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Directed Membrane Transport Is Involved in Process Formation in Cultured Podocytes

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Abstract. Mature glomerular visceral epithelial cells, or podocytes, are unique cells with a complex cell architecture. Characteristically, they possess a highly branched array of major processes and foot processes, which are essential for glomerular filtration in the kidney. A podocyte cell line with the potential to exhibit many features of differentiated podocytes, particularly the formation of cell processes, was recently established. In this study, it is shown that directed membrane transport is involved in process formation in cultured podocytes. The well-characterized vesicular stomatitis virus G was used as a marker protein for the biosynthetic pathway in these cells. It seems that newly synthesized vesicular stomatitis virus G is preferentially delivered into the cell processes of the podocytes, where it is colocalized with known regulators of vesicular transport from the Golgi apparatus to the plasma membrane, such as the small GTPase rab8 and the sec6/sec8 complex. To determine the role of vesicular transport in process formation, cells were treated with brefeldin A, a drug that disrupts the trafficking of post-Golgi transport vesicles. As a result, the podocytes reversibly lost their ability to form processes. These findings suggest that podocytes are dependent on a constant fresh source of lipids and proteins to form their processes.

Podocytes are highly specialized cells, with complex cell morphologic features, that contribute to many functions of the normal kidney glomerulus (1). The differentiation process for podocytes results in cells extending many major processes and foot processes. The foot processes of one podocyte must find foot processes of neighboring podocytes to form filtration slits, leading to the formation of the characteristic pattern of interdigitating foot processes bridged by the slit membrane (1). During glomerular development, podocytes undergo substantial changes in phenotype. Most important is the transition from the S-shaped body stage to the capillary loop stage, when podocytes become postmitotic and begin to establish their complex cell architecture, with the formation of processes (2–4). Little is known regarding what triggers these events and what types of signals are involved, because no *in vitro* model that could reproduce these steps in podocyte differentiation has been available (1).

In a recently established cell line of conditionally immortalized mouse podocytes, the decisive steps of podocyte differentiation from mitotic to postmitotic cells can be induced (5). When cultured under permissive conditions, the cells proliferate at a very high rate and grow as undifferentiated cobblestone cells; under nonpermissive conditions, most of the cells become growth-arrested within 6 d and acquire many characteristics of differentiated podocytes, such as cell processes and synaptopodin expression (5).

In a great variety of experimental and human glomerulopathies, altered morphologic characteristics of the podocytes (for example, foot process effacement) are correlated with clinical symptoms such as proteinuria. It is clear that a recovery state (*i.e.*, reconstitution of the foot process pattern) can only be achieved by the renewed formation of foot processes together with the formation of firm contacts between the processes and the glomerular basement membrane (6). Therefore, it might be speculated that podocytes are equipped with very dynamic cell extensions and that this ability to remodel cell processes represents a facilitating factor in glomerular filtration under both physiologic and pathologic conditions.

Our cell culture system offers a model to study the regulation of podocyte morphologic characteristics, especially the formation of processes, at the molecular level. In this study, we analyzed the events that lead to process formation by studying membrane transport in cultured podocytes. To this end, we analyzed the transport of vesicular stomatitis virus G (VSV-G) protein (a marker of the basolateral pathway in epithelial cells) to the surface of podocyte processes. In parallel, we analyzed the expression of different regulatory molecules of the biosynthetic pathway, such as the small GTPase rab8 and rsec6/rsec8, which are mammalian homologues of the exocyst complex in yeast. In a recent study, we showed that microtubules are necessary for process formation in cultured podocytes (N. Kobayashi, J. Reiser, W. Kriz, and P. Mundel, submitted for

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publication). In addition to cytoskeletal dynamics, we now provide evidence that vesicular membrane transport to specific docking sites is involved in the formation of podocyte processes.

Materials and Methods

Cell Culture of Mouse Podocytes

The generation and initial characterization of conditionally immortalized mouse podocyte clone cells (hereafter referred to as podocytes) from renal glomeruli of transgenic mice harboring a γ-interferon-inducible, temperature-sensitive mutant of the SV-40 large T antigen (7) were recently described (5). Podocytes were maintained in RPMI 1640 (Life Technologies, Eggenstein, Germany) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (Life Technologies) in a humidified atmosphere with 5% CO_2. To propagate podocytes, the culture medium was supplemented with 10 U/ml mouse recombinant γ-interferon (Sigma, Munich, Germany) to enhance the expression of the T antigen, and cells were cultivated at 33°C (permissive conditions). To induce differentiation, podocytes were cultured on type I collagen (Biochrom, Berlin, Germany) at 37°C without γ-interferon (nonpermissive conditions). In this study, podocytes of clone 1 (5) between passages 10 and 20 were used in all experiments.

Virus Infection and Immunocytochemical Analysis

Differentiated podocytes, which had been grown under nonpermissive conditions on coverslips for at least 10 d, were infected with 40 plaque-forming units/cell of VSV ts045 virus (kindly provided by Dr. Kai Simons, European Molecular Biology Laboratory, Heidelberg, Germany) at 32°C in RPMI 1640 containing 0.2% (wt/vol) bovine serum albumin, 10 mM Hepes, and 1% penicillin/streptomycin. Cells were then incubated for 2 h at 39.5°C (8) and for 1.5 h at 19.5°C, in the presence of cycloheximide to synchronize transport of newly synthesized proteins from the Golgi apparatus (9). The coverslips were then transferred to 32°C, and cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at different time points (0, 15, 30, 45, and 60 min).

For surface staining, cells were immunostained without permeabilization. In brief, free aldehyde groups were quenched with 50 mM NH_4Cl in PBS for 10 min. Nonspecific binding sites were blocked for at least 30 min with 2% fetal calf serum, 2% bovine serum albumin, and 0.2% fish gelatin in PBS (blocking solution). Primary and secondary antibodies were diluted in blocking solution and applied to the cells for a 1-h incubation at room temperature. For intracellular staining, cells were permeabilized with 0.3% Triton X-100 before immunostaining. After antibody application, cells were washed with PBS and mounted for observation. Photographs were taken with an Axiophot microscope (Carl Zeiss, Oberkochen, Germany) coupled to a Color Coolview, eight-bit, charge-coupled device, color camera (Photonic Science, Millham, United Kingdom). Primary antibodies included monoclonal antibody (mAb) 17.2.21.4 against VSV-G (dilution, 1:50) and polyclonal rabbit antiserum against VSV-G (1:400) (both kindly provided by Dr. Kai Simons), rabbit polyclonal anti-rab8 P-16 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-rsec8 mAb 2E9 (1:20; Biomol, Hamburg, Germany), anti-rsec6 mAb 9H5 (1:500; kindly provided by Dr. Richard Scheller, Stanford University, Stanford, CA), and mAb against giantin (1:500; kindly provided by Dr. Kai Simons). As secondary antibodies, FITC- or tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit antibodies and FITC- or tetramethylrhodamine isothiocyanate-conjugated rat anti-mouse antibodies (Dianova, Hamburg, Germany) were used.

Brefeldin A Treatment

To disrupt post-Golgi vesicular transport, brefeldin A (BFA; Sigma) was added (at 1 mg/ml) to the medium of differentiated cells (which had been maintained for at least 10 d under nonpermissive conditions) directly after subcultivation. The recovery of the BFA-treated and untreated cells from the "trypsinization stress" was observed 12 h later, using phase-contrast microscopy. The reversibility of the BFA treatment was examined by washing out the BFA.

Results

VSV-G Is Preferentially Routed into Podocyte Processes

Enveloped viruses have been widely used to study the biosynthesis and transport of proteins to the cell surface. After infection by these viruses, host protein synthesis is shut off and large amounts of viral surface glycoproteins are synthesized. This amplification is the key to the use of viral glycoproteins as probes of membrane traffic from the endoplasmic reticulum (ER) to the cell surface (10). In this study, we used the well-characterized VSV, expressing a temperature-sensitive mutant of VSV-G (ts045), to monitor the delivery of newly synthesized VSV-G to the cell surface of differentiated podocytes. This virus was very useful because it not only had a weak cytopathic effect on podocytes, but also allowed the analysis of synchronized transport to the cell surface. It was possible to accumulate VSV-G in the ER at the nonpermissive temperature of 39.5°C. The proteins accumulated in the Golgi apparatus during incubation at 19.5°C (9), and synchronized transport could then be initiated by increasing the temperature to 32°C. The appearance of VSV-G at the cell surface was detected by labeling intact cells with the polyclonal antibody. Intracellular VSV-G was detected in the same cells, using a mAb, after permeabilization with Triton X-100. As early as 20 min after release of the Golgi block, newly synthesized VSV-G was found to accumulate in the processes of the podocytes (Figure 1, A through C, and Figure 2F). Intracellular staining showed the transport route of VSV-G from the Golgi apparatus into the processes (Figure 1, A through C, and Figure 2F). Surface staining showed that fusion of membrane vesicles carrying VSV-G preferentially occurred with the plasma membrane of the processes (Figure 1D). Incubation at 32°C for more than 45 min resulted in staining of the entire cell surface for VSV-G (Figure 3), indicating the absence of a diffusion barrier in the plasma membrane, such as is present in epithelial and neuronal cells (10,11). Therefore, only cells that had been fixed within 20 min after the initiation of post-Golgi transport were used in additional experiments to observe the intracellular transport route for VSV-G in podocytes. For semiquantitative analysis of these cells, infected cells with at least one process longer than the cell body were counted. Approximately 75% of these cells showed increased staining of VSV-G in their processes (data not shown). Accumulation of VSV-G was clearly observed in processes, compared with the rest of the cell, where immunoreactivity was present either in transport vesi-

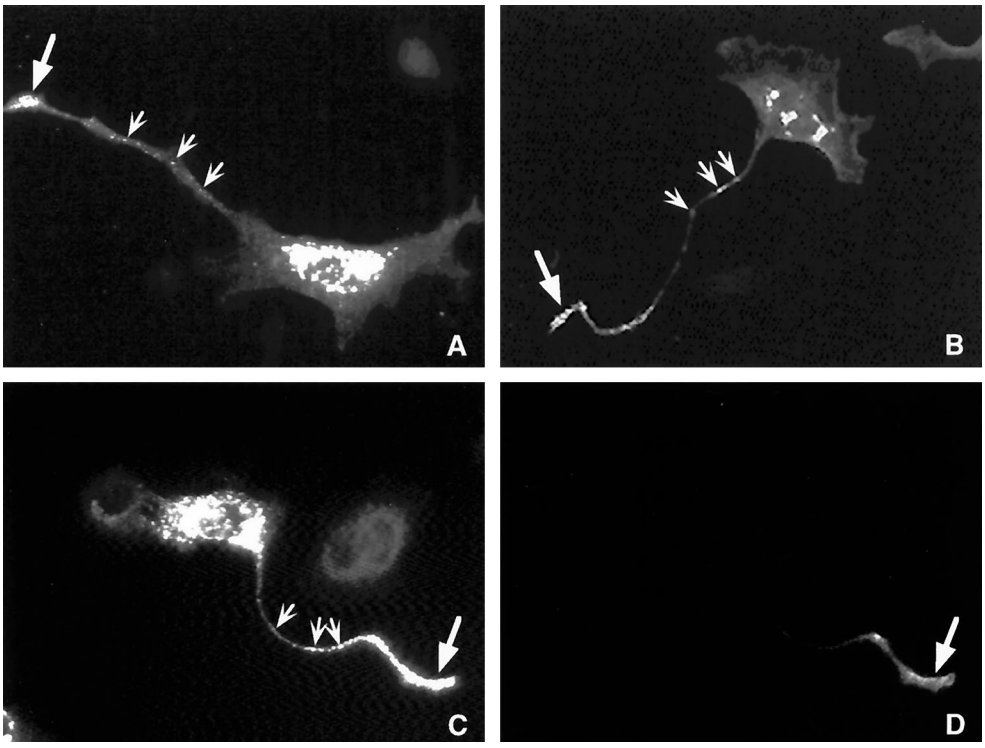

Figure 1. Delivery of newly synthesized vesicular stomatitis virus G (VSV-G) protein from the Golgi apparatus to the cell surface of cultured podocytes. VSV-infected cells were fixed and processed for immunocytochemical analysis 20 min after the initiation of post-Golgi transport. (A through C) After release of the Golgi block, VSV-G is transported into the processes of podocytes. Small arrows, intracellular transport of VSV-G in the form of vesicles; large arrows, accumulation of VSV-G in the tips of the processes. (D) Surface staining for VSV-G (same cell as in C) shows that the tip of the process is the site where newly synthesized VSV-G appears on the surface (arrow).

cles or at the cell membrane. Some cells accumulated VSV-G exclusively in one process (Figure 1, A through C, and Figure 2F), whereas others showed a concentration of VSV-G in several processes.

Rab8 and the Mammalian Sec6/Sec8 Complex Are Localized to the Intracellular Transport Pathway Taken by VSV-G

These results led us to conclude that newly synthesized VSV-G follows a specific pathway to move from the Golgi apparatus to the processes in differentiated podocytes. We were interested in examining this pathway in more detail and in identifying other molecules that might be involved in the regulation of this pathway. In fibroblasts, the activated form of the small GTPase rab8 (*i.e.*, the rab8Q67L mutant) is responsible for the formation of cell protrusions, into which newly synthesized VSV-G is preferentially delivered (12). On the basis of these findings, rab8 seemed to be a good candidate for regulating the transport of VSV-G into the processes of podocytes. To test this hypothesis, immunocytochemical analysis of differentiated podocytes was performed with a polyclonal antibody against rab8. Using this approach, rab8 was shown to be localized in the tips of the processes, whereas the cell body showed only weak staining (Figure 2, A and B). The proteins rsec6 and rsec8 are mammalian homologues of protein subunits of the exocyst complex, which in yeast is known to function downstream of the rab8 homologue Sec4p (13). Immunolabeling of podocytes with mAb against rsec6 and rsec8 showed almost the same distribution pattern as that observed for rab8. Both rsec6 and rsec8 were found around the nucleus and in processes. Like VSV-G, expression of rsec6/rsec8 was seen in either several processes (Figure 2C) or only one process (Figure 2, D and F) in each cell. In double-labeling experiments with VSV-G and rsec8, it was determined that the two proteins were coexpressed in processes, as shown by overlapping of immunoreactivity (Figure 2, E and F).

An Intact Golgi Apparatus Is Required for Process Formation by Podocytes

BFA is an antibiotic that is widely used to study membrane traffic. BFA treatment results in the collapse of the Golgi complex into the ER and the blocking of protein transport from the Golgi complex to the plasma membrane (14,15). To demonstrate that an intact Golgi apparatus is necessary for process formation in podocytes, the effect of BFA was examined. Differentiated podocytes were treated with BFA (1 mg/ml) for 12 h after trypsinization. After the rounding up that occurred during the trypsinization stress, control cells that had not been treated with BFA were able to regain normal morphologic features, forming long processes, within 12 h (Figure 4, B and D). In contrast, BFA-treated cells did not re-extend processes; they retained a round cell shape (Figure 4, A and C). Immunofluorescence staining for the Golgi marker protein giantin showed an intact Golgi apparatus in the control cells (Figure

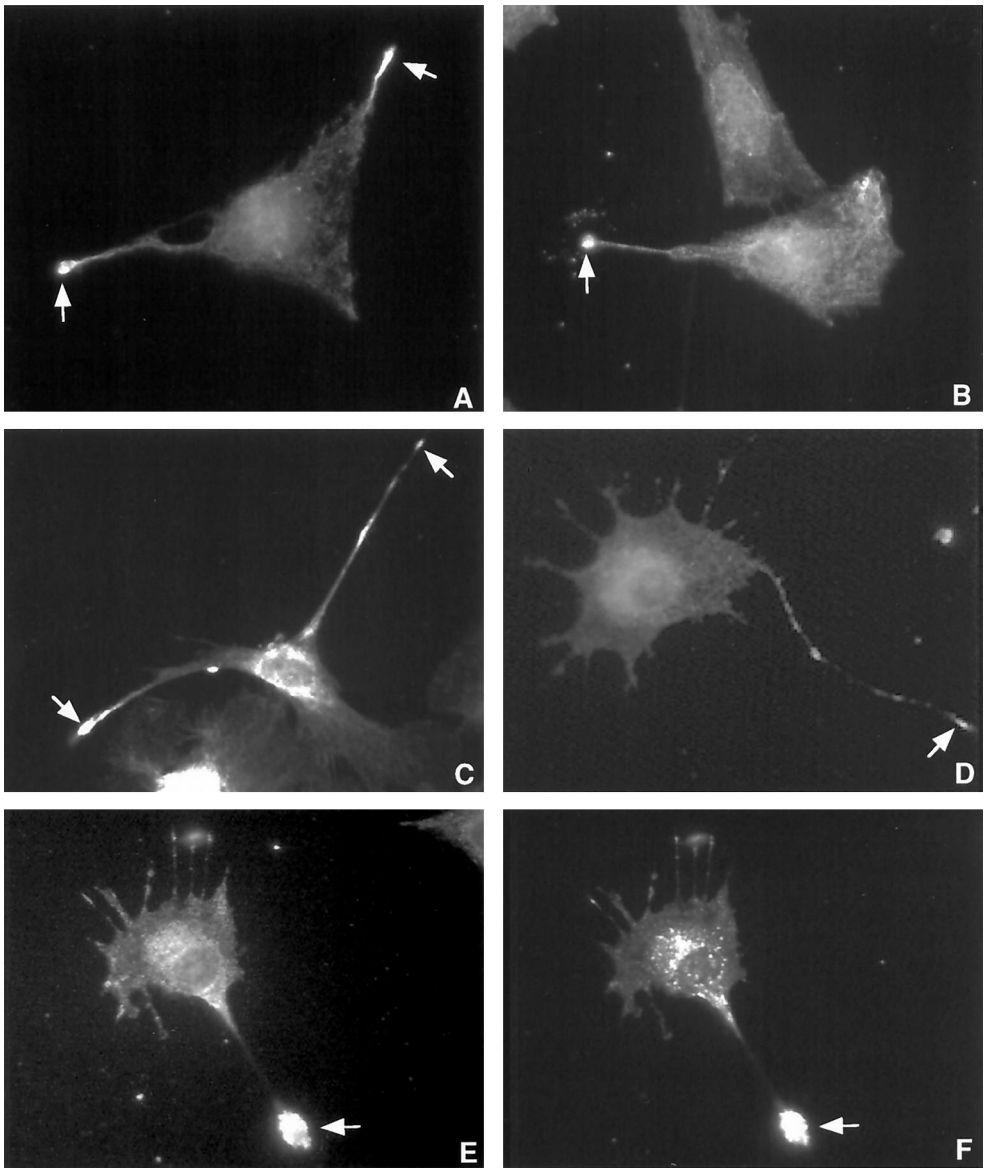

Figure 2. Presence of rab8 and the sec6/sec8 complex in podocyte processes. (A and B) Expression of rab8 at the tips of podocyte processes. Arrows, localization of rab8 in the tips of the podocyte processes. Note that in B only one long process expresses rab8. (C and D) Concentration of two proteins of the exocyst complex, *i.e.*, rsec6 (C) and rsec8 (D), in the processes (arrows), similar to rab8 (B). (E and F) Double-labeling immunofluorescence, showing the colocalization of rsec8 (E) and VSV-G (F) in VSV-infected cells. The overlap of immunoreactivity is strongest at the tip of the process (arrows).

4D) and dispersal of the Golgi apparatus in the BFA-treated cells (Figure 4C). Furthermore, most of the BFA-treated cells failed to transport newly synthesized VSV-G to the cell surface (Figure 5). Washing away of the BFA led to recovery of the cells, with the formation of processes, indicating that the cells were still viable and that the effect of BFA on the cells was reversible (data not shown).

Discussion

This study was performed to analyze the link between biosynthetic membrane traffic and process outgrowth in podocytes. This was achieved by demonstrating that newly synthesized VSV-G is preferentially transported into cell processes, where different regulator molecules of the biosynthetic pathway, such as rab8 and subunits of the sec6/sec8 complex, were detected. Furthermore, application of BFA, a drug that disrupts post-Golgi traffic, induced reversible inhibition of process formation in cultured podocytes. On the basis of these findings, it can be hypothesized that constant transport of vesicles loaded with biosynthetic material to specific docking sites on the plasma membrane is essential for the formation and maintenance of podocyte processes.

How cell morphogenesis occurs and how a specific cell architecture, which allows a cell to perform its specific function, is established are issues that have been difficult to study, in part because of the lack of proper *in vitro* models. These

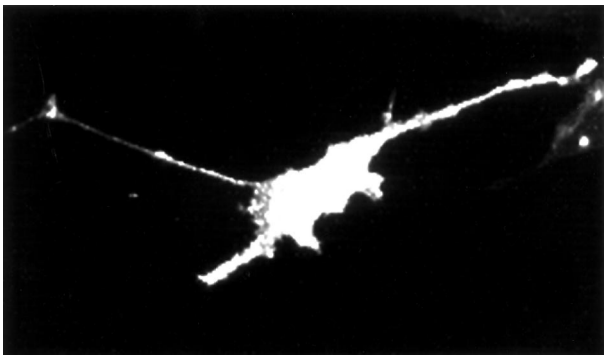

Figure 3. Evidence for the lack of a diffusion barrier in the podocyte cell membrane. Surface staining for VSV-G protein was performed 45 min after release of the Golgi block. At that time, immunolabeling is no longer restricted to processes but is found on the entire surface of the cell membrane, indicating the absence of a diffusion barrier.

events include complex interactions involving cell-cell signaling, signal transduction, gene expression, membrane trafficking, and dynamic cytoskeletal networks. To date, most information regarding cell morphogenesis, including the formation of processes, has been obtained from studies of neurons, with their characteristic polarity of axons and dendrites. In cultured rat hippocampal neurons, the free end of a growing axon (the growth cone) represents a site where preferential addition of newly synthesized membrane proteins occurs (16). Treatment of differentiating neurons with BFA reduces the insertion of proteins into the membrane of the growth cone (16) and inhibits axonal outgrowth (17).

The first molecular link between membrane traffic and process outgrowth was recently provided by Peränen *et al.* (12). In their study, the active mutant of rab8, which is not able to hydrolyze GTP, was shown to be responsible for the extension of long processes in stably transformed fibroblasts. The formation of these processes was accompanied by the reorgani-

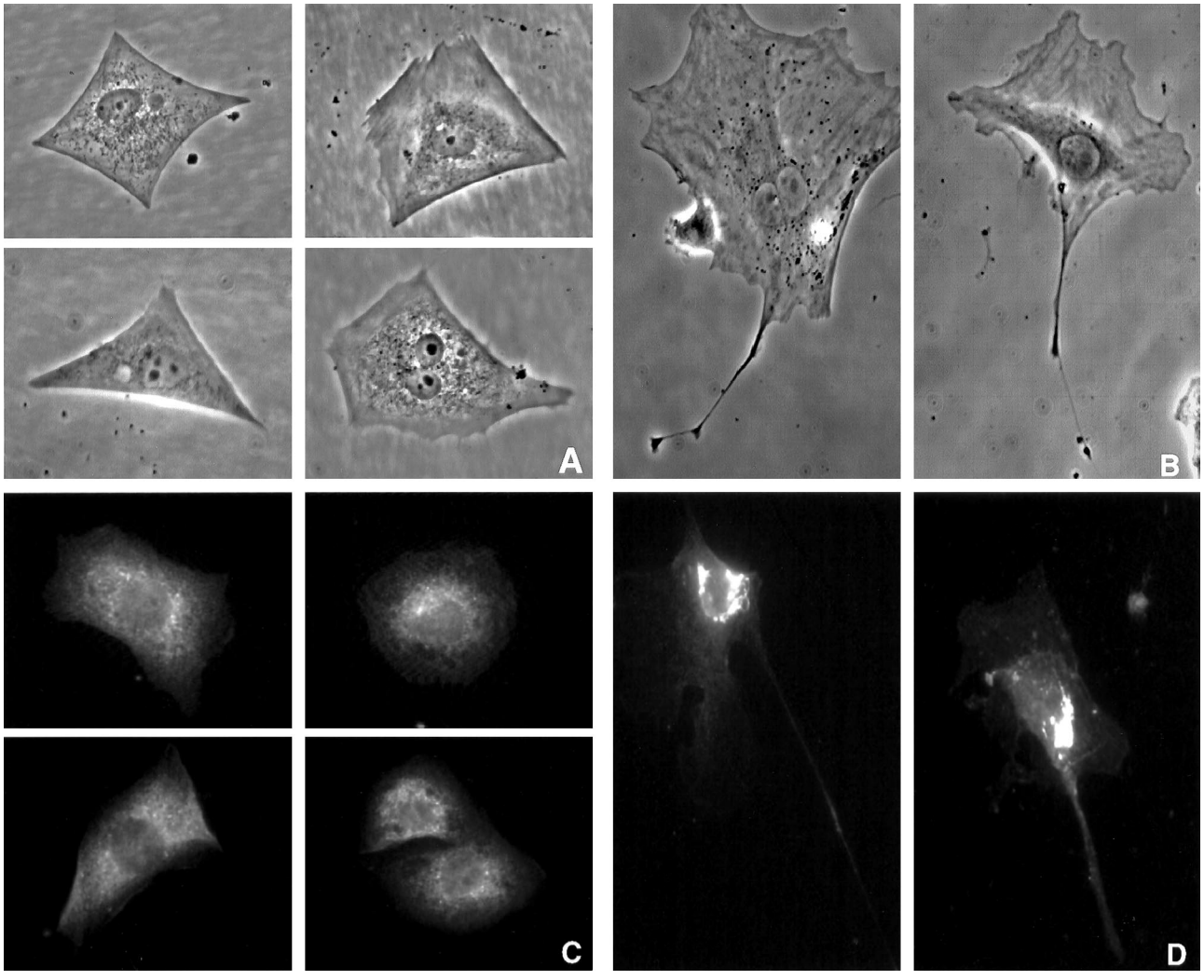

Figure 4. Brefeldin A (BFA) blockade of process formation in cultured podocytes. (A and B) BFA treatment abolished the ability of cultured podocytes to form cell processes (A), compared with untreated control cells with normal arborized morphologic characteristics (B). (C and D) Immunofluorescence staining for the Golgi marker giantin shows dispersal of the Golgi apparatus in BFA-treated cells (C); in untreated control cells, normal distribution of giantin in the intact Golgi apparatus is observed (D).

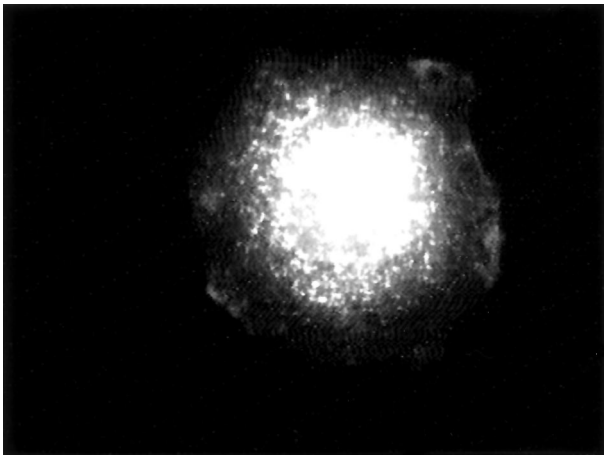

Figure 5. BFA inhibition of surface transport of VSV-G. Immunostaining for VSV-G in BFA-treated cells was performed 45 min after release of the Golgi block. The Golgi apparatus appears to be dispersed, and VSV-G is not transported to the cell surface. Note that the cells have lost their processes. After removal of BFA, the cells recover their normal process architecture and VSV-G is sorted to the processes (data not shown).

zation of the actin/microtubular cytoskeleton, and the processes seemed to be targets for transport vesicles carrying newly synthesized VSV-G (12). The protein rab8 is a ubiquitously expressed small GTPase, which may be involved in membrane transport from the trans-Golgi network to dendrites in hippocampal neurons (18) and to the basolateral surface of Madin-Darby canine kidney (MDCK) cells (19). Antisense treatment against rab8 led to impaired neurite outgrowth (20). The precise role of rab8 (or rab proteins in general) (21) in vesicular delivery is still controversial, although it was proposed that rab molecules might confer specificity to each vesicular trafficking event by regulating SNARE complex assembly (22,23) and/or disassembly after the fusion step (24).

In yeast, the rab8 homologue Sec4p is known to function upstream of a 19.5S particle (13) that represents a complex of proteins, seven of which have been identified (25). This complex was termed the exocyst complex because it was found at the tips of forming yeast buds, where Sec4p-containing exocytosis vesicles dock and fuse (26,27). Some of the mammalian homologues were recently identified; of these, rsec6 and rsec8 were further characterized. These proteins are recruited from the cytosol to specific sites on the plasma membrane, *e.g.*, to the free endings of growing neurites (28) and to cadherin-based cell-cell contact sites on the basolateral domain of MDCK cells (29). The application of antibodies to rsec8 can block transport to the basolateral surface, indicating that these sites may serve as major docking sites for basolateral vesicles (29). The molecular composition of these docking sites and the functional role of the constituting proteins remain to be elucidated. On the basis of studies performed in yeast, however, involvement of the rsec6/rsec8 complex in mammalian cell exocytosis is likely.

In this study, the same molecules were found in processes of cultured podocytes, suggesting that podocyte processes also contain these docking sites. Interestingly, strong immunoreactivity for rab8, sec6, sec8, and VSV-G (Figure 2, B, C, E, and F) was found at the tips of those processes that exhibited growth cone-like morphologic features. It is tempting to speculate that the processes with growth cone-like morphologic features are in a growing state. In fact, phase-contrast videomicroscopy of cultured podocytes revealed great motility of these cells and very dynamic podocyte processes (M. Simons and P. Mundel, unpublished observations). Within 1 h, cells may form and/or retract processes with the length of the cell body. Whether these movements reflect some type of migration potential of the podocytes in the culture dish, leading to the formation of contact sites or junctions with other podocytes, is an interesting question. Similar dynamic behavior of the podocytes may characterize these cells *in vivo*. For example, in the differentiation events leading to the conversion of the epithelial cells of the S-shaped body stage to the process-bearing podocytes of the capillary loop stage, dynamic outgrowth of cell processes is expected.

On the basis of our studies with cultured podocytes, we propose that any event leading to the formation or modulation of podocyte processes, under physiologic or pathologic conditions, requires a mechanism by which biosynthetic material is preferentially transported into the growing processes. We previously showed that microtubules are necessary for process formation and that depolymerization of microtubules leads to inhibition of process formation (N. Kobayashi, J. Reiser, W. Kriz, and P. Mundel, submitted for publication). In this study, we provide evidence that process formation involves the directed transport of vesicles from the Golgi apparatus to specific docking sites in the processes. A specific post-Golgi pathway carrying newly synthesized lipids and proteins, such as integrins, along microtubules into the processes would thus account for the elongation of the processes and finally the fixation of the processes to the substratum. Other cell types, such as MDCK cells, neurons, and fibroblasts, make use of polarized membrane trafficking to generate cell morphologic features (12,30). In the future, it will be interesting to examine whether, in podocytes as well, different post-Golgi pathways, with corresponding sorting machineries in the Golgi apparatus (30), are necessary to create, maintain, or remodel the complex cell architecture. Additional studies will be required to determine whether these or additional transport mechanisms operate in podocytes *in vivo*.

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