 1988). Thus, internalised desmosomes are likely to be of very different ages. The experiments reported below, with two exceptions, were performed upon cells cultured for 5-6 days and without reduction of Ca^{2+} concentration.

Three types of markers for fluid-phase endocytosis were used: HRP, cationised ferritin and BSA/gold particles. Each of these markers were added, separately and in different combinations, to the apical or basolateral compartments for periods varying from 5 minutes to 2 hours and in some cases chased with unlabelled medium for periods up to 18 hours. When HRP was added basolaterally the reaction product did not appear on the external face of the apical surface, confirming the intactness of the cell monolayer.

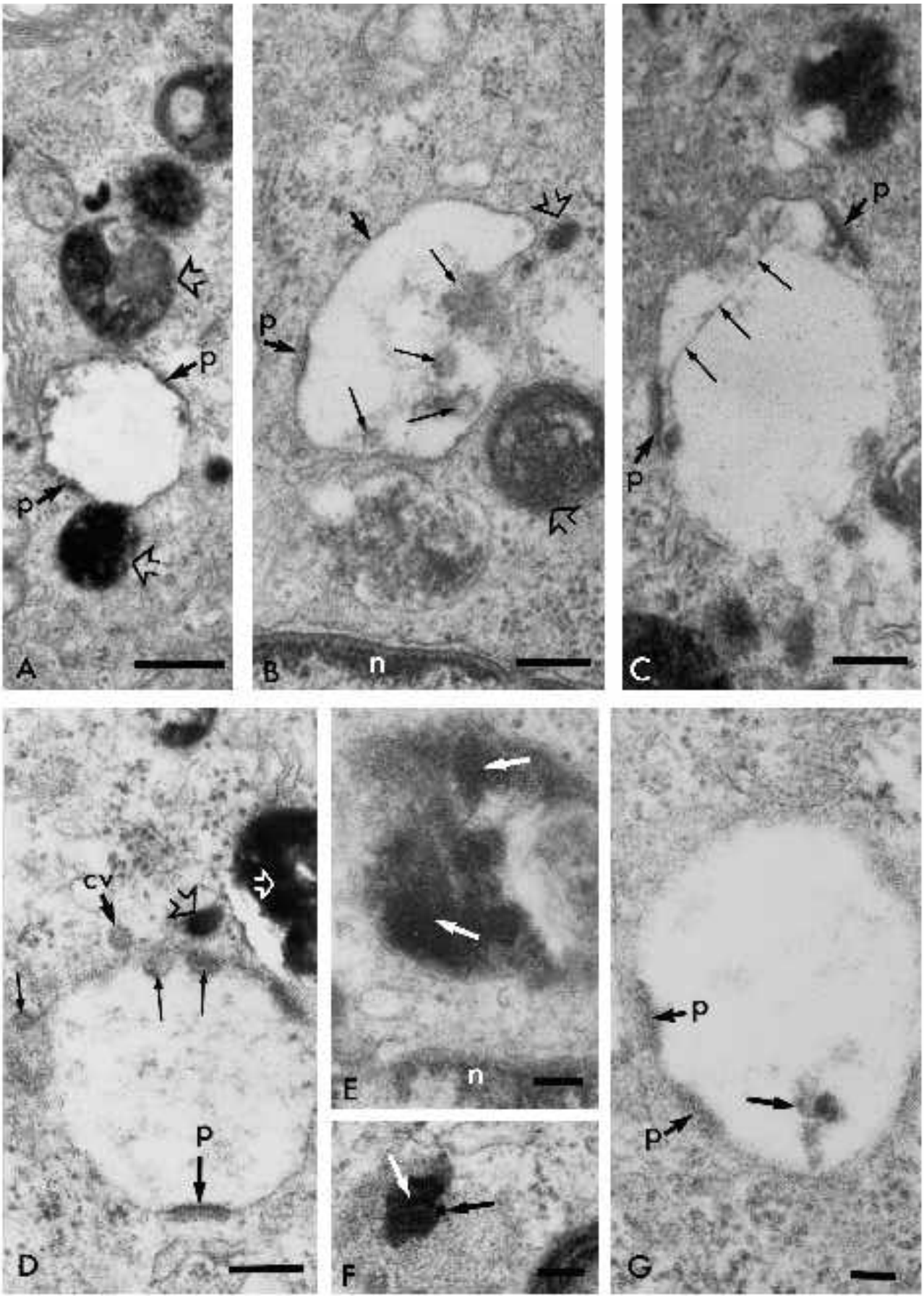
When short pulses (5-10 minutes) of HRP were added from the apical or basolateral surfaces, the reaction product seen after DAB treatment was localised to collections of small vesicles, coated pits, or occasionally to large endosomes, as described by Parton et al. (1989). Studies by Hopkins et al. (1990) and Tooze and Hollinshead (1991) indicate the existence of tubular networks of endosomes in some cell lines, including MDCK cells (Tooze and Hollinshead, 1991), although whether these represent early or late endosomal compartments, or a connection between the two, is not clear at present. Some of the structures observed here may correspond to cross-sections through such a network. No internalised desmosomes were labelled. If HRP added from the apical surface was chased with unlabelled medium for 2-18 hours, the DAVs were again unlabelled. Only when HRP was added from the basolateral surface for 30 minutes and then chased for 2-18 hours, was the dense reaction product seen either within DAVs or with associated structures presumed to be late endosomes or lysosomes (Fig. 2A-D). These labelled structures from presumed late stages of the endocytic pathway were usually seen in close proximity, if not physically contiguous with, internalised desmosomes (Fig. 2A). The reaction product was often seen to comprise vesicular or tubular profiles (Fig. 2B) within the vacuole. Also associated with the reaction product and with the interior face of the plaque was a fine membrane (Fig. 2C). Vesicular profiles apparently 'budding' into the interior of the vacuole, possibly containing HRP reaction product, were also observed (Fig. 2D). In some cases, apparent discontinuities in the profile of the vacuolar membrane were seen (Fig. 2C), suggesting that the membrane was damaged. No endogenous peroxidase was detected in control samples. The structure of internalised desmosomes is considered further below.

The other markers used for following the endocytic pathway, as noted by others (e.g. Parton et al., 1989), did not, in general, yield as high a signal as HRP. When two markers were added separately from the apical and basolateral surfaces, such as cationised ferritin and HRP or BSA/gold, the markers were found to be co-localised in a perinuclear late endosome or lysosome (Fig. 2E,F). This structure was also found to contain the marker when the tracer was added singly to either the apical or basolateral surface. Localisation of an endocytic marker within internalised desmosomes was found to occur only when the tracer was added from the basolateral surface. For example, when HRP was applied apically and cationised ferritin basolaterally, discrete crystals of ferritin (Fig. 2G), comparable to those seen in the late endosome/lysosome meeting compartment (Fig. 2E,F), but no HRP reaction product, were observed within internalised desmosomes. This invariable association of the basolaterally applied marker, but not with that from the apical surface, within DAVs, was found to be true of all combinations of markers tested. In spite of extensive examination of thin sections, no examples of late endosomes/lysosomes containing double markers, and associated with internalised desmosomes, were seen.

Attempts were also made to label desmosomes in the process of internalisation, by adding HRP at the same time as the cells were exposed to low Ca^{2+} medium. When filter-grown cells were so treated, very few of the surface-located desmosomes were internalised efficiently or contained reaction product (data not shown). The same experiment performed with EGTA (added to 4 mM) resulted in rapid junctional separation, but the origins of internalised membranes loaded with HRP were more difficult to interpret, since cell morphology was highly disordered under these conditions.

Structure of internalised desmosomes

Using the results where HRP was applied from the basolateral surface as a pulse for 30 minutes and then chased overnight, additional features of the structure of internalised desmosomes were observed. In suitably sectioned specimens, the vacuole was seen to be surrounded by a region of low electron density in which the profiles of many small vesicles were present (Fig. 3A,B). In no case were these vesicles seen to be labelled with HRP reaction product, not even during short (5-10 minutes) pulses of HRP. Many coated vesicles were observed, although the coat structure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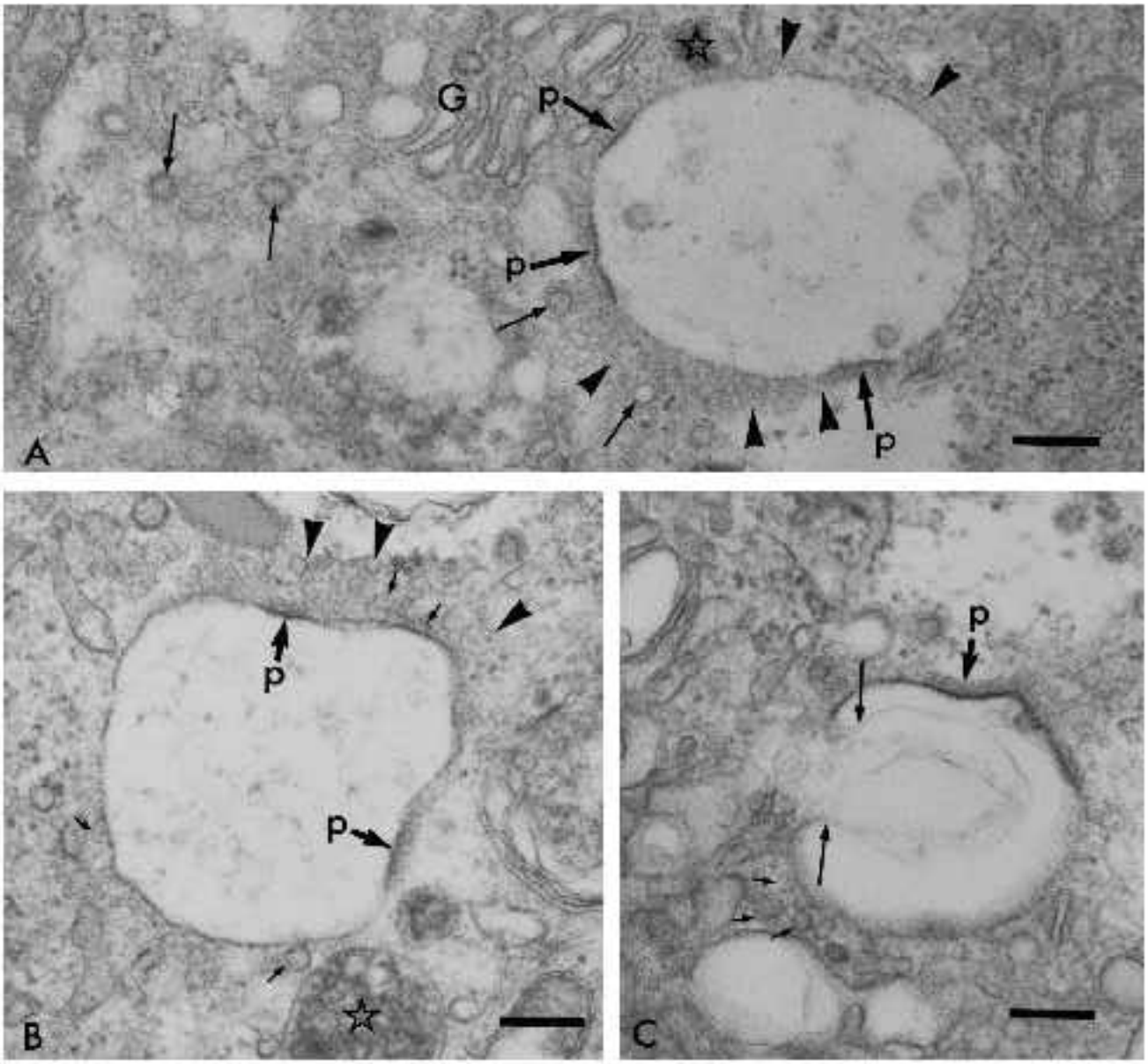

Fig. 3. (A-C) Structure of internalised desmosomes of MDCK cells labelled basolaterally with HRP for 30 minutes and chased overnight (A), or exposed to HRP apically and cationised ferritin basolaterally (B-C), again for a pulse time of 30 minutes and an overnight chase. The micrographs illustrate the presence of a matrix of low electron density (arrowheads in A,B) in which a variety of small vesicles (unlabelled arrows) are embedded or associated, some of which are coated vesicles. An infolded membrane (large arrows in C) is present within the vacuolar membrane of the internalised desmosome, and many smooth-surfaced vesicles (small arrows) are present. Note that no reaction product is present within the vesicles, although HRP−, but not cationised ferritin, is visible in large endosomal/lysosomal vesicles (stars). The plaque (p) in C shows a laminated structure parallel to the membrane together with a transverse periodicity. Plaque (p), Golgi cisternae (G). Bars, 0.2 μm.

Fig. 2. Micrographs of internalised desmosomes labelled with HRP from the basolateral surface for 30 minutes and chased for 120 minutes (A,D) or 18 hours (B,C). Internalised desmosomes, identifiable by their plaques (p), are closely associated with HRP-labelled late endosomes/lysosomes (open arrows). Reaction product is also seen (unlabelled arrows in B) within the vacuolar membrane or adjacent to the plaques. The vacuolar membrane may be disrupted in the lower part of the figure (C). Note the fine internal membrane (arrows) in (C), coated vesicle (cv) and vesicular profiles (unlabelled arrows in D). (E-F) colocalisation of two markers in a peri-nuclear located late endosome/lysosome after (E) supplying cationised ferritin (white arrows) from apical surface and HRP basolaterally, for 30 minutes and chased overnight; the micrograph has been lightly printed to show the crystalline aggregates of ferritin amid a dense matrix of HRP reaction product. (F) Similar to (E), but BSA-gold (black arrow) was supplied apically and cationised ferritin basolaterally, again for 30 minutes followed by an overnight chase. (G) Localisation of cationised ferritin (arrow) within an internalised desmosome (p, plaque). After supplying HRP apically and ferritin basolaterally (30 minutes pulse, overnight chase), no HRP reaction product was seen within internalised desmosomes (see text). n, nucleus. Bars: (A) 0.5 μm; (B-D) 0.2 μm; (E,G) 0.1 μm.

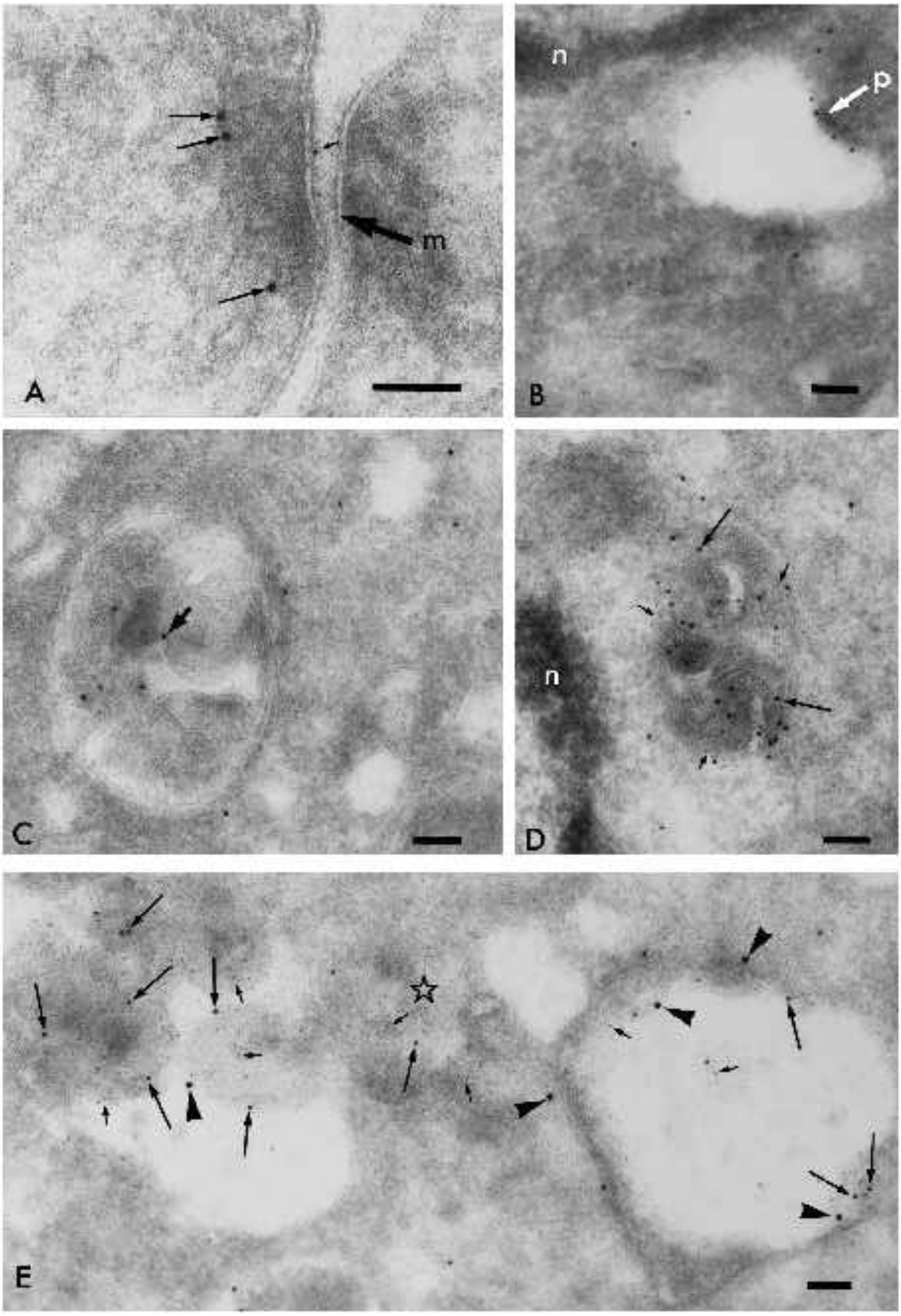
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Fig. 4. (A-E) Micrographs of frozen sections of MDCK cells, not exposed to HRP (A-B) or labelled with HRP for 30 minutes and chased for 120 minutes (C-E). (A) Section of a desmosome labelled with antibodies to DPI/II (10 nm gold, medium arrows) located at edge of plaque and to the cytoplasmic portion of Dsg (5 nm gold, small arrow) associated with membrane (m). (B) Section of internalised desmosome labelled with 10 nm gold for Dsg, located at edge of plaque (p); n, nucleus. Perinuclear endosomes/lysosomes labelled with antibodies to HRP (C) or double-labelled (D) to Dsg (5 nm gold, small arrows) and HRP (10 nm gold, medium arrows). (E) Triple-labelled section, with antibodies to MPR (5 nm gold, small arrows), HRP (10 nm gold, medium arrows) and Dsg (15 nm gold, large arrowheads). Note the spatial separation of the labels, the structures on the left and in the centre (star) containing significantly greater amounts of MPR than the structure on the right. The presence of label to MPR, as well as the multivesicular structure of the vesicles, suggests that they might be identified as late endosomes. Bars, 0.1 μm.

was not always apparent on vesicles surrounded by the low-density matrix (Fig. 3A). In some instances (Fig. 3A), the clusters of vesicles appeared to be associated with, or possibly simply adjacent to, Golgi cisternae. The fine membrane noted above (Fig. 2A), was occasionally seen in the form of an infolding (Fig. 3C), resembling an autophagous vacuole, and where large numbers of vesicles were gathered around the opening to the vacuole. The exterior face of the desmosomal plaque, adjacent to the cytoplasm, often appeared with heightened definition (Fig. 3C), in which the double-layered structure of the plaque and the transversely oriented 'spikes' were clearly discerned (Fig. 3C). The width of the laminations (~14 nm) and lateral spacings (~12 nm), were comparable to those of the plaque within intact cells (~15 nm and ~11 nm, respectively). It is important to note that these structural features observed in samples exposed to endocytic tracers were seen also in material not subjected to any pre-treatment.

Immunolabelling of desmosomes and endocytic structures

Frozen sections of MDCK cells grown on filters were prepared and immunolabelled with Protein A-gold, using control samples and those in which HRP was added to the basolateral surface after pulse times of 5-30 minutes, with or without a subsequent chase of 2-18 hours.

The distribution of labelled antibodies to DPI/II and Dsg on assembled desmosomes (Fig. 4A) was similar in control and HRP-treated cells. The anti-DPI/II antibody labelled the peripheral margins of the plaque whilst the anti-Dsg antibodies were located near the membrane. The specificity of the localisation was the same for either antibody applied to the sections separately; background labelling was not significant when the primary antibody was omitted or when non-immune serum was used (data not shown). Internalised desmosomes were more difficult to recognise in frozen sections but, as in the case of resin sections, the presence of the electron-dense plaque was used as a means of identification. Anti-desmosomal antibodies were shown to have labelling restricted to discrete edges of the vacuole (Fig. 4B), corresponding to the location of the plaque. Material within the vacuole was also occasionally labelled.

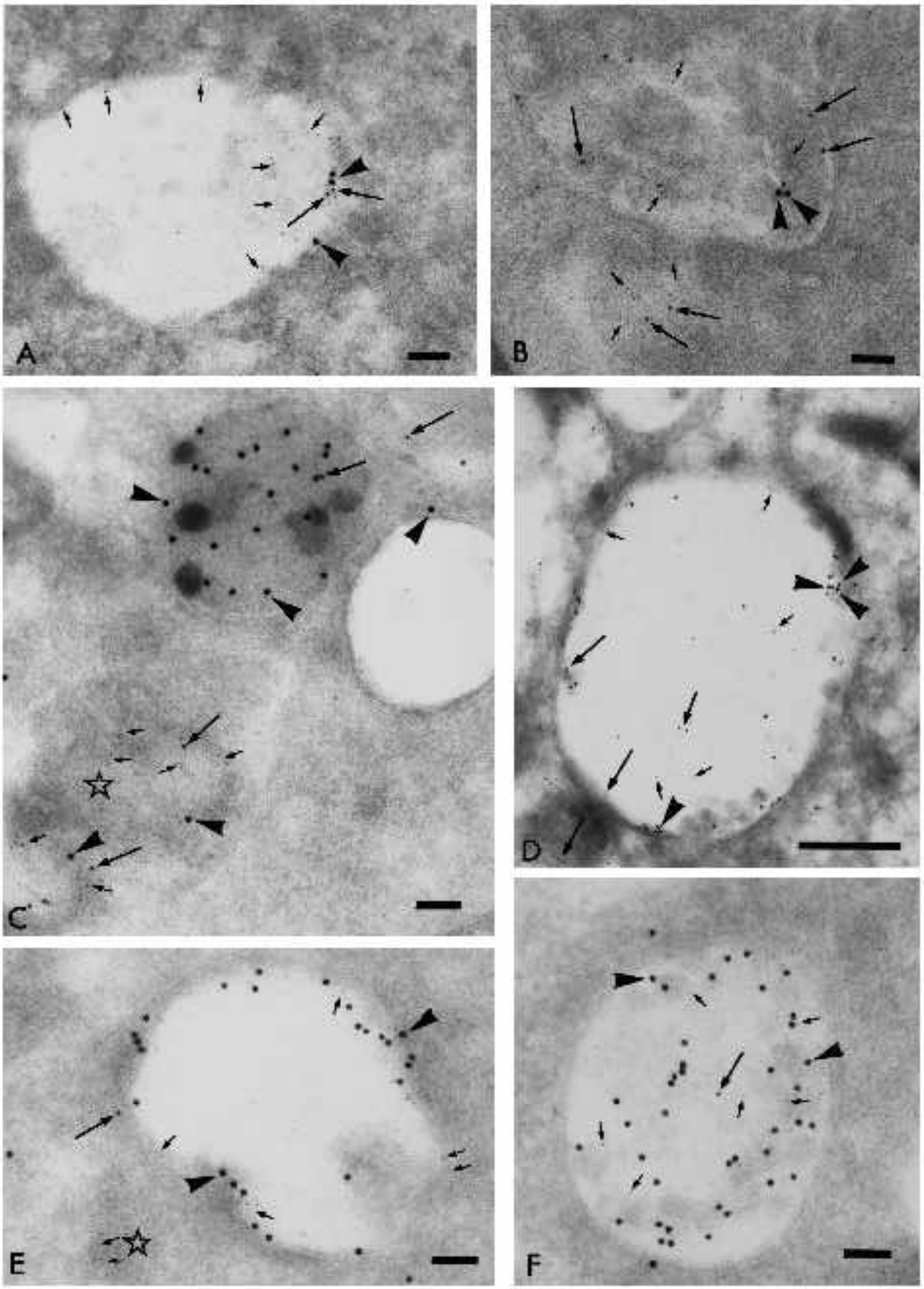
In cells pulse-labelled for 30 minutes and chased for 2-18 hours, antibodies to HRP were localised to peri-nuclear bodies (Fig. 4C) containing small vesicles or whorls of membrane, comparable to the structures seen (Fig. 2E,F) in resin sections. Double-labelling procedures showed that these endocytic structures contained both HRP and Dsg (Fig. 4D). To further establish the identity of these endosomes, an antibody to the cation-independent mannose 6-phosphate receptor (MPR) was used in labelling experiments. This receptor is known to recycle between intracellular compartments, but one major role is to sort newly synthesised lysosomal enzymes from the *trans*-Golgi network and to direct them to an endosomal compartment (for reviews, see Kornfeld and Mellman, 1989; Kornfeld, 1992). Recently, it has been shown that the bulk of the MPR can be localised to a late endosomal compartment (Griffiths et al., 1988). Mature lysosomes are therefore regarded as MPR^- structures (Geuze et al., 1988; Griffiths et al., 1988, 1990).

Triple-labelling experiments were used to investigate both the entry points of internalised desmosomes into the endocytic pathway and to establish the identities of the associated endocytic structures. The antibodies used were directed against MPR, Dsg (and DPI/II) and HRP, or combinations of them, using a range of Protein A-gold antibodies coupled to gold particles (5-15 nm). Preliminary experiments utilised different sizes of gold probe with each antibody to establish that the qualitative pattern of labelling was similar in each case. This was necessary because it is known that the labelling density is greatest for smaller gold probes (Yokota, 1988).

When cells were pulse-labelled with HRP for 5 minutes, populations of 'early' endosomes were labelled solely with HRP, but not with MPR, Dsg or DPI/II. At progressively greater times of incorporation of HRP, all three labels were found in the late endosome compartments (Figs 4C-E, 5A-F). The distribution of Dsg and DPI/II was also studied in HRP^+ but MPR^- structures (putative lysosomes; see Fig. 5C). One feature suggested by these results, and supported by preliminary quantitative studies, is the proportionately greater amount of Dsg rather than DPI/II found in terminal endocytic structures as well as within internalised desmosomes. MPR-enriched structures were also seen in association with DAVs (Fig. 5D), and with endosomes in which cationised ferritin had been localised under conditions like those shown in Fig. 2E,F. The results therefore confirm and extend the picture using endocytic markers and conventional resin sections, that DAVs (or their component molecules) are not associated with early endosomes, but interact decisively with late endosomes en route to lysosomes.

Desmosome structure in developing mouse kidneys

Junctional elements performing an adhesive function might be expected to play an important role in the dynamic circumstances of organ development, where localised growth or change in size could be accommodated by tissue remodelling. Under such circumstances, insertion, and possibly also removal, of desmosomes might provide a mechanism for controlled morphogenesis. I have therefore investigated whether internalised desmosomes of the type described above for MDCK cells, might not also be a feature of devel-


A
B
C
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E
F

Fig. 5. (A-F) Micrographs of frozen sections of MDCK cells after uptake of HRP for 30 minutes and chased for 120 minutes (A-C) or overnight (D) and labelled with antibodies to MPR (5 nm gold, small arrows), HRP (10 nm gold, larger arrows) and Dsg (15 nm gold, arrowheads). The labels are confined to the periphery or to the vesicular contents of structures interpreted to be late endosomes. Note that the structure shown in the upper half of (C) contains no MPR label, and may be a putative lysosome, whereas the lower structure (identified by a star) contains significant levels of MPR. (D) Putative internalised desmosome associated with late endosome (lower left) containing only MPR label; Dsg label confined largely to two sites on vacuolar membrane. (E-F) Sections from cells containing no internalised endocytic tracers but labelled with MPR (5 nm gold), DPI/II (10 nm gold) and Dsg (15 nm gold); the assignments of arrows of different sizes as described for (A-D). Both these late endosomes contain both DPI/II and Dsg, the latter being present in greater abundance, whereas the grazing section of a vesicle (star in E) contains only MPR. Bars: (A-C,E-F) 0.1 μm; (D) 0.5 μm.

oping tissue. For this purpose samples of embryonic mouse kidney were examined and supplementary data obtained from adult rat kidney.

Kidneys were taken from embryos at 12.5 and 13.5 days of gestation, when the shape of the kidney and gonads is sufficiently developed to allow differentiation of tissue morphology (Rugh, 1990; Kaufman, 1992). In conventional resin sections of 13.5-day material, junctional elements suggestive of very small (~0.1 μm) desmosomes were observed (Fig. 6A), although these structures did not possess well-defined plaques or prominent bundles of cytoskeletal filaments. More prominent adhaerens junctions, of the type described by Garrod and Fleming (1990), were also present. Kidneys taken at 12.5 days were much less differentiated. Examination of the cytoplasm of 13.5-day embryonic kidneys showed the occasional presence of structures closely resembling internalised desmosomes (Fig. 6D), containing plaques whose structure was much more sharply defined than that of junctions in situ. In all respects (length, thickness and spacing within the plaque) the dimensions of these structures were similar to those described above for MDCK cells. Within these vacuolar structures were small vesicles, again reminiscent of those observed in MDCK cells (cf. Figs 2, 3). No comparable internalised desmosomes were seen in sections of adult kidneys, including that from rats (data not shown).

When frozen sections of 13.5-day kidneys were immunolabelled with Dsg or DPI/II antibodies, a low but specific localisation was observed (Fig. 6B,C) on structures presumed to be putative desmosomes. Dsg labelling was seen in small cytoplasmic vesicles, but a protracted search was necessary to locate internalised desmosomes labelled with either Dsg or DPI/II antibodies (Fig. 6E,F). As in MDCK cells, the label was confined to one or two patches on the membrane (Fig. 6E,F) or, in the case of Dsg, to gold particles being also associated with the low-density material within the vacuole (Fig. 6E).

DISCUSSION

Structure and general properties of DAVs

As described earlier (Overton, 1968; Kartenbeck et al., 1982, 1991), the dissociation of desmosomes leads to their internalisation into structures that are here termed desmosome-associated vacuoles. These structures have a complex fate and several novel features with respect to the uptake of endocytic tracers. DAVs are distinct from but related to the analogous structures within which adhaerens junctions are engulfed after dissociation of MDBK cells with EGTA (Kartenbeck et al., 1991). The simplest explanation as to why the two junction types should associate with distinct vesicle populations is that this arises merely from their initial spatial separation in the plasma membrane, rather than that any selectivity exists in mechanisms of internalisation. Once internalised, the desmosomal plaque spans the plane of the vacuolar membrane; unlike other endocytic and phagocytic structures, the DAV presents some components on its outer cytoplasmic side as well as having others enclosed within the bounding membrane. The exposed components are progressively, though perhaps never completely, eroded from the cytoplasmic surface, since immunolabelling suggested a gradual decline in the level of DPI/II relative to the integral membrane protein, Dsg. The vacuolar membrane itself persists for long periods of time without fragmenting into smaller vesicles.

Passage of endocytic tracers into DAVs

DAVs do not appear to become competent to fill with endocytic markers until the tracers have reached a late endosome, as characterised by immunolabelling. Short (5 minutes) pulses of HRP do not enter DAVs, although antibodies to MPR can label DAVs, suggesting an association of DAVs with late endosomes in the steady state. Significant labelling was not observed unless HRP was chased for 2-18 hours. When fluid-phase markers were applied simultaneously to the apical and basolateral surfaces, both markers were eventually found in a late endosome compartment occupying a perinuclear location (Fig. 2E-G; see also Bomsel et al., 1989; Parton et al., 1989; Prydz et al., 1990). Although the perinuclear late endosomes act as a meeting compartment for markers ingested from both apical and basolateral surfaces, only the basolateral tracer was internalised into DAVs. Thus the DAVs show selectivity in their fusion with other endocytic structures, suggesting a category of perinuclear late endosomes that is excluded from associating with DAVs and therefore a population of basolateral endosomes that do not merge with those of apical origin. Such a class of late endosomes may possess special properties enabling them to engage internalised organelles such as DAVs. An alternative, and less probable, explanation for the exclusive presence of basolateral markers within DAVs, is that the perinuclear late endosomes are partitioned into separate, but juxtaposed, apical and basolateral chambers even though they act as a common receptacle for tracers endocytosed from the two domains. Transfer of the basolateral marker would then occur by association with DAVs in a second step and markers ingested from the apical surface would not participate in these fusion events with DAVs. Some degree of selectivity is shown by the basolaterally and apically derived fluid-phase vesicles themselves, in that over 70% of solutes entering basolateral endosomes become transferred to late endosomes, whereas only 10% do so from apical early endosomes, the remain-

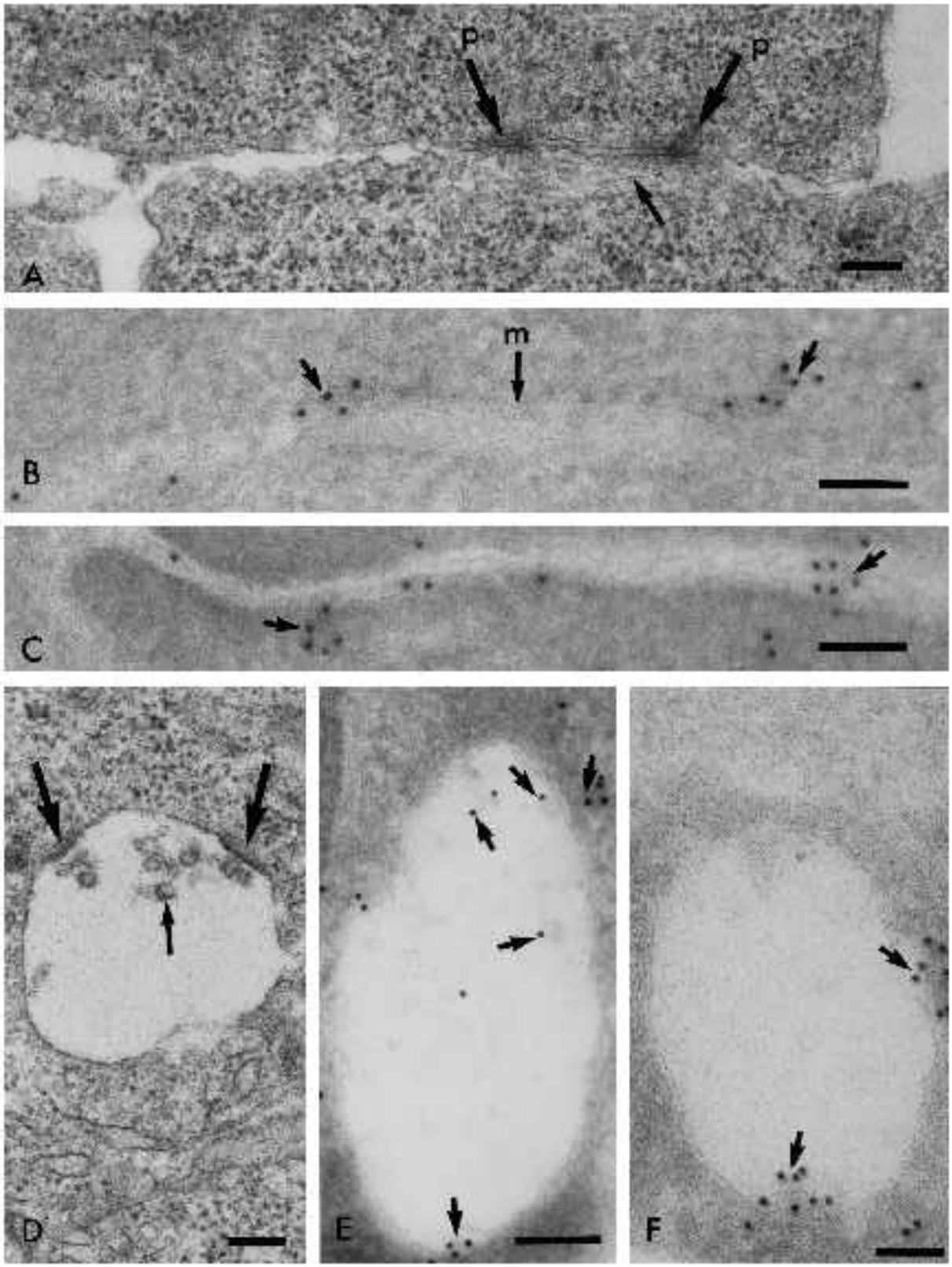

Fig. 6. (A-F) Sections through medullary regions of 13.5-day mouse embryos, showing: (A) section through apposed membranes, with possible plaque-like structures (p, large arrows) and fine filaments (small arrow). (B,C) Cryosections of apposed membranes (m) labelled with DPI/II (B) and Dsg (C) (short arrows). (D) Resin section of a presumptive internalised desmosome, with plaque-like structures (large arrows) and small vesicles (smaller arrow) within a vacuolar membrane. (E,F) Cryosections of putative DAVs after labelling with antibodies to Dsg (E) and DPI/II (F); 10 nm gold particles (arrows) are located in discrete locations on or close to the membrane or within the vacuole. Bars: (A,D) 0.2 μm; (B,C,F) 0.1 μm.

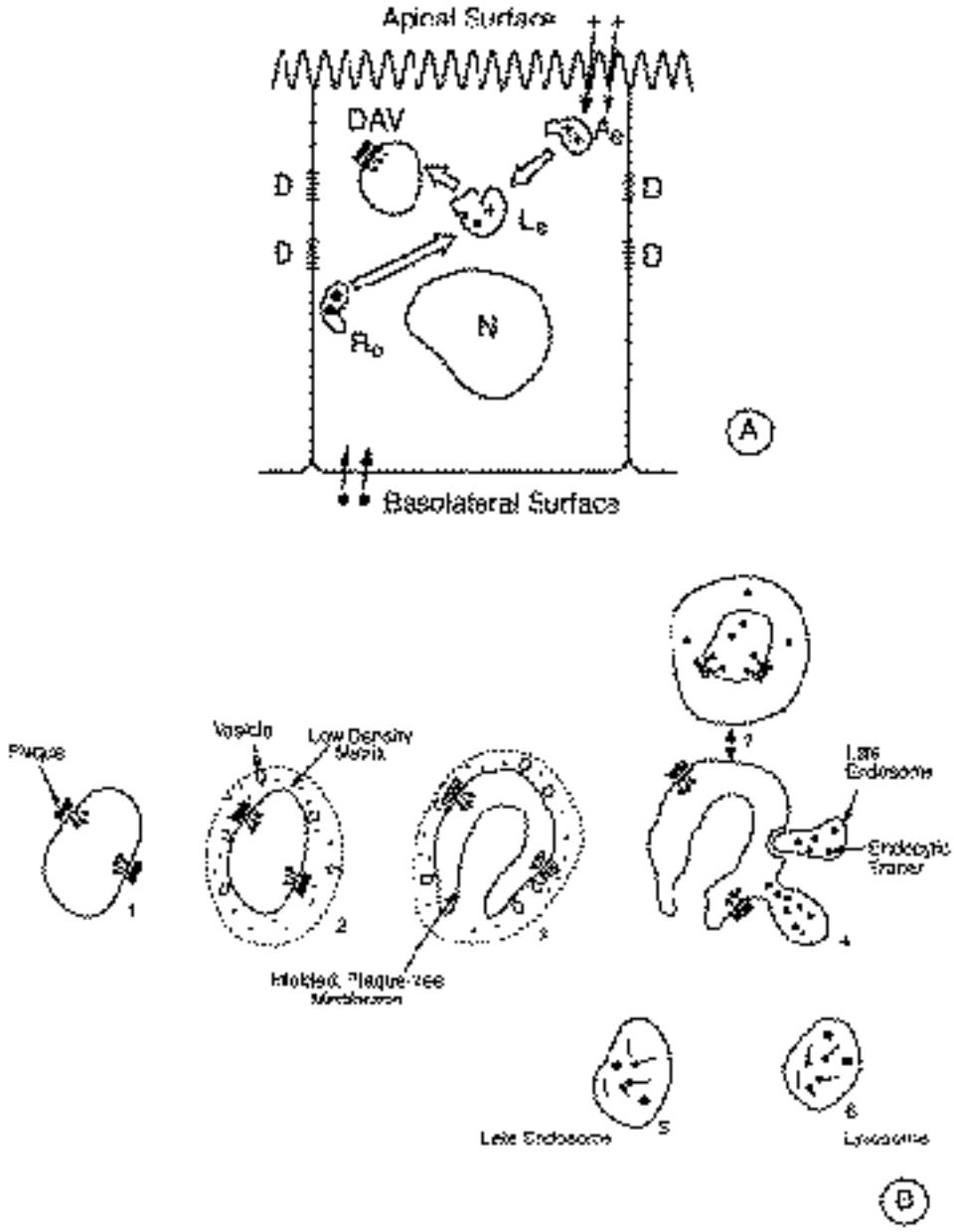

Fig. 7. Diagram illustrating (A) the uptake of endocytic markers from the apical (+) and basolateral (●) surfaces into separate apical (A_e) and basolateral (B_e) early endosomes. These separately administered markers meet in perinuclear located late endosomes (L_e), which engage or fuse with internalised desmosomes (DAV); desmosomes on lateral plasma membranes (D); nucleus (N). The possible subsequent stages of desmosomal endocytosis are shown in (B). 1,2: the vacuolar membrane of the DAV (1) is surrounded by a matrix of low electron density in which vesicles are present, some of which appear to be attached to the vacuolar membrane. An infolded membrane (3,4) is present within the vacuole. Basolaterally administered tracers (●), but not those supplied from the apical surface, are transported to late endosomes. The latter attach or fuse with, internalised desmosomes, transferring the endocytic marker (●) to the interior of the vacuolar membrane (4). Internalisation of the plaque is presumed to occur at stage 4, following the association of the DAV with a late endosome, although the precise mechanism of engulfment has not been established and is indicated by a question mark (?). The constituents of the plaque, together with the endocytic tracer (shown here for convenience as particulate), appear in the terminal stations of the endocytic pathway, the late endosome (5) and lysosome (6). See text for further details.

ing 90% being transcytosed (Bomsel et al., 1989). The finding is also consistent with emerging evidence for selective mechanisms in fusion steps following endocytosis (van der Sluijs et al., 1992; Bucci et al., 1992). The large differential in selectivity or efficiency of uptake, as well as the greater size of endocytic structures in the basolateral domain (Parton et al., 1989), may of course also reduce the threshold for detection of apically applied markers, particularly in thin sections of cells.

Later stages of DAV disassembly

The final stages in the life of the DAV can be identified in those structures in which the desmosomal plaque has been most substantially modified. The dense plaque components are greatly diminished, and the removal of interstitial material has enhanced an underlying periodicity of desmosome fine structure (Fig. 3C). The degradation of internalised proteins is known to begin in early endosomes (Diment and Stahl, 1985), although markers such as HRP survive proteolytic degradation and can be detected in lysosomes (Steinman and Cohn, 1972). A clue to one of the mechanisms involved in the dissolution of DAV components might be found in the recent demonstration that the ubiquitin-activating enzyme, E1, is located on intact desmosomal plaques in HepG2 cells (Schwarz et al., 1992). This enzyme provides the initial activation for transfer to ubiquitin carrier proteins, for subsequent targeting towards lysosomal and non-lysosomal proteolysis. Also at this stage, a non-plaque-bearing membrane frequently appears to penetrate the DAV (Figs 2B,C, 3C) and the vacuole becomes surrounded by small vesicles (Fig. 3) whose spatial distribution would suggest that they may originate in the Golgi. The infolded membrane is reminiscent of features of autophagous vacuoles, where an organelle targeted for degradation may be enclosed within membranes derived from the ER to form an autophagosome as the first stage

towards ultimate delivery to lysosomes (for review, see Seglen and Bohley, 1992). The pattern of immunolabelling seen in late endosomes and lysosomes suggests that the components of the DAV pass first into late endosomes and then proceed to lysosomes. However, much careful searching failed to discover possible intermediates in the conversion of late endosome (MPR^+) - DAVs into (MPR^-) lysosomes.

Whatever degradative route is followed by DAVs, the pathways appear to converge, as in the dissolution of material ingested by phagocytosis and autophagy, at the late endosome. In addition to its role as a meeting point for apical and basolateral endocytic markers, the late endosome may therefore also play a pivotal role in the merging of the endocytic and autophagic pathways (see also Gordon and Seglen, 1988; Griffiths et al., 1990).

A summary of these observations on the internalisation and degradation of desmosomes is shown in Fig. 7. The loss of intermediate filaments may occur relatively soon after internalisation (Kartenbeck et al., 1982), and might arise at least in part from the steady-state exchange, which has been demonstrated to be a normal cellular process (Vikstrom et al., 1992). The diagram shows the passage of endocytic tracers from endosomes, DAVs through to lysosomes, but does not directly address the question of whether passage of ligands through endosomes occurs via a maturation mechanism (Murphy, 1991) or by carrier vesicles (Griffiths and Gruenberg, 1990), although the resolution of the mechanism involved would clearly be of relevance in understanding the selective transfer of basolateral markers into DAVs.

Relationship between DAVs and structures involved in desmosome assembly

The vacuolar membrane of DAVs does not appear to fragment into smaller vesicles until the entire structure is engaged by late endosomes, which are themselves readily identified by immunocytochemical markers. The retention of a largely intact structure would therefore seem to afford a distinction from putative vesicular traffic engaged in desmosome assembly, because some components, such as DPI/II and Dsg1, appear to retain their separate association with cytoskeletal elements until a late stage of assembly (Pasdar et al., 1991). The nature and morphology of these assemblies have yet to be established, but their characterisation can now be approached with confidence that they need not be confused with enitities on the degradative pathway, since the latter, having been characterised, should now be readily recognisable. The structures reported by Duden and Franke (1988) as cytoplasmic half-desmosomes that continuously assemble in mouse keratinocytes and A431 cells grown in low Ca^{2+} media, are now seen to be closely related to DAVs and therefore likely to have been involved with degradation rather than assembly. Jones and Goldman (1985) reported intermediate filament-associated dense bodies staining with antibodies to DPI/II but lacking visible membrane profiles, that formed in mouse keratinocytes during a Ca^{2+} shift. Although Duden and Franke (1988) have suggested that these might also be endocytosis products (see also Mattey and Garrod, 1986b), they do not correspond to any structures seen in the present study and should therefore be considered possible candidates for assembly intermediates.

Possible role of DAVs in tissue development

It is of interest to ask whether the course of desmosome internalisation and degradation characterised above, is a mere artefact of cell culture or whether similar events occur as a normal part of tissue development and remodelling. The plaque-like structures identified as desmosomes (Fig. 6A-C) might correspond to the electron microscopic images described by Garrod and Fleming (1990), who also showed by immunofluorescence microscopy that desmosomal antigens are present from an early stage of mouse kidney development. More striking was the similarity, in the present study, of plaque-bearing vacuoles (see Fig. 6D) to the DAVs of MDCK cells, an impression reinforced by the localisation of Dsg and DPI/II to the bounding membrane and its contents. Such structures are also present in a number of other published reports; for example, in dendritic clustering in rat cerebral cortex (Hirst et al., 1991; for earlier references, see Kartenbeck et al., 1982). In the deeper proliferative layers of stratified epidermal tissue, Allen and Potten (1975) have described the complete engulfment of desmosomes by finger-like extensions of cytoplasm, in contrast to the mechanisms of desmosome removal in the superficial, cornified layers, which would appear to involve extracellular proteolysis (King et al., 1987).

It is therefore proposed that the process of desmosome internalisation into DAVs and eventual fusion with late endosomes for delivery to lysosomes for final degradation, is a general mechanism for removing these junctions from areas of cell-cell contact in normal physiological remodelling of tissue architecture.

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