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Association of Poliovirus Proteins with the Endoplasmic Reticulum

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Poliovirus proteins, except P3-7c, are associated with the endoplasmic reticulum after extraction of the cytoplasm and centrifugation of membranes to equilibrium in sucrose gradients. Proteins P3-2, P2-X, and P3-9 are found preferentially among the rough endoplasmic reticulum, whereas P3-7c is located in smooth endoplasmic reticulum fractions. P3-7c is probably not membrane associated, since it can be separated from membranes after centrifugation in buffer. However, P3-4a, P2-5b, P2-X, and P3-9 are avidly bound to membranes and cannot be dislodged with high-ionic-strength buffer containing EDTA or 4 M urea. These proteins are digested by trypsin, indicating peripheral rather than internal localization.

Electron microscopic (2, 5) and cell fractionation (6–8, 11, 14, 16, 17, 22) studies show that smooth and rough endoplasmic reticula (SER and RER, respectively) are involved with the replication of picornaviruses. Poliovirus polymerase is associated with a membranous complex (4, 8), and specific enzyme activity appears to be highest in the SER (8, 22). Most viral proteins are distributed among both the SER and RER (6), but recent data indicate that P3-9, a potential precursor to genome linked protein VPg, is found in highest concentrations in the RER (22). Several viral proteins such as P2-X, PX/9, and P3b/9 appear to be membrane associated (22).

Poliovirus is sensitive to guanidine, and the initiation step of RNA synthesis is the site of inhibition (9, 23). Although earlier studies implicated capsid proteins as determinants of guanidine sensitivity (and resistance among guanidine-resistant mutants), current experiments with foot-and-mouth disease virus show that a protein coded by the middle region of the genome is responsible for the guanidine trait (19). This protein is comparable to poliovirus protein P2-X, and we have recently shown that 12 of 18 guanidine-resistant (g^r) mutants produce modified P2-X as evaluated by isoelectric focusing and peptide mapping of protease digests (3). It has been suggested that P2-X (p34 with foot-and-mouth disease virus) is essential for the continued synthesis of viral RNA, and a critical function would involve binding of viral polymerase to membranes (19). The following experiments were undertaken to evaluate the association of P2-X with the endoplasmic reticulum by employing procedures that normally dislodge extrinsically bound proteins (1, 20, 24). The data show that P2-X, its precursor P2-5b, P3-4a (a polymerase precursor; 10), and P3-9 are more tenaciously associated with cellular membranes than other virus-specific proteins. P3-7c, a virus coded protease (12), is found in highest concentrations in the SER, whereas P2-X and P3-9 are most prominent in the RER, after fractionation of cellular components by equilibrium contrifugation in sucrose. However, guanidine does not dissociate P2-X or its precursor P2-5b from membranes at concentrations 20- to 100-fold higher than virus-inhibitory levels.

MATERIALS AND METHODS

Virus. A plaque-purified, g^s type 1 Mahoney poliovirus designated g^s1 along with a g^r1 variant derived from g^s1 were used. Selection of virus has been described previously (3).

Cells and medium. HeLa-Ohio cells from Flow Laboratories were used as monolayer cultures in 150-cm Corning flasks. The medium was minimal essential medium with Earle salts, glutamine, 7% fetal bovine serum, and standard levels of penicillin, streptomycin, and fungizone obtained from GIBCO Laboratories.

Infection of cells, labeling of viral proteins, and sucrose gradient centrifugation. Flasks with 5×10^7 HeLa cells were customarily inoculated with virus to provide 20 to 50 PFU/cell. After 1 h at 36°C cells were washed with warm Hanks balanced salt solution, removed from flasks with a 0.2% solution of trypsin, and suspended to 4×10^6 cells per ml in Hanks balanced salt solution containing 1% fetal bovine serum and antibiotics. Flasks were incubated in a water bath shaker at 36°C, and cells were pulse-labeled with [^{3}H]leucine at 8 to 15 μCi/ml in various experiments for periods ranging from 15 to 75 min as designated in the figure legends. Radioactivity was added 2 h and 50 min after initial infection, and no compensation was allowed for the period of trypsin treatment. After the labeling period, cells (usually 2.0×10^8) were poured into 125 ml of partially frozen Hanks balanced salt solution and pelleted by low-speed centrifugation. After three washes with cold Hanks balanced salt solution, cells were suspended in 6 to 7 ml of reticulocyte standard buffer (RSB; 10 mM Tris [pH 7.4], 10 mM KCl, and 1.5 mM $MgCl_2$) and ruptured with 20 strokes in a Dounce homogenizer after swelling for 7 min. Nuclei and debris were removed at 1,000 × *g* for 10 min; 1 ml of the supernatant was processed for total cytoplasmic viral proteins, and the remainder was diluted 1:1 with 60% sucrose in RSB for equilibrium centrifugation. Discontinuous sucrose gradients were prepared as described previously and centrifuged for 18 to 19 h at 4°C in a Spinco SW27 rotor at 25,000 rpm (7).

Isolation and treatment of the SER and RER. Membrane fractions were removed from gradients with a syringe and distributed equally among SW56 or SW40 cellulose nitrate tubes. The samples were then diluted fourfold with RSB or various solutions to provide final concentrations of 0.5 M KCl, 10 mM EDTA, 2 or 4 M urea, or 5 to 100 mM guanidine as indicated in the figure legends. Immediately after dilution, the membranes were pelleted at 120,000 or 140,000 × *g* for 75 min at 10°C. Pellets were rinsed three times with distilled water and disolved in gel sample buffer at 75°C for 90 min. Proteins in the supernatants were precipitated with 3% final trichloroacetic acid overnight at room temperature. Precipi-

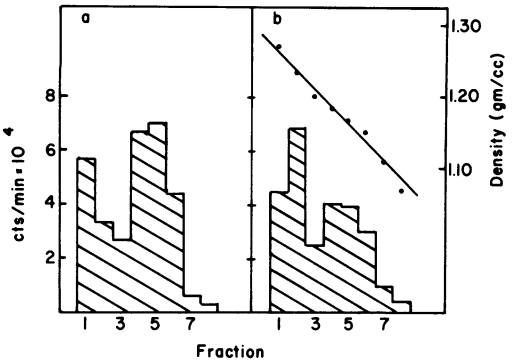

FIG. 1. Equilibrium sucrose gradients of membranes from poliovirus-infected cells. HeLa cells (2×10^8) were infected with 50 PFU of g^s Mahoney poliovirus per cell and pulse-labeled for 15 min at 36°C with 15 μCi of [^{3}H]leucine per ml at 2 h and 50 min after infection. The suspension culture was then divided into two equal portions; in one set cells were sedimented and washed, and cytoplasmic membranes were extracted (a), whereas in the second set cells were chased 15 min with a 100-fold excess of non-radioactive leucine before harvesting and processing (b). After centrifugation of the cytoplasmic membranes to equilibrium for 18 h at 4°C in discontinuous sucrose gradients, 5-ml fractions were collected, and radioactivity was measured. The bottoms of gradients are to the left. Fractions were collected with a Buchler Auto-densi Flow apparatus with the probe at the bottoms of gradients, and densities were determined with a Bausch & Lomb refractometer.

tates were pelleted at 15,000 × *g* for 10 min at 4°C, dispersed with 0.9 ml of water, and mixed with 0.4 ml of 25% trichloroacetic acid in 1.5 ml Fisher Micro-Centrifuge tubes. Precipitates were pelleted with a Fisher Micro-Centrifuge, washed seven times with a 1:1 solution of ether-ethanol, dried, and dissolved in gel sample buffer. The washed membrane pellets and their respective supernatant proteins were dissolved in equal volumes of gel sample buffer. To measure incorporation, 10 μl portions of all samples were mixed with 10% trichloroacetic acid and carrier protein, and precipitates were collected on Whatman GF/A glass fiber filters and washed with 2% trichloroacetic acid. After dissolving precipitates in Scinti Verse 1 fluid (Fisher Scientific Co.), radioactivity was measured. Samples of total cytoplasmic proteins underwent the same precipitation and washing procedures.

To measure the percentage of viral proteins lost from the SER and RER after various treatments, samples of pellets that were dissolved in gel sample buffer and samples of supernatants were treated with 10% TCA (final concentration). Protein precipitates were collected on GF/A filters, and radioactivity was measured. From the total combined counts in the pellets and supernatants, the percentage distribution of counts in pellets and supernatants was determined. Because trypsin degraded viral proteins (see Fig. 6 and 7), it was not possible to examine supernatants for total counts. Consequently estimations of the percent digestion were based on comparisons of pellet fractions obtained from untreated and trypsin-treated samples.

Polyacrylamide gel electrophoresis. Denaturing gels of 13.5% and occasionally 12 to 18% were used as described previously (13). Electrophoresis was conducted at 160 to 170 V for 4.5 to 5.5 h with 1.5-mm slab gels. Gels were stained for 1 h with 0.1% Coomassie blue in 40% methanol–10% acetic acid and destained overnight with 30% methanol–10% acetic acid. Gels were then impregnated 45 min with Enlightening (New England Nuclear Corp.), dried under vacuum, and covered with Kodak XAR-5 X-ray film for 3 to 7 days before developing.

^{14}C-labeled proteins were frequently used as standards and consisted of myosin (200 kilodaltons [kd]) β-galactosidase (116 kd), phosphorylase B (94 kd), bovine serum albumin (68 kd), ovalbumin (43 kd), α-chymotrypsinogen (25.7 kd), β-lactoglobulin (18.4 kd), lysozyme (14.3 kd), cytochrome *c* (13.3 kd), and bovine trypsin inhibitor (6.2 kd).

Nomenclature of viral proteins. The designation of poliovirus-specific proteins is in accordance with standard nomenclature (15). However, a more recent system of nomenclature, designated L-434, was recommended and appeared in press at the time this manuscript was submitted for publication (18). The newer system is listed below for the various proteins discussed in this report. The older nomenclature is designated first, followed by the L-434 convention: Pla, 1 or Pl; P3-2, 3CD; P3-4a, no change; P3-4b, 3D; P3-7C, 3C; P3-9, 3AB; X/9, 2C-3AB; 3b/9, no change; P2-3b, 2 or P2; P2-5b, 2BC; P2-X, 2C; VP0, 1AB; VP1, 1D; VP2, 1B; VP3, 1C; VP4, 1A.

Materials. L-[3,4,5-^{3}H(N)] leucine (120 Ci/mmol) and Enlightening were obtained from New England Nuclear Corp. All electrophoresis chemicals were purchased from Bio Rad Laboratories. Molecular weight standards were purchased from Bethesda Research Laboratories, Inc. The trypsin inhibitor aprotinin was purchased from Sigma Chemical Co.

RESULTS

Distribution of viral proteins among cellular membranes. The initial experiments were undertaken to determine the distribution of viral proteins among the RER and SER. Cells were infected with guanidine-sensitive poliovirus and administered [^{3}H]leucine for 15 min at 2 h and 50 min after infection. After the pulse, one-half of the culture was poured over ice-cold Hanks balanced salt solution, and the remainder was incubated an additional 15 min with a 100-fold excess of non-radioactive L-leucine. After extraction of the cytoplasm and centrifugation in discontinuous sucrose gradients, 5-ml fractions were collected and assayed for acid precipitable radioactivity. Because infection severely inhibits its synthesis of cellular proteins, virus-specific proteins are predominantly measured. The date in Fig. 1 show that during both the pulse (part a) and chase (part b) periods approximately 30 to 40% of the viral proteins were distributed among fractions 1 and 2. These fractions have densities ranging from 1.27 to 1.23 g/ml and correspond to the RER as described by others (7, 14, 16). Fractions 5 and 6 contain the SER with densities of 1.17 to 1.12 g/ml and 25 to 40% of the virus-specific proteins. In several studies short labeling periods of 15 to 20 min resulted in an approximately equal distribution of viral proteins between the RER and SER. With labeling periods of 60 to 75 min the RER contained 65 to 80% of the incorporated precursor (data not included). At least part of this change in distribution of viral proteins as well as the loss of viral peptides from the SER following a chase period (Fig. 1) can be attributed to assembly of capsid proteins into virions, which have been shown to cosediment with the RER (16). It should also be noted that negligible amounts of viral proteins were located in the upper 8 ml of gradients, i.e., fractions 7 and 8, in agreement with data obtained by others (7).

Extraction of viral proteins from the endoplasmic reticulum with high-salt buffer. Many cellular proteins are extrinsically associated with the endoplasmic reticulum and can be dislodged with buffers of high ionic strength and EDTA (1, 20, 24). Some of these proteins probably bind non-specifically

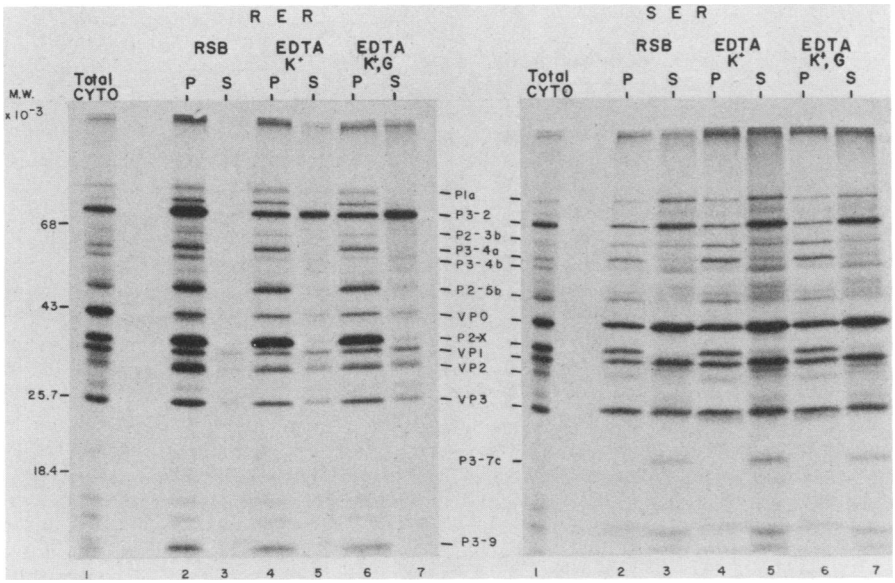

FIG. 2. Extraction of viral proteins from membranes with high-ionic-strength buffer and EDTA. A culture of 10^8 HeLa cells was infected with g^s Mahoney poliovirus at 20 PFU/cell and fed [^{3}H]leucine at 10 μCi/ml for 1 h starting at 2 h and 50 min after infection. After extraction of the cytoplasm and centrifugation of membranes to equilibrium, the RER and SER were carefully withdrawn and distributed equally among SW40 tubes. One tube from each membrane fraction was diluted fourfold with RSB, another was diluted with RSB containing high salt-EDTA, and the third was diluted with high salt-EDTA buffer containing 5 mM guanidine (final concentration). Tubes were immediately centrifuged at 120,000 × *g* for 75 min at 10°C, and pellets were washed with distilled water and prepared for polyacrylamide gel electrophoresis. A final concentration of 3% trichloroacetic acid was added to supernatants, and protein precipitates were collected at 15,000 × *g* for 10 min, processed, and dissolved in gel sample buffer equivalent to the amount used to dissolve pellets. Electrophoresis was performed 5.5 h at 160 V with 13.5% polyacrylamide gels. P, Pellets; S, supernatants. Molecular weight standards were included for reference in an alternate lane.

during isolation procedures, but others such as the signal recognition protein transiently associate with membranes during protein synthesis (24). Numerous poliovirus (7, 22) and foot-and-mouth disease virus proteins (11) have been demonstrated to associate with cellular membranes. To determine whether poliovirus proteins adventitiously bind to the reticulum both the RER and SER were obtained from infected cells and subjected to various treatments such as washing with RSB, high salt-EDTA (0.5 M KCl–10 mM EDTA) in RSB, and high-salt buffer containing 5 mM guanidine. Nine separate studies were performed; although there were variations, several findings were consistent (Fig. 2). Proteins in the RER fractions of equilibrium sucrose gradients were not readily released from membranes after pelleting in RSB. Washing the RER with buffer alone dislodged 5% or less of the viral proteins, whereas high salt-EDTA buffer removed 20 to 30%. Viral proteins that were found among SER fractions of gradients were more readily separated from membranes during pelleting of the latter. SER samples that were diluted with RSB before sedimentation of membranes lost 30 to 50% of their virus-specific proteins to the supernatants, whereas high salt-EDTA buffer dislodged 50 to 70% of the proteins from the SER. The only virus-specific protein that was completely washed free of membranes in the majority of experiments was P3-7c, a virus-coded protease (12). This protein, along with VP0, was found in the highest concentrations with SER fractions. However, it should be emphasized that the SER contained one-fourth as much viral protein as the RER in this study, and autoradiograms were developed for the SER after twice the exposure time employed for the RER. In contrast the RER contained the highest levels of P3-2, P2-X, and P3-9. P3-9 was previously demonstrated to associate predominantly with the RER (22). In many of our experiments this protein was run off the gels during electrophoresis, as can be noticed with the SER in Fig. 2. With shorter periods of electrophoresis (Fig. 3), P3-9 was found to associate with both membrane fractions, but was found in greater abundance among RER fractions.

Proteins most conspicuously found in the wash fluids of the RER were P3-2 and capsid peptides. The high level of VP2 among the RER is indicative of mature virions, which cosediment with these membranes (16). Capsid proteins in the supernatants probably reflect incomplete sedimentation. After dilution of the RER with various buffers the sucrose concentration is still 12%, a level that could hinder pelleting of virions. This supposition is supported by data in Fig. 3, where centrifugation of washed membranes was conducted with higher gravitational forces and all capsid peptides were cleared from supernatants.

In all nine experiments to date P3-4a, P2-5b, P2-X, and P3-9 were most recalcitrant to washing with high salt-EDTA buffer. The low levels of these proteins in the wash fluids of the SER (Fig. 2) were not a consistent observation. Some of these proteins could be of cellular origin, since cells were infected with only 20 PFU in this particular study and blockage of host protein synthesis might have been incomplete.

Treatment of microsomal membranes with guanidine. Guanidine at molar concentrations is a denaturant that disrupts complexes of proteins and nucleic acids and aggregates of proteins. Picornavirus growth is blocked at much lower concentration of 1 to 5 mM and cellular metabolism does not appear to be affected (9). It has been hypothesized that P2-X might be critical for binding of viral replicase to cellular membranes and that guanidine interferes with viral growth

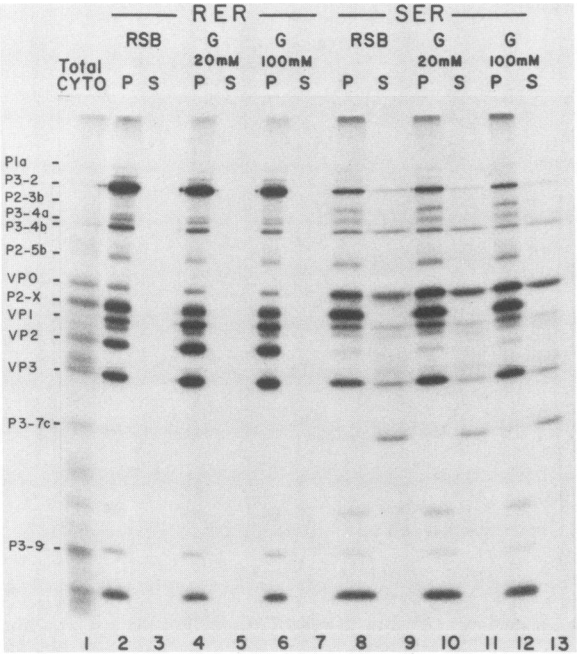

FIG. 3. Extraction of viral proteins from the RER and SER with guanidine. The experiment was similar to that described in Fig. 2, with several modifications. Cells were infected with 50 PFU of g^s Mahoney virus per cell and pulse-labeled with 8 μCi of [^{3}H]leucine per ml for 75 min starting 2 h and 50 min after initial infection. The membranes that were obtained by equilibrium centrifugation were treated with 20 and 100 mM guanidine in RSB and sedimented at 140,000 × g for 75 min. Electrophoresis was performed for 4 h at 160 V with 13.5% polyacrylamide gels.

by disrupting this bond (19). In the study reported in Fig. 2, standard virus-inhibitory concentrations of guanidine were used to treat cellular membranes. However, P2-X and its precursor P2-5b were not disassociated from the membranes by this treatment. Because the preparations contained high concentrations of cellular components, low levels of guanidine could have been ineffective. Consequently, membranes were obtained from cells that were infected with g^s and g^r poliovirus and treated with 20 or 100 mM guanidine. The data in Fig. 3 show these levels of guanidine to be ineffective for washing proteins of g^s virus from both the RER and SER. Similar results were obtained with g^r virus (data not included).

The concentration of P2-X appears to be relatively high in the SER in Fig. 3. in comparison with data in Fig. 2. The SER contained only 30% of the virus-specific proteins in this experiment, and the SER samples added to gels were twice as large as RER samples. P2-3b, the precursor of P2-5b, and ultimately P2-X were evident in Fig. 3. P2-X was detected in only 50% of the studies, probably because of proteolytic processing. In general P2-3b was most prominent in SER fractions of sucrose gradients.

Effects of sonic vibration on association of viral proteins with cellular membranes. The RER contains membrane-bound ribosomes and for this reason is denser than smooth-surfaced membranes. From the data in Fig. 2 it is apparent that high-salt-EDTA buffer did not effectively dissociate viral proteins from the RER compared with the SER. Part of this differential effect could be attributed to protein aggregates that cosedimented with the SER during equilibrium centrifugation, but remained in the supernatants during pelleting of membranes. However, the likelihood that viral proteins are within vesicles or that ribosomes interfered with the salt-EDTA wash procedure warrants consideration. Experiments were undertaken to determine whether sonic vibration could release polysomes and virus-specific proteins from the endoplasmic reticulum.

Sonic vibration in buffer alone had virtually no effect on the density of the SER (Fig. 4b), but caused a shift of 23% of the radioactivity of the RER to densities characteristic of the SER (Fig. 4). In contrast, sonic vibration in the presence of high salt-EDTA buffer cause 80% of the radioactivity of the RER to shift to densities comparable to those of the SER (Fig. 4). High-salt buffer appears to effectively strip ribosomes from the RER (1). The residual 20% virus-specific proteins that banded with an average density of 1.27 g/ml after treatment of the RER were most likely a combination of

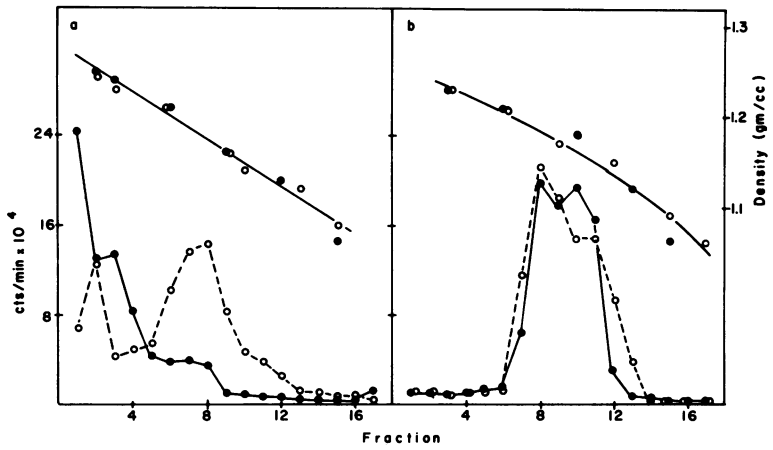

FIG. 4. Effects of sonication on membranes from poliovirus-infected cells. Cells were infected and incubated with 15 μCi of [^{3}H]leucine per ml for 75 min as in Fig. 3. After isolation the RER and SER were diluted twofold with RSB or RSB containing 0.5 M KCl and 10 mM EDTA (final concentrations). Samples were then administered five 10-s pulses with an ultrasonic W-10 high-intensity sonicator, and membranes were centrifuged to equilibrium in sucrose gradients. Fractions (2 ml) were collected, and the radioactivity in 0.5 ml of each was determined after precipitation of proteins with trichloroacetic acid and washing on GF/A filters. (a) RER exposed to sonic vibration in RSB (●) or RSB containing high-ionic-strength buffer and chelating agent (○). (b) SER treated in RSB (●) or RSB containing high salt-EDTA (○).

coat proteins of virions and proteins associated with membranes. Polyacrylamide gel electrophoretic analysis of RER fractions after sonication in the presence of high salt-EDTA buffer demonstrated a high level of VP1, VP2, and VP3 among the denser fractions (Fig. 5; compare RER, 1.19 g/ml with RER, 1.27 g/ml, after high salt-EDTA extraction, i.e., lane 5 versus lane 4). After sonication in high-ionic-strength buffer and equilibrium centrifugation, VP2 was not located among membranes that shifted to lighter densities (Fig. 5, lane 5) reinforcing the previous conclusion that virions cosediment with the RER. The selective distributions of P3-2, VP0 and P2-X noted above are again evident.

The change in density of the RER after treatment with high-ionic-strength buffer and chelating agent can be attributed to the effective release of membrane-associated polysomes (1). Interestingly, virus-specific proteins did not redistribute to the top fractions of the gradients. At least 50 to 70% of radioactivity associated with the SER was removed after exposure to high salt-EDTA buffer and pelleting of membranes (Fig. 2). The absence of radioactivity in the upper portions of equilibrium gradients was probably due to aggregation and banding of virus-specific proteins with smooth membranes. Because of this the sonic vibration study cannot determine whether viral proteins were compartmentalized within vesicles. However, digestion of viral proteins with trypsin suggests the absence of internalization (Fig. 6 and 7).

Treatment of membranes with urea, NP-40, trypsin, and RNase. Even though proteins such as P3-4a, P2-5b, P2-X, and P3-9 are more tenaciously associated with cellular membranes than other virus-specific proteins, only P3-7c was completely released during pelleting of membranes after treatment with high salt-EDTA buffer. In fact, P3-7c was found in the supernatants after treatment with RSB alone, indicating the absence of membrane linkage (Fig. 2). Additional experiments were undertaken to determine whether other agents could completely or selectively release viral proteins from the RER and SER. Membranes were isolated and washed with urea or low concentrations of Nonidet P-40 (NP-40) or incubated with trypsin and RNase. The studies presented in Fig. 6 and 7 were performed with g^s virus, but other experiments with g^r virus provided analogous data (studies not included).

The results of a representative study with the RER are shown in Fig. 6. Urea at concentrations of 2 and 4 M only partially removed viral proteins from membranes, and a low level of NP-40 (0.03%) was virtually ineffective. Urea partially dislodged P2-5b, P2-X, and P3-9 from the RER, but had no effect on P3-4a, even at 4 M. Urea increased the levels of VP1, VP2, and VP3 very noticeably in the supernatants, an expected result due to disruption of virions. The distribution of P1a and P1b (between P1a and P3-2) after exposure to a denaturing agent is not characteristic. These proteins were usually partially released from both the RER and SER with urea as well as high-ionic-strength buffer containing EDTA. Trypsin digested most viral proteins except VP1, VP2, and VP3, which are present in virions that are resistant to trypsin. RNase had no effect on the association of viral proteins with the RER. Proteolysis of viral proteins indicates surface association with membranes and the absence of internalization within vesicles. Studies by others also demonstrated trypsin sensitivity of membrane-bound viral peptides (22). Similar results were obtained with the SER (Fig. 7). Although data with NP-40 are not included, a low level of the detergent was no more effective than RSB during the washing procedures. Trypsin only digested 50% of the

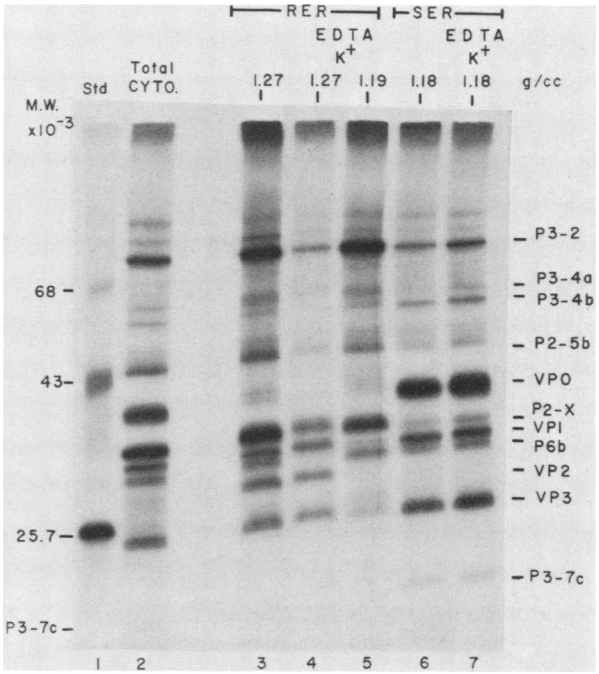

FIG. 5. Viral proteins associated with the RER and SER after sonic treatment. Proteins from peak fractions in the experiment described in Fig. 4 were examined by polyacrylamide gel electrophoresis. RER lanes: 1.27, fractions 1 to 3 in RSB; 1.27 with EDTA and K^+, fractions 1 to 3 in high salt-EDTA buffer; 1.19 with EDTA and K^+, fractions 7 to 9 in high salt-EDTA buffer. SER lanes: 1.18, fractions 8 to 11 treated in RSB; 1.18 with EDTA and K^+, fractions 8 to 11 treated in high salt-EDTA buffer.

proteins in SER samples, but the SER preparations contained more than twice the amount of protein as the RER. The important observation is the absence of selectivity. Among the RER and SER viral proteins, except capsid proteins in mature virions, were not selectively degraded. Even though P3-4a, P2-5b, P2-X, and P3-9 were membrane associated, they remained sensitive to digestion with trypsin.

DISCUSSION

Synthesis of foot-and-mouth disease virus RNA and poliovirus RNA is severely blocked by millimolar levels of guanidine, and the viral gene product responsible for this effect is translated from the central region of the genomes (3, 19). It has been suggested that p34 of foot-and-mouth disease virus and P2-X of poliovirus might function via a membrane association that is critical for polymerase activity and is disrupted by guanidine (19). Because experiments indicate that P2-X is membrane associated (22), studies were undertaken to determine the extent of this association and whether virus-inhibitory concentrations of guanidine could disrupt the protein-membrane complex. The overall conclusions from this report are that many viral proteins are bound to the RER and SER; P3-4a, P2-5b, P2-X, and P3-9 appear to be more tenaciously associated with membranes than are other viral proteins. High-ionic-strength buffer and 4 M urea dislodge, on the average, 50% of viral proteins from membranes, but have negligible or slight effects on the membrane association of P3-4a, P2-5b, P2-X, and P3-9. These virus-coded proteins are not compartmentalized within vesicles since they are degraded by trypsin, an observation made by

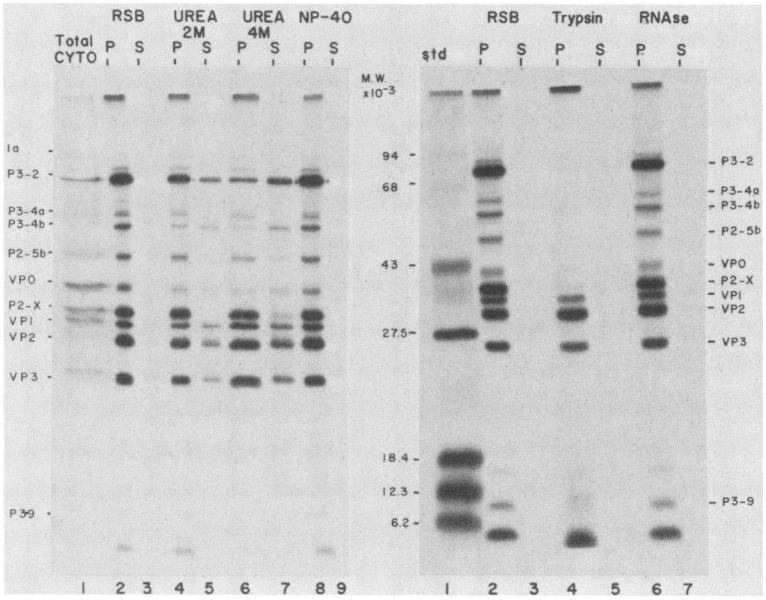

FIG. 6. Treatment of the RER from infected cells with urea, NP-40, and enzymes. HeLa cells were infected as in Fig. 3, and the RER was isolated and distributed equally into tubes. Final concentrations of 2 or 4M urea, 0.03% NP-40, 50 μg of trypsin per ml, or 60 μg of RNase per ml were added. Trypsin-treated samples were incubated for 15 min at 35°C, and 4 trypsin-inhibitory units of aprotinin was then added while samples digested with RNase were held at room temperature 20 min. All samples were then centrifuged at 120,000 × *g* 75 min, and polyacrylamide gel electrophoresis was performed with pellet (P) and supernatant (S) fractions after processing. Electrophoresis was conducted with 13.5% polyacrylamide gels at 160 V for 5 h (left autoradiogram) or 4 h (right autoradiogram).

others (22). Whether they are partially embedded in the lipid component, bind hydrophobically with intrinsic membrane proteins, or both is open to conjecture. These data were obtained with cell-free membranes. The possibility that internalization of viral proteins occurs in vivo has not been ruled out.

Guanidine at 20 to 100 times the standard levels employed to inhibit poliovirus growth had no discernible effect on the binding of P2-X or any viral proteins to the endoplasmic reticulum. The effects of physiological levels of the inhibitor during in vivo viral growth are probably due to mild conformational changes in P2-X that do not disassociate this protein from membranes. It could be argued that guanidine only blocks the initial bonding of P2-X to membranes and has no effect once association is established. However, experiments were performed where viral protein synthesis was inhibited 50 and 80% by guanidine. Extracts were prepared, and membranes were separated and treated with

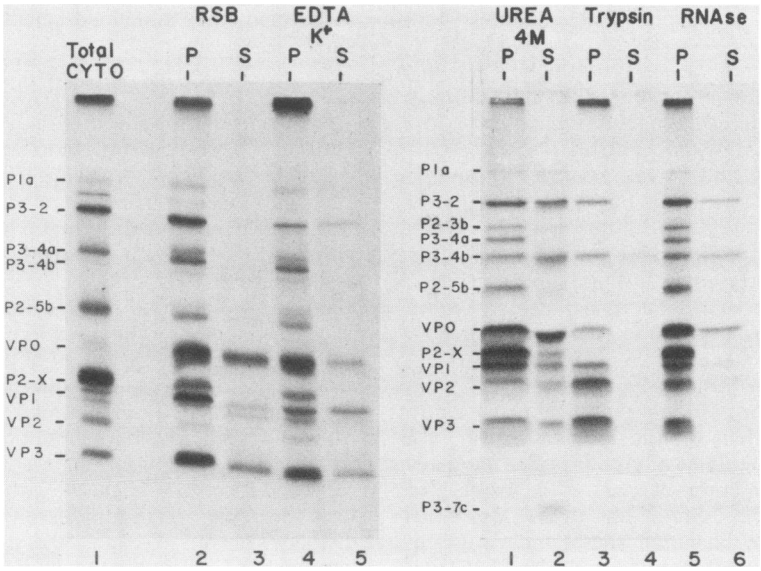

FIG. 7. Treatment of the SER from infected cells with urea, trypsin, and RNase. The experiment was identical to that of Fig. 6. Electrophoresis was conducted for 5 h at 170 V with a 12 to 18% gel polyacrylamide gel (left autoradiogram) and for 5.5 h at 160 V with a 13.5% polyacrylamide gel (right autoradiogram).

the various reagents employed in this study. There were no differences in membrane association of virus-specific proteins in comparison to data presented in this report. P3-4a, P2-5b, P2-X, and P3-9 were avidly associated with membranes when synthesized in the absence or presence of guanidine (data not included). The residual synthesis of these proteins in the presence of guanidine was 50 and 20% in two separate studies.

There are several possible mechanisms whereby PX-2 could participate in viral RNA synthesis. As a component of P9/X (22) membrane association might be critical for cleavage of VPg and subsequent initiation of RNA synthesis. P9/X is a peptide containing, P2-X, P3-9b, and VPg (22). Seventy six percent of the amino terminal region of P9/X is P2-X, which might provide a critical membrane linkage before proteolytic processing. If this were the case the membrane-associated P2-X observed in this report and by others (22) would only represent a nonfunctional byproduct of processing. A second possibility is that a stereospecific complex of P2-X and other viral proteins occurs that is mandatory for the initiation of viral RNA synthesis. Guanidine could disaggregate or structurally alter the polypeptide complexes in vivo during viral growth, but have no effect on extracted membranes because of distortions caused by isolation procedures. P2-X lacks a hydrophobic domain, whereas P3-9 has a hydrophobic region of 22 amino acids (21). P3-9 is membrane bound, has an affinity for P2-X (22), and might serve as an anchor for P2-X and other membrane-associated viral polypeptides as they are generated by cleavage from a common precursor. Conformational changes in this membrane aggregate, induced by guanidine, could restrict the initiation of viral RNA synthesis.

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