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Uukuniemi Virus Maturation: Accumulation of Virus Particles and Viral Antigens in the Golgi Complex

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We studied the maturation of Uukuniemi virus and the localization of the viral surface glycoproteins and nucleocapsid protein in infected cells by electron microscopy, indirect immunofluorescence, and immunoelectron microscopy with specific antisera prepared in rabbits against the two glycoproteins G1 and G2 and the nucleocapsid protein N. Electron microscopy of thin sections from infected cells showed virus particles maturing at smooth-surfaced membranes close to the nucleus. Localization of the G1/G2 and N proteins by indirect immunofluorescence at different stages after infection showed the antigens to be present throughout the cell interior but concentrated in the juxtanuclear region. The G1/G2 antiserum also appeared to stain the nuclear and plasma membranes. Double staining with tetramethylrhodamine isothiocyanate-conjugated wheat germ agglutinin, which preferentially stains the Golgi complex, and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G, which stained the G1/G2 or N proteins, showed that the staining of the juxtanuclear region coincided. Similarly, double staining for thiamine pyrophosphatase, an enzyme activity specific for the Golgi complex, showed the fluorescence and the cytochemical stain to coincide in the juxtanuclear region. Immunoperoxidase electron microscopy of cells permeabilized with saponin revealed that the viral glycoproteins were present in the rough endoplasmic reticulum and the nuclear and Golgi membranes; the latter was heavily stained. With this method, the N protein was localized to the cytoplasm, especially around smooth-surfaced vesicles in the Golgi region. Taken together, the results indicate that Uukuniemi virus and its structural proteins accumulate in the Golgi complex, supporting the idea that this compartment rather than the plasma membrane is the site of virus maturation. This raises the interesting possibility that deficient transport of the glycoproteins to the plasma membrane and hence their accumulation in the Golgi complex determines the site of virus maturation.

A common feature of the viruses belonging to the large Bunyaviridae family (3) is that they appear to mature intracellularly by a budding process at the smooth-surfaced vesicles in the Golgi region (2, 18, 21, 37). If this finding is indeed true, the maturation of the bunyaviruses differs from that of other enveloped viruses, which receive their envelopes either by budding at the plasma membrane (e.g., alpha-, myxo-, paramyxo-, and rhabdoviruses [4, 12, 13]), nuclear membrane (e.g., herpesviruses [5]), or the rough endoplasmic reticulum (e.g., coronaviruses [20]). The factors determining the site of maturation of enveloped viruses have remained largely unknown.

The evidence that the bunyaviruses mature at the membranes in the Golgi complex has so far been based exclusively on electron microscopy studies (2, 18, 21, 36, 37). Because of limitations in interpreting electron microscopic results, we used immunological techniques to be able to localize viral antigens within the infected cell at different stages of infection. The antigens were localized by indirect immunofluorescence and immunoelectron microscopy with highly specific antisera directed against the structural proteins of one particular bunyavirus, Uukuniemi virus, the prototype of the *Uukuvirus* genus of Bunyaviridae (3). This virus consists of a lipoprotein membrane containing two envelope glycoproteins, G1 and G2 (M_r, about 70,000 and 65,000, respectively [27, 36]), and a nucleocapsid, containing three single-stranded RNA segments (L, M, and S [25, 28]), to which multiple copies of the N protein (M_r, 25,000) and a few copies of the L protein (M_r, 200,000) are associated (27). The two glycoproteins, which are encoded by the M RNA segment, are synthesized as a precursor (p110), which is cleaved cotranslationally roughly in the middle to yield G1 and G2

(34). The glycan structure of G1 and G2 from virions has recently been determined (23). The N protein, together with a nonstructural protein, NS (M_r, 30,000), is encoded by the S RNA segment (34). We prepared specific antisera against G1/G2 and N and used them to localize the viral antigens in infected cells. The results strongly support the idea that the site of Uukuniemi virus maturation is at the smooth membranes of the Golgi region.

MATERIALS AND METHODS

Cells and virus. All experiments were carried out on secondary cultures of chicken embryo cells grown as monolayers either in plastic dishes (20 cm^2) or on cover slips. The origin and cultivation of the cells and the preparation of stock virus from the prototype strain S23 of Uukuniemi virus after several successive plaque purifications have been described previously (26). The titer of the stock virus was 10^8 PFU/ml. A multiplicity of infection of about 10 PFU/cell was used in all experiments.

Purification of viral proteins. To prepare specific antisera against the glycoproteins G1/G2 and the nucleocapsid protein N, we purified the proteins as follows. Uukuniemi virus (4 mg), purified as described previously (26), was mixed with [^{35}S]methionine-labeled virus. The virus was disrupted with 2% (vol/vol) Triton X-100 at 20°C for 10 min, and the nucleocapsid and envelope fractions were separated on CsCl gradients as described previously (25, 27). Centrifugation was for 20 h at 45,000 rpm and 4°C in a Spinco SW50.1 rotor. Fractions (0.3 ml) were collected from the bottom, and samples from each fraction were assayed for radioactivity. The ribonucleoproteins banding at a density of 1.31 g/cm^3 (27) were dialyzed against TN buffer (0.1 M NaCl–0.05 M Tris-hydrochloride, pH 7.4) at 4°C and used for immunization. This fraction was free of any detectable glycoproteins as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The top fractions of the CsCl gradient containing the envelope glycoproteins were pooled and dialyzed against TN buffer. At this stage, the glycoproteins were still contaminated with the N protein. The glycoproteins were therefore purified further as described by Helenius and von Bonsdorff (9). To 2.7 mg of viral glycoproteins in 1.4 ml of the pooled top fractions, 50 mg of Triton X-100 was added. The samples were split into two equal fractions and layered on two identical sucrose gradients consisting, from the bottom, of: (i) 0.5 ml of 60% sucrose; (ii) a 20 to 50% sucrose gradient; and (iii) 0.3 ml of 15% sucrose containing 1% Triton X-100. The sucrose was made in TN buffer. The glycoproteins were then complexed into rosettes by centrifugation for 25 h at 39,000 rpm and 20°C in a Spinco SW41 rotor. Fractions (0.6 ml) were collected from the bottom, and samples of each fraction were assayed for radioactivity. The peak fractions containing the glycoprotein complexes were pooled, dialyzed against 1:10-diluted TN buffer, and lyophilized to dryness. The proteins were finally suspended in distilled water in 1/10 the original volume. This material, which by SDS-PAGE was shown to contain a roughly equimolar ratio of G1 and G2 and no traces of the N protein or any other contaminating proteins, was used for immunization.

Preparation of antisera. Antisera against purified nucleocapsid and G1/G2 complexes were prepared in rabbits by injecting 100 μg of protein in Freund complete adjuvant. Two booster injections of 100 μg each of G1/G2 and three injections of 80, 50, and 50 μg, respectively, of nucleocapsid in Freund incomplete adjuvant were given subcutaneously at 2-week intervals. Rabbits were bled before immunization (control serum) and 2 weeks after the last injection. The specificity of the antisera was checked by immunoblotting (33) as described below.

Immunofluorescence. For immunofluorescence microscopy, cells grown as nonconfluent monolayers on cover slips in 35-mm dishes were infected with Uukuniemi virus or mock infected as described previously (25). At different times postinfection (p.i.) the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS) and fixed with 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 25°C for 15 min. These cells were used to stain for surface immunofluorescence. For intracellular staining, fixed cells were permeabilized with 0.05% Triton X-100 in PBS at 25°C for 30 min (15). For indirect immunofluorescence, cells were incubated for 30 min at 37°C with antiserum diluted 1:400, washed three times with PBS, and incubated again for 30 min at 37°C with sheep anti-rabbit immunoglobulin G (IgG) (diluted 1:30) conjugated with fluorescein isothiocyanate (FITC; Wellcome, Beckenham, U.K.). The cells were finally washed three times with PBS. In double-labeling experiments, the cells, treated as described above, were also labeled with wheat germ agglutinin (WGA) conjugated with tetramethylrhodamine isothiocyanate (TRITC; Vector Laboratories, Burlingame, Calif.) at a lectin concentration of 100 μg/ml in PBS. WGA has been shown to stain predominantly the Golgi apparatus in intracellular immunofluorescence (35). The fluorescence was examined with a Leitz Dialux 20 microscope fitted with a 100× oil immersion objective and filters for FITC and TRITC fluorescence. Photomicrographs were taken on Agfapan 400 film.

Electron microscopy. (i) Negative staining. Viral glycoprotein spike protein complexes (rosettes) were visualized from dialyzed sucrose gradient fractions by applying a drop of the sample on a Formvar-coated grid and then staining it with 2% neutral potassium phosphotungstate.

(ii) Thin sections. Thin sections of infected cells concentrated from dishes by centrifugation were processed as described previously (36). The specimens were examined in a Siemens Elmiskop 1 A, and photographs were taken at original magnifications of 20,000 or 40,000 at 80 kV.

Immunoperoxidase electron microscopy. Virus-infected cell cultures on plastic dishes were briefly rinsed with PBS at 8 and 16 h p.i. and fixed with a mixture of 4% paraformaldehyde, 0.01 to 0.025% glutaraldehyde, and 0.05% saponin for 60 min at 4°C, and then rinsed with 0.1 M lysine-hydrochloride, 0.02% bovine serum albumin, and 0.05% saponin in Dulbecco PBS. The samples were then treated with antisera (diluted 1:100 in PBS-albumin-saponin) against the virus envelope glycoproteins or the nucleocapsid protein for 60 min, rinsed, treated with peroxidase-conjugated staphylococcal protein A, and proc-

essed further for immunoelectron microscopy as previously described (8). The glutaraldehyde concentration used in the prefixative was selected in preliminary light microscopic experiments to give staining patterns comparable to those of conventional immunofluorescence staining and also to give acceptable ultrastructural preservation (not shown).

Cytochemical staining for TPPase activity. The thiamine pyrophosphatase (TPPase) activity was used in double-staining experiments to demonstrate that the strong virus-specific immunofluorescence in the juxtanuclear region was localized to the Golgi apparatus. Uukuniemi virus-infected cells were fixed with 3% paraformaldehyde at 8 h p.i., incubated in TPPase medium (2 mM thiamine pyrophosphate, 80 mM Tris-maleate [pH 7.2], 3.6 mM lead nitrate, 5 mM manganese chloride [22]) for 20 min at 25°C, and then treated with mild ammonium sulfide solution for 3 min. After being stained for TPPase activity, the cells were counterstained by indirect immunofluorescence with anti-G1/G2 or anti-N serum as described above.

SDS-PAGE and electrophoretic transfer of proteins to nitrocellulose sheets. To check for cross-reactivity between the anti-G1/G2 and anti-N sera, we used the immunoblotting procedure (Western blotting) described previously (33). The viral proteins from purified [^{35}S]methionine-labeled virions were separated on SDS-polyacrylamide gels by the method of Laemmli (14), with a 10% separating and a 5% stacking gel. Viral protein (0.5 μg) was used in each lane. The proteins were then electrophoretically transferred to nitrocellulose sheets (0.45 μm; Schleicher & Schüll BA-85). The sheets were saturated with bovine serum albumin and then stained by the indirect immunoperoxidase method. Individual strips were incubated with 1:400-diluted rabbit antiserum. Anti-rabbit IgG peroxidase conjugate (peroxidase-conjugated swine anti-rabbit; DAKO, Copenhagen, Denmark) was used at a dilution of 1:200 in Tris-buffered saline. The dried filter strips were photographed and then autoradiographed to localize the viral proteins.

Other methods. Protein concentration was determined by the method of Lowry et al. (17). Radioactivity was determined in a xylene–Triton X-100 scintillation cocktail with a Wallac 81 000 scintillation counter.

RESULTS

Preparation of antisera against the envelope glycoproteins and the nucleocapsid. To be able to study the localization of the G1/G2 and N proteins independently, we raised antibodies specific for these proteins in rabbits. A glycoprotein-free N protein preparation was obtained by disrupting purified virions with Triton X-100 and separating the three ribonucleoproteins from the envelope fraction on a CsCl gradient (Fig. 1A). Since the ribonucleoproteins banding at a density of 1.31 g/cm^3 were free of any detectable glycoproteins (data not shown), this material was used for immunization. The top fractions (nos. 13–15, Fig. 1A) still contained some N protein, and further purification was thus needed. A convenient method for obtaining membrane glycoproteins in pure form has been described by Helenius and von Bonsdorff (9); this procedure was applied here. The envelope fractions, treated with Triton X-100, were layered on a sucrose gradient containing at the top a cushion of 15% sucrose and 1% Triton X-100. When the spike proteins are centrifuged through the cushion into the detergent-free gradient, the membrane glycoproteins will form soluble complexes due to their hydrophobic terminal segments. These rosettes are recovered as a homogeneous peak sedimenting faster than the monomeric spikes. A sedimentation profile similar to the one obtained for Semliki Forest virus (SFV) glycoproteins (9) was also obtained for Uukuniemi virus glycoproteins (Fig. 1B). SDS-PAGE analysis of the fast-sedimenting peak revealed the presence of only G1 and G2 in about equimolar ratio. No N or other proteins were found in this peak (data not shown). Electron micrographs taken from the dialyzed peak fractions revealed star-shaped particles roughly 20 nm in diameter. This material was used to prepare anti-G1/G2 serum.

Immunoblotting was used to exclude cross-reactivity between the two sera. Total viral proteins labeled with [^{35}S]methionine were fractionated by SDS-PAGE, and the proteins were

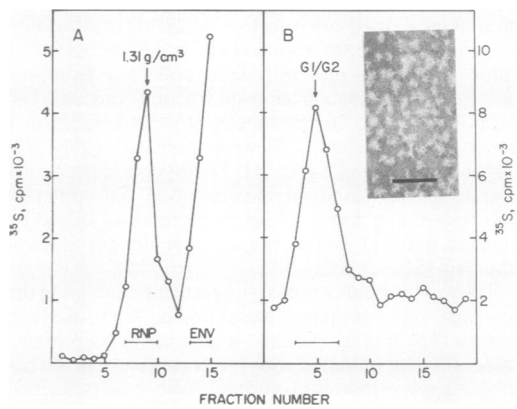

FIG. 1. Purification of viral proteins. (A) Purified Uukuniemi virus, containing [^{35}S]methionine-labeled virus as a marker, was disrupted with 2% (vol/vol) Triton X-100 at 20°C for 10 min and centrifuged in a CsCl gradient (initial density, 1.31 g/cm^3) for 20 h at 45,000 rpm and 4°C in a Spinco 50.1 rotor. Samples (0.3 ml) were collected from the bottom. The ribonucleoproteins (RNP) banded at a density of 1.31 g/cm^3. The top of the gradient contained the envelope glycoproteins (ENV). (B) Pure glycoproteins were obtained by complexing them into rosettes in a sucrose gradient consisting, from the bottom, of 0.5 ml of 60% sucrose, a 20 to 50% sucrose gradient, 0.3 ml of 15% sucrose containing 1% Triton X-100, and 0.7 ml of the sample. Fractions (0.6 ml) were collected from the bottom. The inset shows negatively stained spike glycoprotein complexes obtained from the peak fraction. Bar, 100 nm.

electrophoretically transferred to nitrocellulose filters. The specificity of the anti-N serum as revealed by the indirect immunoperoxidase staining method is shown in Fig. 2, lane a. No staining was apparent at the position of the glycoproteins, whereas the N protein was heavily stained. An autoradiogram of the same filter strip confirmed that all proteins had been equally well transferred (Fig. 2, lane b). Similarly, the anti-G1/G2 serum reacted only with G1 and G2 (lane c). Since G1 and G2 were not separated under reducing conditions (lanes a–d), electrophoresis was also carried out under nonreducing conditions (lanes e and f). Immunoblotting showed that anti-G1/G2 reacted equally well with both glycoproteins. The slowly migrating band (lanes c and e) represent dimeric forms of the glycoproteins (R. F. Pettersson, unpublished data). Thus, we conclude that there is no cross-reactivity between the anti-G1/G2 and anti-N sera.

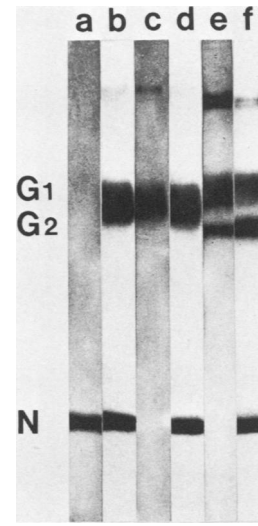

FIG. 2. Specificity of anti-G1/G2 and anti-N sera as analyzed by immunoblotting. The structural proteins of Uukuniemi virus were separated in a 10% SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose sheet. The virus was a mixture of unlabeled and [^{35}S]methionine-labeled virus. Viral protein (0.5 μg) was applied to each slot. The proteins were separated under reducing conditions (lanes a–d) and under nonreducing conditions (lanes e and f). The immunoblotting was carried out as described in the text. Lanes: (a) immunoblotting with anti-N serum; (b) autoradiography of the strip in lane a; (c) immunoblotting with anti-G1/G2 serum; (d) autoradiography of the strip in lane c; (e) immunoblotting with anti-G1/G2 serum; (f) autoradiography of the strip in lane e.

Thin sections of infected cells. Results previously obtained in our laboratory indicated that Uukuniemi virus particles appear to be formed intracellularly by budding at smooth-surfaced vesicles in a juxtanuclear region which shows the morphology of the Golgi complex (36, 37). Electron microscopic images of such cellular regions at 9 h p.i. are shown in Fig. 3A and B. Numerous particles were seen inside large smooth-surfaced vesicles near the nucleus. Virus particles in the process of budding are indicated by arrows. A series of particles at different stages of maturation are shown in Fig. 3C–F. As noted previously (36, 37), no indication of electron-dense nucleocapsids was seen in the cytoplasm. Also, no budding profiles were seen at the plasma membrane, although extracellular virus particles could be seen in abundance close to the plasma membrane (not shown).

Intracellular localization of viral proteins. To study the distribution of the structural proteins of Uukuniemi virus, we stained infected cells by indirect immunofluorescence at various stages of infection with either anti-G1/G2 or anti-N serum and FITC-conjugated anti-rabbit IgG. The growth curve of Uukuniemi virus is rather slow. The first extracellular virus particles were detected at 5 to 6 h p.i., and the production of extracellular virus reached a plateau at about 16 to 18 h p.i. No apparent cytopathic effect is detectable until about 40 h p.i., and the host cell macromolecule synthesis is not shut off (24). The N protein is first seen in cell extracts from pulse-labeled cells at about 6 h p.i., whereas the G1 and G2 glycoproteins are detectable somewhat later (34).

Cells were stained for G1/G2 at 5 h (early), 10 h (midcycle), and 15 h (late) p.i. (Fig. 4a–c). In all cases a perinuclear reticular staining typical for the rough endoplasmic reticulum was seen. The intensity of this fluorescence increased during the infection. The nuclear periphery was also clearly stained. At all stages, intense fluorescence was apparent in a region close to the nucleus. This juxtanuclear region enlarged as the infection progressed, and the fluorescence became more intense. In many cases, the fluorescence appeared to be localized to vesicular structures. Occasionally, heavily stained vesicles were also seen at the cell periphery.

Staining the cells for the N protein revealed a different type of fluorescence. Instead of a reticular pattern, there was a granular fluorescence diffusely scattered throughout the cytoplasm, and the nuclear periphery was not stained (Fig. 4d–f). With anti-N serum, the juxtanuclear region was also intensively stained, particularly at midcycle to late in the infection. The juxtanuclear staining was similar in appearance to that observed with anti-G1/G2 serum. Early in the infection (Fig. 4d) the concentration of the N protein in the juxtanuclear region was not as prominent as it was at later stages.

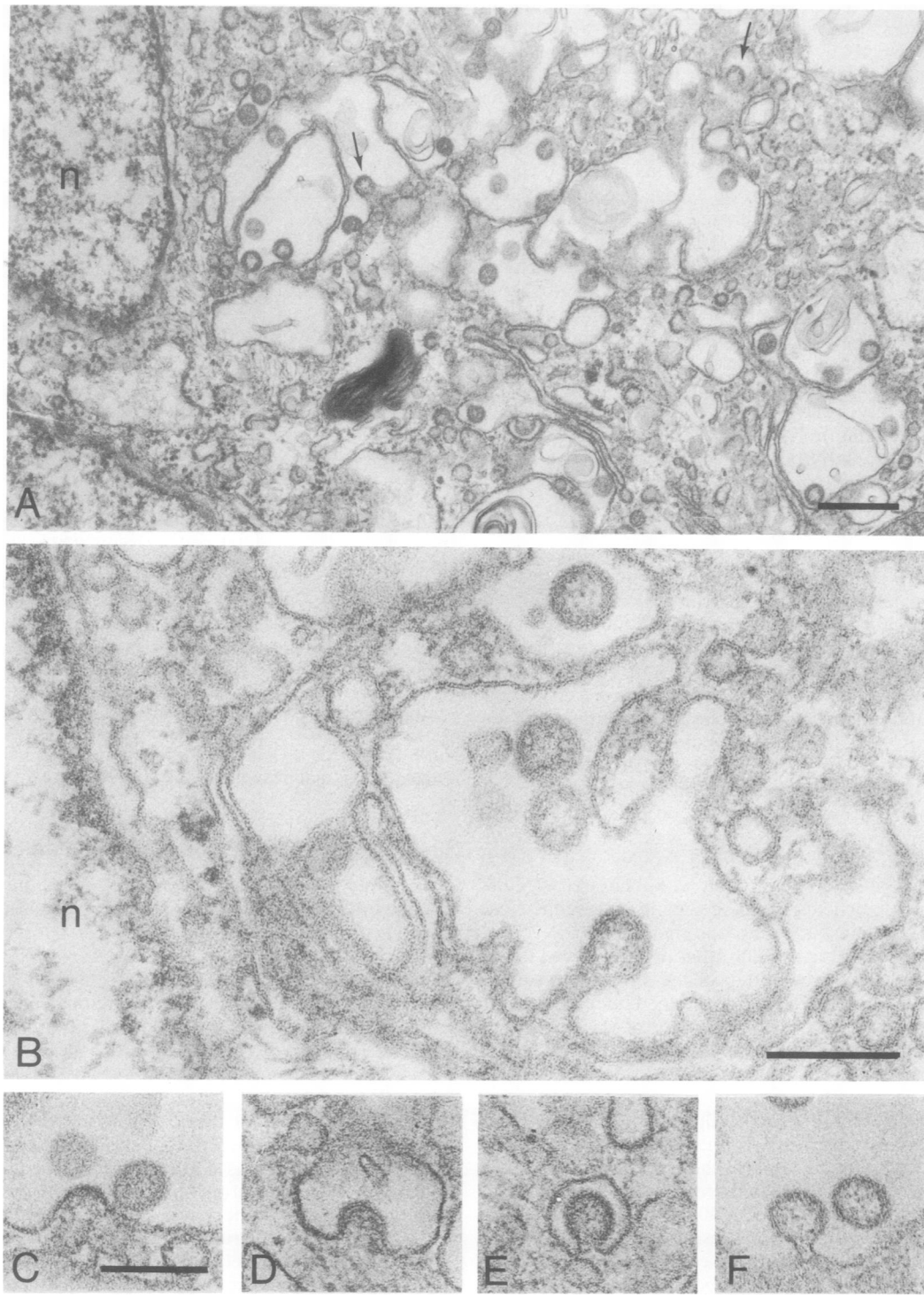

FIG. 3. Electron micrographs of thin sections of Uukuniemi virus-infected cells at 9 h p.i. (A and B) Virus particles are seen in the lumen of smooth-surfaced vesicles close to the nucleus (n). The arrows indicate particles in the process of budding. (C–F) Virus particles at different stages of budding. Note that the nucleocapsid is not detectable. Bars: (A) 400 nm (33,000×); (B) 200 nm (100,000×); (C–F) 200 nm (80,000×).

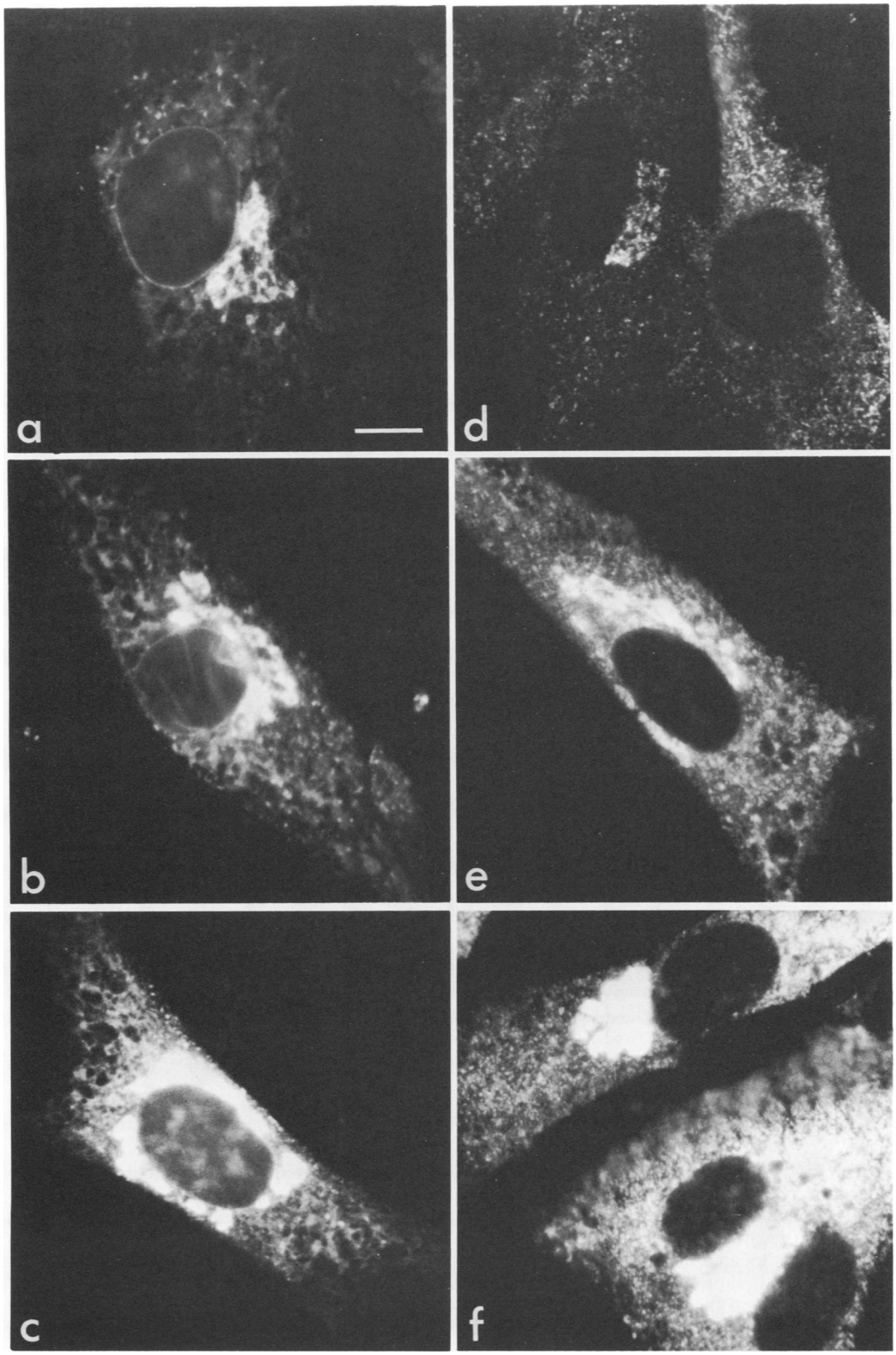

FIG. 4. Intracellular indirect immunofluorescence of Uukuniemi virus-infected cells. Staining with anti-glycoprotein serum at 5 h (a), 10 h (b), and 15 h (c) p.i., respectively. Staining with anti-nucleocapsid serum at the same time points (d, e, and f). Bar, 10 μm.

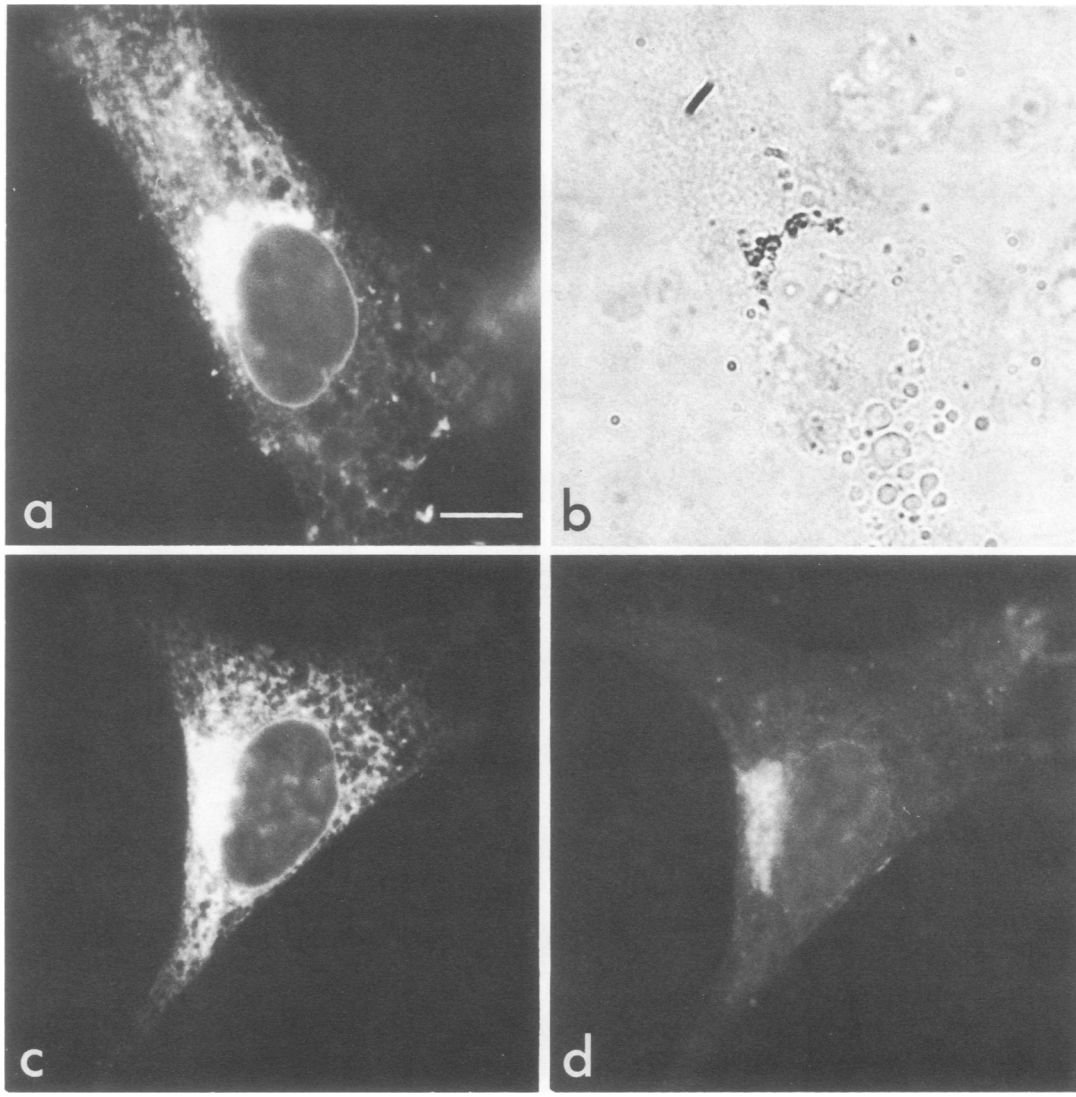

FIG. 5. Double staining of Uukuniemi virus-infected cells with anti-G1/G2 serum and with WGA or the TPPase reaction. The cells were fixed at 9 h p.i. and permeabilized with Triton X-100. (a and c) Uukuniemi virus-infected cells stained with anti-glycoprotein serum and FITC-conjugated anti-rabbit IgG. (b) Cytochemical staining for TPPase activity of the cell shown in a. (d) Double staining of the cell shown in c with TRITC-conjugated WGA. Bar, 10 μm.

Viral proteins are concentrated in the Golgi apparatus. The electron microscopic and immunofluorescence results discussed above suggested that virus maturation occurs at smooth-surfaced vesicles located close to the nucleus and that viral structural proteins also are concentrated in this region of the cells. To study whether this region represents the Golgi apparatus, we used two methods to localize this compartment: (i) staining for TPPase, which under appropriate assay conditions results in the labeling of the Golgi apparatus (6), and (ii) staining with TRITC-conjugated WGA, a lectin which has been shown to stain predominantly the Golgi membranes due to its affinity to the distal glucosamine residues present in incompletely glycosylated glycoproteins (35). Figure 5a shows an infected cell at 9 h p.i. after indirect immunofluorescence staining for G1/G2 with FITC-conjugated anti-rabbit serum; a strong juxtanuclear fluorescence was seen. The same cell was then cytochemically stained for TPPase activity (Fig. 5b). The lead precipitate was seen only in the juxtanuclear region coincident with the fluores-

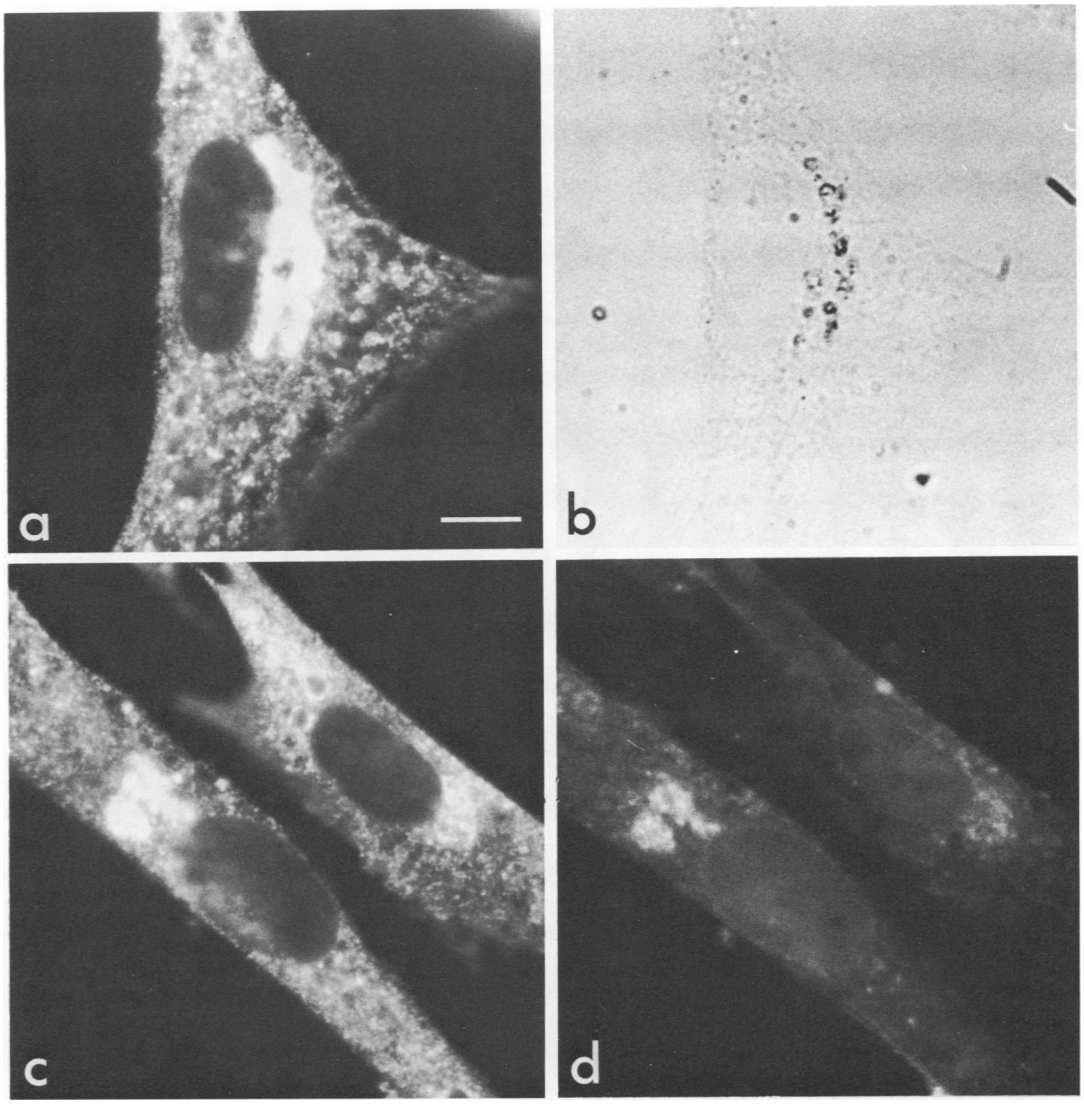

FIG. 6. Double staining of Uukuniemi virus-infected cells with anti-N serum and with WGA or the TPPase reaction. (a and c) Intracellular immunofluorescence with anti-N. (b) TPPase staining of the cell shown in a. (d) WGA staining of the cell shown in c. Bar, 10 μm.

cence, although it appeared to occupy a somewhat smaller area than the strong fluorescence. An infected cell was again stained for G1/G2 and then double stained with TRITC-WGA (Fig. 5c and d, respectively). The juxtanuclear region stained with WGA was coincident with the area strongly stained with anti-G1/G2 and FITC-conjugated anti-rabbit IgG.

When the cells were stained with anti-N and FITC-conjugated anti-rabbit IgG and then double stained either cytochemically for TPPase activity or with TRITC-WGA, essentially the same results were obtained as with anti-G1/G2 serum (Fig. 6). The juxtanuclear region showing strong FITC fluorescence for N protein (Fig. 6a and c) was coincident with both the TPPase activity (Fig. 6b) and the TRITC-WGA fluorescence (Fig. 6d).

Immunoperoxidase electron microscopy. To examine the subcellular location of the structural proteins of Uukuniemi virus at the ultrastructural level we used immunoperoxidase electron microscopy. Virus-infected cells were fixed with a combination of glutaraldehyde and formaldehyde in the presence of the permeabilizing agent saponin for staining the intracellular viral membrane and nucleocapsid proteins (8). When fixed at 8 h p.i. and immunoperoxidase stained with

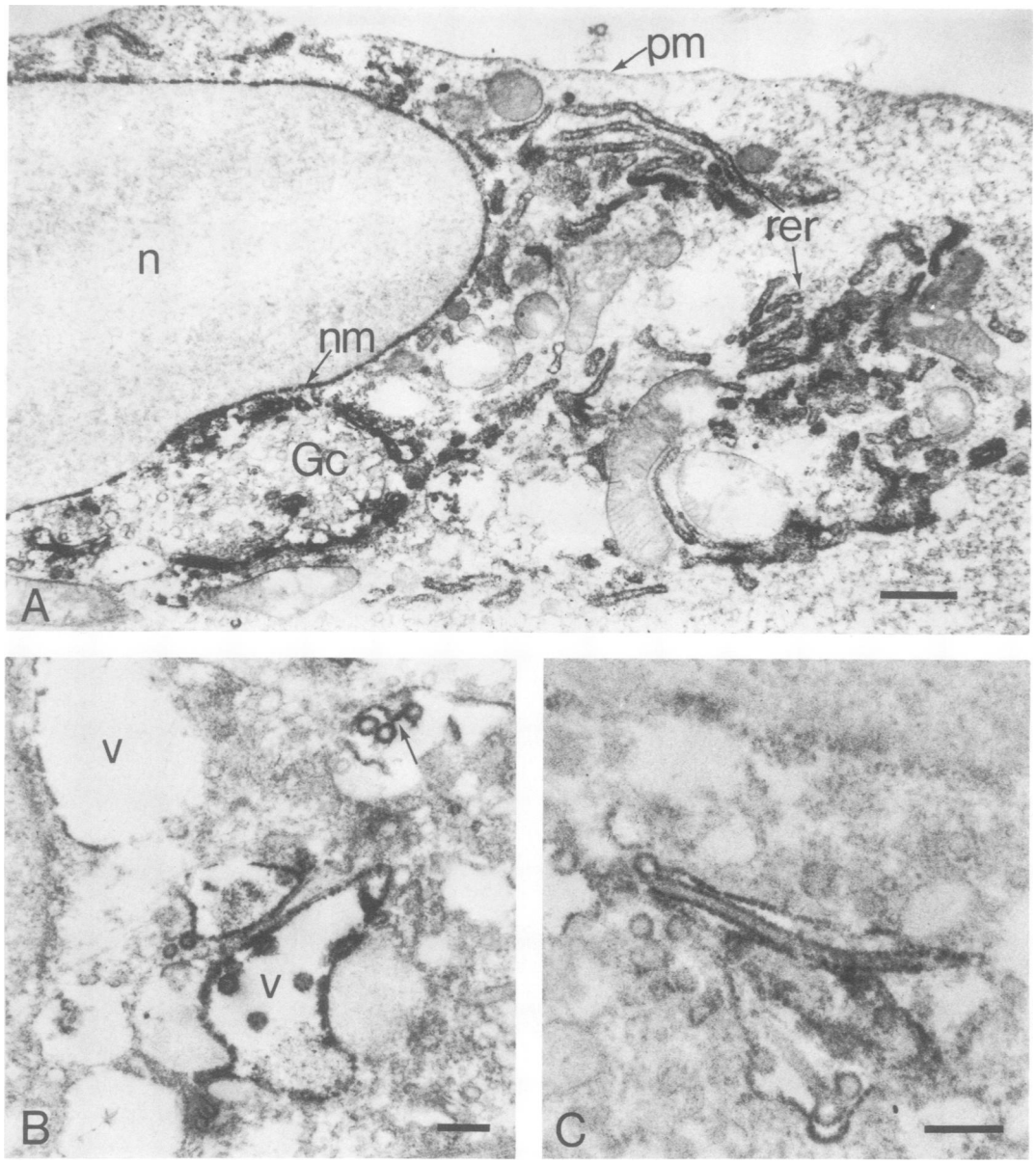

FIG. 7. Intracellular localization of Uukuniemi virus glycoproteins by immunoperoxidase electron microscopy. (A) Staining of the nuclear periphery (nm), the rough endoplasmic reticulum (rer), and the Golgi complex (Gc) is evident. (B and C) Stained virus particles are seen inside smooth-surfaced vesicles. The arrow in B indicates a budding profile with associated peroxidase stain. n, Nucleus; pm, plasma membrane; v, juxtanuclear vacuoles. (A) Bar, 1 μm (10,500×); (B) bar, 0.3 μm (24,000×); (C) bar, 0.3 μm (35,500×).

antibodies against the virus envelope glycoproteins, the membrane network of the rough endoplasmic reticulum and the nuclear membrane were stained with the peroxidase reaction product (Fig. 7a). In the juxtanuclear region, the Golgi complexes were stained; the peroxidase reaction product decorated the membranes of the stacked cisternae and large vacuoles of the Golgi region (Fig. 7a–c). Stained virus particles were frequently observed in the luminal space of the Golgi vacuoles (Fig. 7b and c), and apparent budding profiles were occasionally observed with associated staining of the membrane of the budding virus (Fig. 7b, arrow). Tubular structures, which are occasionally seen inside smooth-membraned vacuoles (37), were labeled as well.

A different staining pattern was obtained with

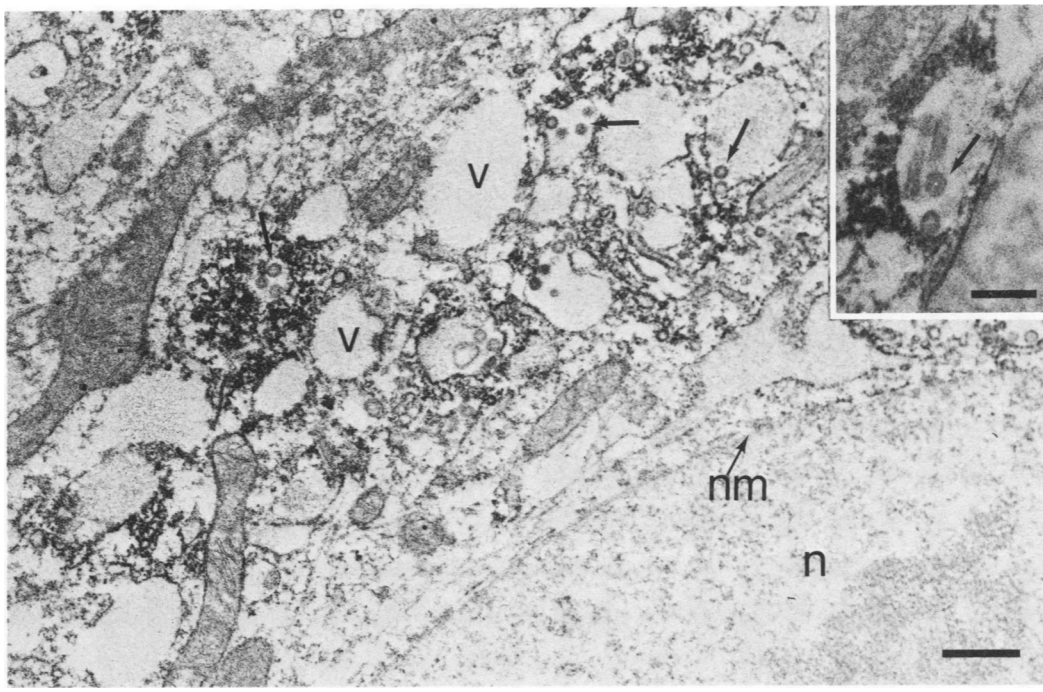

FIG. 8. Intracellular localization of Uukuniemi virus nucleocapsid protein by immunoperoxidase electron microscopy. The peroxidase reaction product is found in the cytoplasm surrounding the vacuoles (v). The arrows indicate virus particles in the lumen of vacuoles. The inset shows staining of nucleocapsid protein close to the cytoplasmic surface of the vacuole. n, Nucleus; nm, nuclear membrane. Bar, 0.5 μm (20,000×); inset bar, 0.2 μm (34,000×).

antiserum against the N protein. In general, the staining was weaker than that seen with anti-G1/G2 serum, probably due to the loss of antigenicity caused by glutaraldehyde fixation. Peroxidase stain was observed in the cytoplasmic space in the form of granular deposits. The heaviest staining was observed in the juxtanuclear area close to the elements of the Golgi complex (Fig. 8). Virus-containing vacuoles were frequently surrounded by peroxidase-stained cytoplasmic material (Fig. 8, inset). In contrast to anti-glycoprotein antibodies, the nucleocapsid antibodies gave only occasional staining of the virus particles seen in the lumen of the vacuoles (Fig. 8, arrows).

Viral glycoproteins are transported to the plasma membrane. To study whether the glycoproteins of Uukuniemi virus are transported to the plasma membrane, we stained paraformaldehyde-fixed infected cells with anti-G1/G2 serum and then with FITC-conjugated anti-rabbit IgG. Examples of the surface immunofluorescence seen at early and late stages of infection are shown in Fig. 9. The staining pattern was unusual and clearly differed from the fluorescence seen at the surface of cells infected with SFV, a virus which is formed at the plasma membrane (30). The staining was in general rather weak, particularly early in the infection, but intensively stained dots (Fig. 9a and b) and patches (b) were seen in most cells. These patterns were absent from mock-infected cells (Fig. 9d) or virus-infected cells stained with preimmune serum (not shown). Later in infection many of the cells displayed more uniform surface staining, with the cellular projections clearly visible. Such cells also showed strongly fluorescent patches (Fig. 9c). The immunofluorescence staining thus showed that the glycoproteins of Uukuniemi virus reach the cell surface during infection.

Localization of SFV capsid protein in monensin-treated cells. Johnson and Schlesinger (10) have recently shown that when cells infected with vesicular stomatitis virus (VSV) or Sindbis virus are treated with the carboxylic ionophore monensin, the cellular location of virus maturation is markedly changed. Monensin treatment leads to the vacuolization of the Golgi apparatus (32) and causes the arrest of virus membrane proteins at this site of the cell (10, 11, 31). Normally these two viruses mature at the plasma membrane, but in monensin-treated cells extensive budding occurs intracellularly in association with the dilated membranes of the Golgi

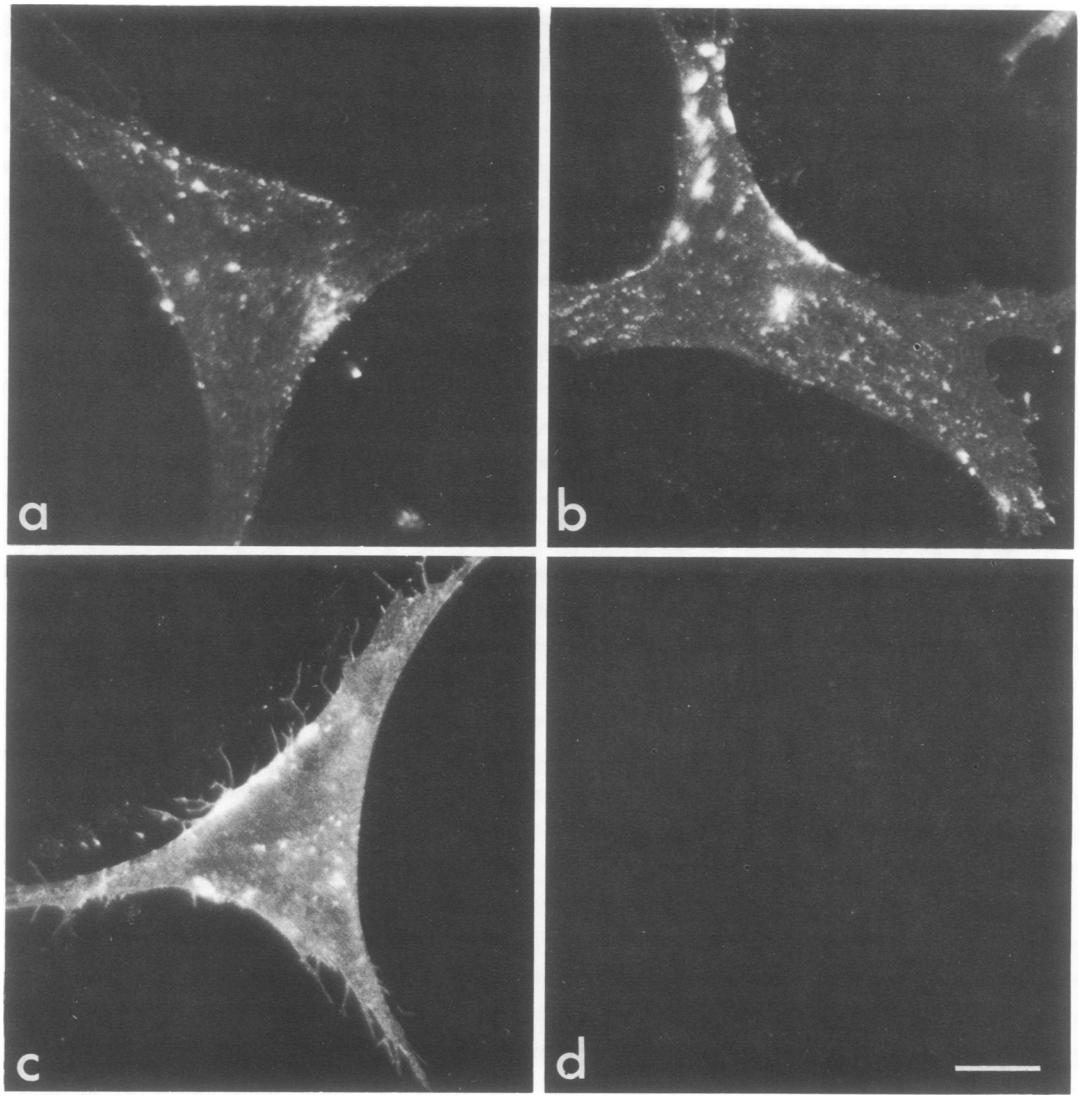

FIG. 9. Surface immunofluorescence of Uukuniemi virus-infected cells stained with anti-glycoprotein serum early (a) and late (b and c) in the infection. (d) Surface staining of mock-infected cells. Bar, 10 μm.

complex. Our own electron microscopic results for SFV-infected cells treated with monensin have also shown that the spherical nucleocapsids of SFV bind extensively to the cytoplasmic surface of virus-containing Golgi vacuoles and that virus maturation occurs at this site (22a).

To compare the location of the SFV nucleocapsid protein in the presence of monensin with the observed location of N protein in Uukuniemi virus-infected cells, we used intracellular immunofluorescence with antibodies to the capsid protein of SFV. In nontreated cells, staining with the anti-capsid serum gave a homogenous, punctuate fluorescence to the entire interior of the cell (Fig. 10a). In contrast, bright staining of numerous juxtanuclear vesicles was observed in monensin-treated cells (Fig. 10b). Thus, the distribution of the SFV capsid protein in monensin-treated cells resembled that of the N protein in Uukuniemi virus-infected cells (Fig. 4 and 6).

DISCUSSION

During the 15 years that have passed since a number of arthropod-borne viruses were found to mature intracellularly in a region of smooth-surfaced vesicles close to the nucleus (for a review, see reference 2), surprisingly little attention has been paid to this interesting observa-

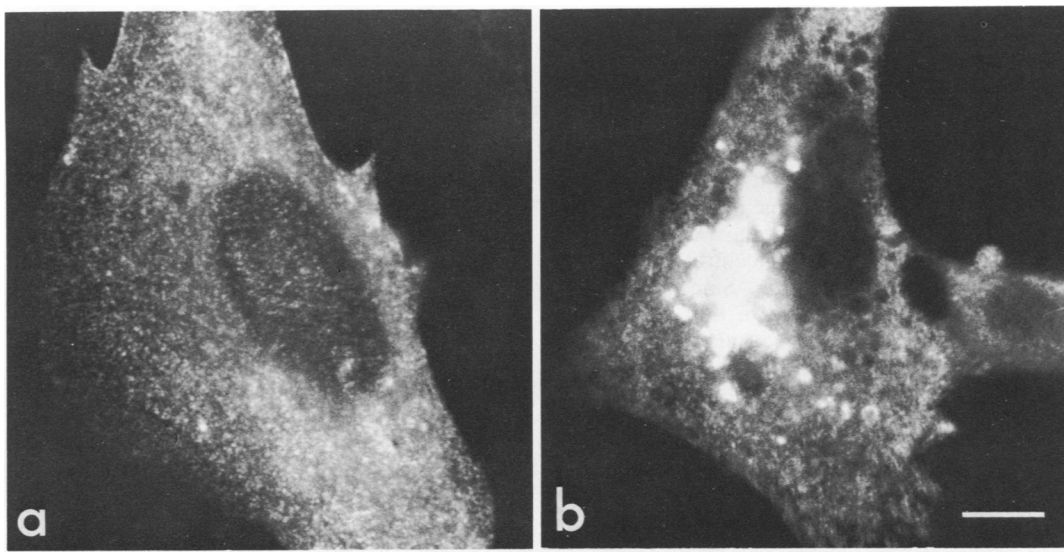

FIG. 10. Intracellular localization of SFV nucleocapsid protein by indirect immunofluorescence in the presence and absence of monensin. (a) Control cell not treated with monensin. (b) After absorption, the cells were incubated in the presence of 10 μM monensin for 2.5 h before being stained. The staining was done with antibodies directed against the nucleocapsid protein of SFV followed by TRITC-conjugated anti-rabbit IgG. Bar, 10 μm.

tion. Partly based on their similar site of maturation, these viruses were later classified as members of the Bunyaviridae family (3). It has been suggested that the smooth-surfaced vesicles in which virus budding occurs represent the Golgi apparatus. Since the current view of bunyavirus maturation has been based exclusively on electron microscopy of thin sections of infected cells (2), we have reinvestigated this question with additional methods for determining the morphogenesis of bunyaviruses and the biosynthesis of the viral glycoproteins in more detail. Here we studied the intracellular location of the viral structural proteins in Uukuniemi virus-infected chicken embryo fibroblasts with immunocytochemical techniques for light and electron microscopy.

The idea that Uukuniemi virus indeed matures in the Golgi apparatus was supported by our observations. (i) Virus particles, as well as budding profiles, were found in large smooth-surfaced vesicles in a juxtanuclear region typical for the Golgi complex. (ii) The viral glycoproteins and, more importantly, the nucleocapsid protein were found by immunofluorescence to be concentrated in a juxtanuclear region. (iii) This region was identified as the Golgi apparatus by double-staining experiments which used two different methods: cytochemical staining for TPPase activity, an enzyme activity specific for the Golgi apparatus under the conditions used here (6), and staining with TRITC-conjugated WGA, a lectin that can be used as a marker for the Golgi complex (35). (iv) Immunoperoxidase electron microscopy confirmed, at the ultrastructural level, the presence of viral glycoproteins and the nucleocapsid protein in association with the membranes of the Golgi complex.

As shown by the immunolocalization methods, the distribution of Uukuniemi virus glycoproteins in infected cells was similar to that of the p62 and E1 glycoproteins of SFV and the G protein of VSV, two viruses known to mature at the plasma membrane. The glycoproteins of SFV and VSV have been extensively used as model systems for studying the transport of eucaryotic membrane glycoproteins from the rough endoplasmic reticulum to the plasma membrane via the Golgi complex (1, 7, 11, 16, 29, 31, 38, 39). As with SFV and VSV, the Uukuniemi virus glycoproteins were localized to the rough endoplasmic reticulum as well as to the nuclear membrane, reflecting the synthesis of viral membrane-bound glycoproteins at these sites. A prominent feature of Uukuniemi virus-infected cells was the strong concentration of glycoproteins in the juxtanuclear region as shown by intracellular immunofluorescence. At light microscopic resolution this area was coincident with the Golgi complex, as shown by the TPPase assay and WGA staining. Part of the G1/G2-specific fluorescence in the Golgi region was due to the presence of membrane-associated proteins and part was due to virus particles, as shown by immunoperoxidase electron microscopy.

The N protein was localized in the cytoplasm as a punctuate fluorescence different in appearance from the reticular pattern of glycoprotein fluorescence. Also, no staining of the nuclear periphery was seen with the N-specific antiserum. Like the viral glycoproteins, the N protein was also heavily concentrated in the Golgi region, especially at the time of efficient virus production. We assume that most of the fluorescence was due to the concentration of viral nucleocapsids in this region. Since the helical nucleocapsid is not visible in thin sections of infected cells (36, 37), direct microscopic demonstration that nucleocapsids are in fact concentrated in the Golgi region has not been possible. Our immunoelectron microscopic results indicated that the N protein is scattered in the cytoplasm but is also concentrated on the cytoplasmic side of vesicles in the Golgi region, supporting the view that nucleocapsids line up underneath the membrane to faciliate budding.

In contrast to Uukuniemi virus-infected cells, no concentration of viral nucleocapsids was seen in the Golgi region of cells infected with SFV. However, when SFV-infected cells are treated with monensin, an ionophore that inhibits the transport of viral glycoproteins from the Golgi complex to the plasma membrane (11), a strong concentration of SFV nucleocapsids in the Golgi area and budding of virus particles into Golgi vesicles are observed (22a). As shown here by immunofluorescence with anti-capsid serum, the Golgi region is heavily stained under these conditions. This result indirectly supports our view that the maturation of Uukuniemi virus occurs at the Golgi complex.

Based on the immunofluorescence data, the size and staining intensity of the Golgi region increased, and progressive vacuolization appeared to occur during the infection. Electron microscopy of thin sections of infected cells has shown that the area of virus maturation proliferates and dilates, and the cells become extensively vacuolized late in infection (37). The data from both immunofluorescence and electron microscopy studies thus are consistent with the idea that the Golgi region expands due to virus infection.

In a recent report, Madoff and Lenard (19) found that the G1 protein of La Crosse virus, a member of the *Bunyavirus* genus of Bunyaviridae (3), accumulates inside the cell and is apparently transported to the plasma membrane and into mature virions two to three times more slowly than the VSV G protein is. The glycans of the intracellular G1 were mostly of the immature high-mannose type (endoglycosidase-H [endo-H] sensitive), whereas the G1 of the cell surface and in extracellular virions contained exclusively complex type (endo-H resistant) glycans. Based on these findings, the authors preferred to interpret their results to mean that La Crosse virus matures at the plasma membrane, although an intracellular site of maturation could not be ruled out. We have recently, by analysis of the monosaccharide sequence, found three types of glycans in extracellular particles of Uukuniemi virus (23) and Inkoo virus (a close serological relative of La Crosse virus [2]) (M. Pesonen, R. Rönnholm, E. Kuismanen, and R. F. Pettersson, J. Gen. Virol., in press): complex glycans, high-mannose-type glycans, and a novel small intermediate type (endo-H resistant) glycan. We have suggested that these intermediate types of glycans represent processing intermediates between high-mannose and complex glycans and that their presence in the virus may reflect the site of virus maturation in the Golgi apparatus (23; Pesonen et al., in press).

What then determines the unusual site of maturation of Uukuniemi virus and how do the mature particles reach the extracellular space? At present we do not know the answer to these questions. One possibility is that the viral glycoprotein(s), as suggested by Madoff and Lenard (19), is not efficiently transported to the plasma membrane, but instead accumulates in the Golgi complex. This, perhaps in combination with a strong affinity of the nucleocapsid for a cytoplasmic extension of one or both of the glycoproteins, would then result in budding into Golgi vesicles. The SFV and the VSV glycoproteins have been shown to contain a signal for transportation to the plasma membrane (30, 39). Whether such a transport signal is absent from or inefficient in the bunyavirus glycoproteins remains to be seen. As shown here, some Uukuniemi virus glycoproteins do reach the cell surface. At present we do not know what fraction of the glycoproteins reach the plasma membrane, but based on the immunofluorescence data it appears to be rather small. Madoff and Lenard (19) also found that only a small fraction of the cell-associated G1 of La Crosse virus was present on the cell surface. The surface fluorescence of Uukuniemi virus-infected cells was unusual in that strongly stained dots and patches could be seen against a weak background of more uniform staining. These may represent aggregated virus particles or clustered glycoproteins.

It is likely that the viruses are transported to the cell surface in vesicles, which fuse with the plasma membrane and thereby expel the virus into the medium. These vesicles would probably contain a large number of "left-over" glycoproteins which have not participated in the budding. After fusion of the vesicles with the plasma membrane, these glycoproteins could be responsible for the cell surface fluorescence observed.

The nature of the vesicles that transport the virus to the cell surface and the route they take is as yet unknown. It would be interesting to know whether these vesicles represent a normal cellular pathway by which material—such as the lipids needed for plasma membrane expansion—is delivered to the exterior of the cell.

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