Alteration of the Cytoplasmic Domain of the Membrane-spanning Glycoprotein p62 of Semliki Forest Virus Does Not Affect Its Polar Distribution in Established Lines of Madin-Darby Canine Kidney Cells

Laura M. Roman and Henrik Garoff

European Molecular Biology Laboratory, Postfach 10-2209, 6900 Heidelberg, Federal Republic of Germany. Dr. Roman's present address is Howard Hughes Medical Institute, University of Texas Health Science Center at Dallas, Dallas, Texas 75235.

Abstract. Expression of the Semliki Forest virus p62/E2 protein was studied in the polarized epithelial cell line Madin-Darby canine kidney (MDCK). After infection this transmembrane protein, together with the other spike subunit E1, accumulates at the basolateral surface of MDCK cells (Fuller, S. D., C.-H. von Bonsdorff, and K. Simons, 1985, EMBO (Eur. Mol. Biol. Organ.) J., 4:2475–2485). The cDNAs encoding truncated forms of the protein were used to stably transform MDCK cells to examine the role of (a) subunit oligomerization (E1-E2) and (b) the cytoplasmic domain of p62/E2 in directed transport to the basolateral surface. The biochemical characteristics and polarity of the expressed proteins were studied using cell monolayers grown on nitrocellulose filters. A wild-type form of p62/E2, in the absence of E1, and two forms having either 15 or 3 of the wild-type 31-amino acid carboxyl cytoplasmic domain were all localized to the basolateral surface. These results indicate that the cytoplasmic domain of E2 does not contain the information essential for directed transport to the plasma membrane, and imply that this information resides in either the lumenal and/or membrane-spanning segments of this transmembrane protein.

In eukaryotes, the biosynthesis of proteins destined for the diverse array of cellular compartments is restricted to a few sites (e.g., cytosol, endoplasmic reticulum [ER]); for review see Sabatini et al., 1982). This implies that sorting mechanisms must exist which ensure the transport of molecules to their site of function. For secretory proteins and integral membrane proteins of the ER, Golgi complex, endosomes, lysosomes, and plasma membrane, it has been established that the first sorting event occurs at the time the nascent polypeptide is targeted to the membrane of the ER (Blobel and Dobberstein, 1975a; b; Hortsch, M., and D. I. Meyer, manuscript submitted for publication). The specificity of this event is a consequence of the expression of the signal sequence(s) of the polypeptide and the subsequent interaction of the signal recognition particle (Walter and Blobel, 1980) and docking protein (Meyer and Dobberstein, 1980). Further compartmentalization requires other processing events and most likely involves additional sorting signals.

The specific targeting of a number of enzyme activities and receptors to either the apical or basolateral surface of epithelial cells is reflected in the morphological and biochemical polarity of this cell type (Rodriguez-Boulan, 1983; Simons and Fuller, 1986). We have used the epithelial Madin-Darby canine kidney (MDCK) cell line to examine the structural features of plasma membrane proteins involved in their sorting into a specific surface domain. The usefulness of this cell line for these studies stems from the observation of Rodriguez-Boulan, Sabatini, and others (Fuller et al., 1984, 1985; Matlin and Simons, 1983; Misek et al., 1984, 1985; Rodriguez-Boulan, 1983; Rodriguez-Boulan and Sabatini, 1978) that MDCK cells infected with enveloped viruses display a polarized distribution of the viral spike glycoproteins as illustrated for apical influenza hemagglutinin, basolateral Semliki Forest virus (SFV) E1 and E2, and vesicular stomatitis virus (VSV) G-protein. It has been demonstrated that the carbohydrate moieties of the viral proteins are not directly involved in this segregation (Roth et al., 1979; Green et al., 1981). Therefore some other feature located in one of the three topological domains of transmembrane proteins must be recognized by the sorting machinery. Recently, the introduction and expression in eukaryotic cells of cloned genes that encode for foreign or altered forms of membrane proteins has allowed a more precise evaluation of the peptide signals involved in traffic to the plasma membrane (Doy et al., 1985; Florkiewicz et al., 1983; Garoff et al., 1983; Gething and Sambrook, 1982; Kondor-Koch et al., 1983, 1985; Poruchynsky et al., 1985; Rose and Berg-
mann, 1982, 1983; Roth et al., 1983; Stephens et al., 1986; Zuniga et al., 1983; Zuniga and Hood, 1986). The expression of wild-type influenza hemagglutinin and VSV G-protein in MDCK cells has shown that the information necessary for polar transport to the cell surface is contained within the amino acid sequence of the membrane protein examined; i.e., no other viral proteins are required (Roth et al., 1983; Stephens et al., 1986).

In this study we have used the cDNA encoding for a viral membrane protein to examine the role of putative peptide signals in the transport of the p62 spike glycoprotein (E2 and E3 precursor) of SFV to the basolateral surface of MDCK cells. This protein is a transmembrane glycoprotein having an ectoplasmic domain with two N-linked sugar groups, a transmembrane segment (anchor) and a 31-amino acid cytoplasmic domain (tail). During infection all of the structural proteins of SFV (capsid-p62-EL) are derived from a polymersome precursor through co-translational cleavage events (Garoff et al., 1977). The membrane proteins p62 and E1 form a complex in the ER which is efficiently transported to the plasma membrane (Ziemiecki et al., 1980); cleavage of p62 to E2 and E3 is a late, post-Golgi event. In nonpolar baby hamster kidney (BHK) cells it has been demonstrated that p62 expressed from cDNA is transported to the plasma membrane in the absence of E1, whereas E1 alone failed to leave the ER (Garoff et al., 1983; Kondor-Koch et al., 1983). Furthermore it was shown that the cytoplasmic domain of p62 was not essential for movement to the cell surface in BHK cells. The present study examines whether p62/E1 oligomerization and the cytoplasmic domain of p62 are involved in the targeting of this protein to the basolateral domain of MDCK cells. To address these questions, lines of polar MDCK cells that stably express various forms of p62 were established. A preliminary report of this data has been presented in abstract form (Roman and Garoff, 1985).

Materials and Methods

Materials

Restriction endonucleases, DNA polymerase I (Klenow), Sal I linkers, and calf alkaline phosphatase were purchased from Boehringer Mannheim Biochemicals (Mannheim, FRG) and T4 ligase was from New England Biolabs (Schabach/Taunus, FRG). [3H]-Methionine (1,000 Ci/mmole) was from Amersham Buchler GmbH (Braunschweig, FRG); [3H]palmitic acid and [4C]-methylated protein mixture (0.27 μCi/μg) were obtained from New England Nuclear (Bedford, MA). Lysiscotamine, N,N,N',N'-tetramethyl ethylene diamine, ammonium persulfate, and molecular weight standards 

The Journal of Cell Biology, Volume 103, 1986 2608

Figure 1. Expression plasmids. The construction of the pSV-SFV plasmids containing the truncated forms of E2 is described in Garoff et al. (1983). Construction of the d4 mutant in the plMTSFVd4/SVNeo vector is under the control of the human metallothionein promoter as detailed in Materials and Methods. The functional regions of the plasmids are indicated as follows: (hatched) SV-40 early promoter, t-splice site, and polyadenylation signals; (cross-hatching) the neo sequence encoding for G-418 resistance; (dots) the metallothionein promoter; (striping) SFV-cDNA; (unshaded) pBR322 ampicillin resistance and origin of replication. Arrowheads indicate the action of DNA polymerase.

formed as in Hanahan (1983). Preparation of DNA was as described in Garoff et al. (1983) and Kondor-Koch et al. (1983).

Construction of Plasmids Containing p62 Proteins

pSV-SFV-dBd4 and d5 are from the series of deletion mutants described by Garoff et al. (1983). An example of one of these vectors is seen in Fig. 1. To construct the plasmid pMT/SVneo (no E2 insert), the Pvu I-Bam H1, 4.1-kb fragment from the plasmid pMT/SVneo (Roman and Hubbard, 1983; a generous gift of U. Rüther) containing the DNA sequences for the SV-40 early promoter, neo, and the human metallothionein II promoter, was ligated to a 33-bp fragment from the polylinker region of pGem-1 (Promega Biotec, Madison, WI; Bam H1-Hind III) and the Hind III-Pvu I fragment from pSV-SFV which contains the termination and polyadenylation signals. To construct the plasmid pMT-SFVd4/SVneo (pMTNd4), the plasmid pL1-SFVd4 (Cutler and Garoff, 1986; Cutler et al., 1986) was digested with Bam H1 and the 2.3-kb fragment containing SFV sequences isolated from an agarose gel. The ends of this fragment were repaired with DNA polymerase I (Klenow) and ligated to Sal I linkers. After chromatography over an S-300 column and digestion with Sal I, the DNA was ligated to the plasmid pMT/SVNeo that had been digested with Sal I and treated with calf alkaline phosphatase.

Cells

MDCK cells, strain II (Louvard, 1980; Richardson et al., 1981) were grown in MEM with Earle's salts supplemented with 10 mEq Hepes, pH 7.3, 2.2 mM glutamine, 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). When grown on filters, the cells were seeded onto 0.45-μm pore size nitrocellulose filter (HATF 02500, Millipore S.A., Molsheim, France) clamped into mini Marbrook chambers (Hendley Engineering, London, England) as described by Fuller et al. (1984). The electrical resistance was measured using a device similar to that described by Perkins and Handler (1981) applying a current of 100 μA in Hank's balanced salt solution containing Ca++ and Mg++ at 25°C.

Virus

A prototype of SFV was grown in BHK cells and purified as previously described (Kääriäinen et al., 1969).

Immunological Reagents

LAP. The purification of dog kidney leucine aminopeptidase (LAP) was based on the protocol of Roman and Hubbard (1983). Antibodies to LAP (0-200 fraction, 267% pure by Coomassie Blue staining) were raised in rabbits according to the immunization program of Louvard et al. (1982). SFV. Antibodies to SFV proteins were raised in rabbits. Briefly, 150 μg
of purified virus was mixed with 1 × 10⁶ cpm of [35S]methionine-labeled SFV and electrophoresed on a 10% acrylamide gel under nonreducing conditions. E1, E2, and p62 were located using autoradiogram and cut from the unfixed, dried gel. The proteins were eluted from the gel by the procedure of Bravo et al. (1983) and mixed with complete Freund’s adjuvant. A portion of this mixture (25–50 μg/0.5 ml) was injected into the popliteal lymph nodes of anesthetized rabbits and an equal portion injected intradermally. The rabbits were subsequently boosted according to the methods by Lowenthal et al. (1982). The specificity of the antibodies was confirmed by Western blot analysis using extracts from SFV-infected and control cell lysates.

Protein A was isolated from the chloramine-T procedure of Greenwood et al. (1963) to a specific activity of 3.6–6.8 × 10⁶ cpm/μg.

**DNA Transfection of MDCK Cells and Selection of Transformed Clones**

Transfection of MDCK strain II cells was by a modification of the calcium phosphate procedure described by Kondor-Koch et al. (1985). The ratio of selectable-to-nonselectable DNA (neo/E2) used in these studies was 1:10. For the pMTN4 plasmid, which also contained the selectable neo gene, co-transfection was not required. Briefly, 0.5 ml of calcium phosphate-DNA precipitates formed using 10–50 μg plasmid DNA was added to a suspension of MDCK cells (0.5–1.0 × 10⁶) in a 10-cm dish. After a 30-min incubation at room temperature, 3.5 ml of MEM containing 10% FCS and 200 μM chloroquine were added and the cells were incubated for 6 h at 37°C under 5% CO₂. The cells were then treated for 2 min at 37°C with a 15% (wt/vol) glycerol solution, washed, and incubated for 72 h at 37°C in 5% CO₂ atmosphere before being split 1:6 into culture medium containing 10% FCS and 200 μM chloroquine and the purity of the batch of G-418. After 8–10 d under selection, the individual colonies were picked, transferred to 24-well multiplates (NUNC, Alagade, Denmark) and screened for the expression of E2 protein by indirect immunofluorescence. The cells transformed with the pMTN4 vector were incubated for 3 h in 10 μM CdCl₂ before analysis.

**Immunofluorescence**

Indirect immunofluorescent screening of MDCK transformants grown on microchips (3 × 3 mm, Tecnomara Deutschland GmbH, FRG) was as described by Ash et al. (1977). This procedure was modified to facilitate the screening of a large number of clones by placing the antibody solution (5 μl) directly on top of the coverslip. Immunofluorescence of MDCK cells grown on filters was carried out by the technique of Fuller et al. (1984) with the following modifications: (a) 10% newborn calf serum was used in place of gelatin; (b) all solutions were incubated for 30 rain; and (c) all washes were for 10 min.

Indirect immunofluorescent staining of 0.5-μm frozen sections of MDCK monolayers grown of collagen-coated filters (Chambard et al., 1981) was as described in Roman and Hubbard (1983).

**Lysis and Carbonate Extraction of MDCK Cells**

**Lysis.** MDCK cells (1.0 × 10⁶ cells) were harvested using a rubber policeman in ice-cold Dulbecco’s phosphate buffered saline (PBS) and washed once in this buffer (2,000 rpm, 5 min, 4°C). They were then resuspended in 5 ml 10 mM Tris–HCl, pH 8.0, 1 mM MgCl₂ containing a protease inhibitory cocktail (1 μg/ml antipain, leupeptin, and pepstatin), 10 μM benzamidine, 20 kU aprotinin, and 0.2 μM PMSF, and immediately centrifuged (3,000 rpm, 5 min, 4°C). The pellet (50 μl) was resuspended in 0.5 ml 0.2 M citrate buffer, pH 5.5, for 4 h at 37°C. The cells were then washed, extracted with the TDS extraction buffer, and processed for immunoprecipitation as described above.

**Alkaline Extraction.** The PNS fraction was divided in half. To one portion (initial), Triton X-100 (TX-100) and TCA were added to a final concentration of 1% and 10%, respectively; this sample was then stored on ice at 4°C until the other samples were prepared. The other half of the lysate was mixed with an equal volume of 100 mM sodium carbonate, pH 11.0, and incubated at 0°C for 15 min (Fujiki et al., 1982). The suspensions were centrifuged at 4°C for 1 h at 50,000 rpm in polycarbonate tubes in a Beckman 75Ti rotor. The supernatants were transferred to 1.5-ml Eppendorf tubes on the ice, and the pellets resuspended in 0.2 ml 50 mM carbonate buffer and recentrifuged. The resulting soluble fraction was pooled with the first supernatant. The pellets were then resuspended and solubilized in 0.5 ml 1% TX-100, 10 mM Tris–HCl, pH 7.4, 0.15 M NaCl and then TCA was added (10% final). All samples were incubated on ice for at least 1 h and then centrifuged 2 min at 4°C in an Eppendorf Microfuge 5414S (Eppendorf Gerätebau, Hamburg, FRG). The pellets were washed once in -20°C methanol (70%) and prepared for electrophoresis followed by immunoblotting as previously described (Roman and Hubbard, 1984). The recovery and distribution of antigen between the supernatant and pellet fractions was quantitated by direct scanning (Desaga Densitometer, Heidelberg, FRG) of the autoradiograms of the immunoblots or by the silver grain elution technique of Suisa (1983).

**Immunological Analysis of Labeled E2 Proteins**

**Pulse-Chase Experiments.** Subconfluent monolayers (5.0 × 10⁵ cells) were infected for 4 h at 37°C with 100–200 pfu/cell in MEM containing 0.2% BSA, 10 mM Hepes, pH 7.3 (Matlin and Simons, 1983). Before labeling, the cells (infected and transformed cell lines) were incubated at 37°C with 0.5 ml/dish warm MEM lacking methionine and containing 10 mM Hepes, pH 7.3, and 2 mM glutamine. After the labeling period (10 min, 100 μCi/dish [35S]methionine, except where stated in text), the radioactivity was removed and 5 ml/dish warm chase medium (MEM, 10% FCS, 10× methionine (150 mg/liter), 100 μg cycloheximide) was added. Samples were taken at various times by placing individual dishes on a metal plate fitted over an ice bath, and washing three times with ice-cold PBS. The cells were extracted with cold extraction buffer (1% TX-100, 0.5% deoxycholate, 0.05% SDS, in 10 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 2 mM EDTA, 1 mM iodoacetic acid, and the protease inhibitor cocktail described above [TDS buffer]). Immunoprecipitation was essentially as described by Balcarova-Stander et al. (1984) except that 60 μl of a 10% Pasnorbpin slurry was used instead of protein A-Sepharose, antibody incubations were carried out overnight at 4°C, and the bacterial pellets were washed additionally with 10 mM Tris–HCl, pH 7.4 (2×). The samples were eluted from the bacteria by heating (5 min, 95°C) in sample buffer (60 mM Tris–PO₄, pH 6.7, 4.5 mM EDTA, 18 mM dithiothreitol (DTT), 3.6% SDS, 8.8% sucrose).

For endo H treatment, the washed bacterial pellets were suspended in 0.2 ml autoclaved 0.2 M citrate buffer, pH 5.5, with a protease inhibitor cocktail. Each fraction was divided; to one half 10 μl of endo H was added. The samples were incubated at 37°C for 8–12 h with constant rotation. The tubes were centrifuged for 1 min in a microfuge, the supernatants discarded, the pellets washed twice with 10 mM Tris–HCl, pH 7.4, and finally resuspended in 60 μl sample buffer.

**Fatty Acid C.** The samples were incubated with 0.5 μCi [3H]palmitate acid in MEM with Earle’s salts containing 1% FCS, glutamine, and Hepes, pH 7.3, for 4 h at 37°C. The cells were then washed, extracted with the TDS extraction buffer, and processed for immunoprecipitation as described above.

**[^125]I-Protein A Binding Assay of E2 and LAP Proteins**

The assay was adapted from that described by Pfeiffer et al. (1985). Three binding parameters were measured for each sample: "apical," "surface," and "total" (surface + internal). To assay antigens in the apical plasma membrane, MDCK cells grown on filters were fixed directly, and antibody was applied to the apical side of the filter. To assay "surface" and "total" antigen, the basolateral surface was first made accessible by incubating the filters for 20 min at 4°C with 2 mM BETA in PBS(-) before fixation (opened). "Total" filters were subsequently treated with 0.2% TX-100 to expose internal antigens. For "surface" and "total" samples, antibodies were applied to both the apical and basolateral sides of the filter. Fixation was in 3% formaldehyde, freshly prepared from paraformaldehyde overnight at 4°C; all solutions used for these experiments were filtered through a 0.45-μm Millipore filter before use. For the binding assay, wedges equivalent to one-eighth the filter were cut, the basolateral surface was marked, and the filters quenched for 1 h in 50 mM NH₄Cl in PBS(−). This, and all subsequent treatments, were carried out at room temperature and all washes were in 2 ml in 6-well multi-dished (NUNC) with gentle agitation. The filters were then washed with PBS(−) and the "total" sample was made permeable by incubation with 0.2% TX-100 for 5 min at 37°C. All filters were then washed twice with 0.2% gelatin in PBS(−), and then incubated with either anti-E2, anti-LAP, or control antibodies applied to either the apical, or both sides of the filter as described above. The filters were washed three times with PBS-gelatin and then...
Figure 2. The deletion mutants of SFV p62/E2 glycoprotein. Schematic representation of the SFV polyprotein synthesized by infected and the established lines of MDCK cells. During infection the structural proteins of SFV are synthesized as a polyprotein (NH$_2$-Capsid-E3-E2-6K-E1-COOH). Cleavage between capsid/E3, E2/6K, and 6K/E1 occur co-translationally, while processing of the E3/E2 precursor (p62) is a late Golgi event/plasma membrane event. In the mutants the entire gene region coding for E1 and variable amounts of the 6K peptide and the cytoplasmic domain (stippled) of E2 were deleted. The number of amino acids in E2, as deduced from the DNA sequence, is given on the right. In all of the constructs the coding region is terminated by a stop translation codon present in all three reading frames. This oligonucleotide together with the Cla I linker add 5-7 amino acids to the truncated proteins (hatched). Note that in the d9 construct the entire p62/E2 coding region is preserved. This DNA is expected to yield a wild-type form since the E2/6K junction is maintained. The d4 construct encodes for a form of p62/E2 with only half of the cytoplasmic domain while with d1 a protein with only three amino acids of the wild-type tail is generated. The d5 mutant lacks both the cytoplasmic and membrane-spanning domains (solid box). The exact nucleotides present in each of these truncated forms has been confirmed by sequencing analysis (Garoff et al., 1983).

2. Since we usually observed partial processing of p62 to the mature E2 in MDCK cells, we will refer to these proteins as p62/E2. Application of antibodies to the surface of the MDCK lines at 4°C, followed by immunoprecipitation, demonstrated that there was no correlation between surface expression of the antigen and precursor processing (data not shown). The major form of the protein precipitated from each of the cell lines in these experiments migrated as the p62 form; variable amounts of processing were detected. The reduced efficiency of p62 processing to E2 and E3, in the absence of E1, has previously been reported (Kondor-Koch et al., 1983).
Table 1. Efficiency of Transformation*

<table>
<thead>
<tr>
<th>Line</th>
<th>Promoter†</th>
<th>Efficiency‡</th>
<th>Positive§</th>
<th>Inducibility</th>
</tr>
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<tbody>
<tr>
<td>MDCK-neo</td>
<td>–</td>
<td>1 colony/5.0 × 10⁴ cells</td>
<td>–</td>
<td>NA</td>
</tr>
<tr>
<td>d9</td>
<td>SV-40</td>
<td>1 colony/2.5 × 10⁵ cells</td>
<td>3-5</td>
<td>NA</td>
</tr>
<tr>
<td>d4</td>
<td>MT</td>
<td>1 colony/1.6 × 10⁵ cells</td>
<td>50-70</td>
<td>3-5</td>
</tr>
<tr>
<td>d1</td>
<td>SV-40</td>
<td>1 colony/5.0 × 10⁵ cells</td>
<td>7-15</td>
<td>NA</td>
</tr>
<tr>
<td>d5</td>
<td>SV-40</td>
<td>1 colony/5.0 × 10⁵ cells</td>
<td>15-20</td>
<td>NA</td>
</tr>
</tbody>
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* The plasmids (pSV + pSVneo or pMTN) were linearized with Pvu I which cuts all three DNA molecules within the portion of the plasmid coding for the resistance to ampicillin. MDCK II cells (0.5-1.0 × 10⁶ cells) were transfected in suspension using 10 μg DNA. The ratio of selectable/nonselectable DNA for the pSV vectors was 1:10. Colonies resistant to the antibiotic G418 were analyzed for the expression of p62/E2 by indirect immunofluorescence staining of cells grown on coverslips.
† The DNA encoding for the d9, d1, and the d5 forms of p62/E2 were placed downstream of the SV-40 early promoter, while in d4 the p62/E2 coding sequences were placed downstream of the human metallothionein II promoter. After incubation of the d4 line with 10 μM CdCl₂ (3 h at 37°C) a three- to fivefold induction of the protein was achieved as assayed by both Northern blots and immunoblotting techniques. No induction was seen after similar treatment of the SV-40 constructs.
‡ The number of p62/E2 expressing colonies is given per cells used in the transfection.
§ In all cases, the clones obtained were mixed; that is, while all of the cells were neo positive only the percent given were also positive for p62/E2. These numbers were determined at the microscope by counting the number of p62/E2⁺ cells in a given field and expressing this value as a percent of the number of cells in that field.
NA, Not applicable.

SV-40 early promoter or the inducible human metallothionein IIA promoter. MDCK cells were transfected in suspension with this DNA along with a selectable marker conferring resistance to the antibiotic G-418 provided in trans or cis, respectively (Fig. 1). G-418-resistant colonies were then screened for the presence of p62/E2 by indirect immunofluorescence. G-418-resistant colonies were obtained with an efficiency of one clone per 5 × 10⁴ cells per μg DNA (Table I); and 6-30 % of the G-418-resistant clones coexpressed the nonselectable DNA. By immunofluorescence, these clones were mixed, that is, while all the cells were G-418-resistant, they were not all reactive for p62/E2 protein. The percent p62/E2-positive cells ranged from 3-70% depending upon the clone examined (Table I; see Discussion for possible reasons for this mixed phenotype).

Biochemical Characterization of the Established Lines

The p62/E2 proteins synthesized by SFV-infected MDCK cells and the transformed cell lines were analyzed by SDS PAGE followed by immunoblotting. The pattern obtained (Fig. 3 a) indicates that the proteins expressed by the stable lines were of the size predicted relative to the number of amino acids deleted. The cleavage between p62 and the truncated 6K peptide seems to have occurred since d9 migrates with the p62 band from infected cells; the uncleaved product is expected to migrate 3,500 D larger. The loss of nine amino acids (~1,000 D) in the d4 mutant is not resolved. The removal of 20 amino acids in d1 (~2,500 D) and 82 residues in d5 (~30,000 D) generated proteins with slightly faster mobilities than wild-type p62. The level of expression in the d4 clones could be enhanced three- to fivefold by adding Cd²⁺ to the culture medium (Fig. 3 c). Northern blot analysis (data not shown) demonstrated that this induction occurred at the transcriptional level as expected for the metallothionein promoter (Rüther et al., 1985).

The biochemical properties of the p62 protein and its mutants were further characterized by alcalike treatment and partitioning into the detergent phase after Triton X-114 extraction.

Alkaline Extraction. Treatment with 0.1 M Na₂CO₃ at 0°C has been used as an assay to operationally define membrane-bound proteins (Steck and Yu, 1973; Davis and Model, 1985; Fujiki et al., 1982). For these studies, half of the PNS was mixed with 0.1 M Na₂CO₃, pH 11.0, whereas the other portion of the PNS served as the "initial" fraction. After sedimentation, the pellet was re-extracted with carbonate, the supernates from both extractions were pooled, and the distribution of p62/E2 between the membrane pellet and the supernate fractions was examined by immunoblotting (Fig. 3 b). Greater than 95 % of the proteins expressed by the d9, d4, and d1 cell lines was found associated with the membrane fraction (the recovery in these experiments [initial = supernate + pellet] was 90-100%). D5 however was quantitatively recovered in the supernate fraction (>97%).

Partitioning in TX-114. To further characterize the proteins synthesized by the established cell lines, we used phase separation in the detergent Triton X-114 (TX-114). Partitioning of a protein between the aqueous and the TX-114 detergent phase has been proposed as a means to distinguish peripheral from membrane-bound proteins (Bordier, 1981). For these experiments, the cells were extracted with TX-114, subjected to three rounds of phase partitioning, and the resulting supernate and detergent phase was examined by immunoblotting. The results obtained (data not shown) agreed with those of the carbonate extraction. That is, >90% of the membrane-bound forms of p62/E2 synthesized by SFV-infected cells and the cell lines partitioned into the detergent phase while >97% of the d5 form was recovered in the supernate.

Biochemical Analysis of Transport to the Plasma Membrane

After core glycosylation, newly synthesized glycoproteins are processed (e.g., carbohydrate modification, fatty acylation, sulfation, etc.). These modifications begin in the ER and are completed in the Golgi complex. A number of the enzymes involved in these events have been identified and localized to specific intracellular compartments (Kornfeld and Kornfeld, 1985; Berger and Schmidt, 1985). Thus the extent of processing at any point in the biosynthesis of a glycoprotein reflects, in part, the compartments it has been transported through. We have used two such processing events, the acquisition of complex carbohydrates and fatty acylation, as an attempt to monitor transport of the p62/E2
proteins from the ER to the plasma membrane. In addition, we have examined the medium for the secreted d5 form of p62/E2.

**Analysis of the Culture Medium for Secreted Forms of p62/E2.** In other viral systems, removal of the cytoplasmic and membrane-spanning segments has resulted in the secretion of hemagglutinin, G-protein, and the rotovirus VP7 (Florkiewicz et al., 1983; Gething and Sambrook, 1982; and Poruchynsky et al., 1985). Since our d5 construct partitioned as a peripheral protein, we examined the apical and basolateral medium for the secreted protein. However, under all conditions tested, released p62/E2 was never detected. By indirect immunofluorescence a strong reticular pattern was observed suggesting that the antigen was concentrated in the ER (data not shown). The exact reason why d5 failed to be transported is not known. Since the polarity of secretion could not be examined, we focused on the membrane-bound forms of p62/E2.

**Carbohydrate Processing.** The acquisition of resistance to the enzyme endo H (Tarentino and Maley, 1974), indicative of the addition of complex oligosaccharides, has been used to monitor the transport of newly synthesized glycoproteins from the ER to the Golgi complex. To assess the rate at which the various forms of p62/E2 were transferred to the Golgi complex, pulse–chase experiments were carried out on the established MDCK lines and SFV-infected cells. For all time points examined (0 min to 6 h chase), p62/E2 from infected and the stable MDCK lines was found to be sensitive to endo H, whereas the E1 synthesized by the infected cells did become partially endo H resistant (data not shown). While the simplest explanation for these observations is that our mutants do not leave the ER, other data (see below) indicated that the proteins are transported to the plasma membrane. The presence of endo H–sensitive forms of mature p62/E2 has previously been reported (Kääriäinen and Pessonen, 1982). The failure of our mutant p62/E2 proteins to be converted to an endo H–resistant form precluded our use of this assay to measure their transport kinetics from the ER to the Golgi complex and from the Golgi complex to the plasma membrane.

**Attachment of Fatty Acids.** The addition of fatty acids to newly synthesized proteins is believed to be a late ER event (Berger and Schmidt, 1985). Berger and Schmidt (1985) have shown that the glycoproteins of SFV contain covalently attached fatty acids which are detected after labeling infected cells with [3H]palmitic acid. To determine if fatty acids were added to the p62/E2 proteins expressed by the various MDCK lines, SFV-infected MDCK-neo and the transformed lines were labeled with [3H]palmitic acid or [35S]methionine, and the p62/E2 protein was precipitated and analyzed by SDS PAGE. The E2 synthesized by SFV-infected MDCK-neo and the transformed lines were labeled with [3H]palmitic acid or [35S]methionine, and the p62/E2 protein was precipitated and analyzed by SDS PAGE. The E2 synthesized by SFV-infected MDCK-neo cells contained the palmitate label as did the membrane-bound forms of p62/E2 expressed by the stable lines (d9, d4, d1; Fig. 4). D5, however, which lacks the membrane-spanning segment, was not fatty acylated. Evidence that the amount of d5 protein present was equivalent to the other MDCK lines was obtained from parallel experiments carried out on [35S]methionine-labeled samples.
Fatty acylation of p62/E2 proteins in transformed and infected MDCK cells. Cells (1 x 10^6) were labeled for 4 h at 37°C with [3H]palmitic acid. The lysates were incubated with anti-E2 antibodies, and the immunoprecipitate analyzed by SDS PAGE followed by fluorography (40 d). Equal volumes from the immunoprecipitated samples were loaded. (Lane 1) SFV-infected cells immunoprecipitated with anti-E2 antibodies; (lane 2) MDCK-d9; (lane 3) MDCK-d4; (lane 4) MDCK-dl; (lane 5) MDCK-dl, from [35S]methionine-labeled d1 cells; (lane 6) MDCK-d5, palmitate label; (lane 7) MDCK-d5, methionine label. The arrows indicate the positions of p62 and E2; the mobilities of the molecular weight standards are given on the right. The membrane-bound forms of the viral proteins are acylated while the d5 mutant lacking the membrane-spanning segment is not labeled with [3H]palmitic acid. Comparison of lanes 4 and 5 and lanes 6 and 7 confirm that our failure to detect acylation of d5 is not the result of there being a lower amount of this protein in this cell line relative to the other MDCK-transformed cell lines. The methionine-labeled samples were electrophoresed on the same gel as the samples described above; exposure 21 d. Note that some processing of d4-p62/E2 to the mature E2 form is evident.

Truncated Forms of p62/E2 Are Expressed at the Basolateral Surface of Filter-grown MDCK Cells

For the analysis of polarity of the p62/E2 expression by the established cell lines, MDCK cells were seeded onto nitrocellulose filters fitted into mini-Marbrook chambers at a density of 1 x 10^6 cells/filter. For all of the cell lines, tight monolayers with electrical resistances of 150-200 ohms/cm^2 were formed. These resistance values were identical to those of the untransformed parental MDCK II line demonstrating the tightness of the junctions between individual cells (Cerjido et al., 1978). The filter system is particularly amenable to localization studies since antibodies can be used to selectively probe one cell surface or the other. This permits the basolateral cell surface to be visualized without interference from apical staining.

Immunofluorescence. Fig. 5 shows the characteristic staining pattern of SFV-infected cells. Immunofluorescent staining of the cell surface reveals that the p62/E2 protein is expressed at the basolateral surface in agreement with the results obtained by Fuller et al. (1985). When antibodies were applied to the apical surface of SFV-infected cells, staining was never observed (data not shown). In contrast, LAP, an apical membrane protein, was only detected when antibodies were applied to the apical side of the filter (compare LAP-A and LAP-B). Examination of the transformed cell lines grown on filters confirmed that the cells formed a continuous monolayer (Fig. 5 c). The fluorescent pattern seen after application of the anti-E2 antibodies to the apical or the basolateral surface is seen in Fig. 5, a and b, respectively. Only a fraction of the cells in the monolayer is positive for p62/E2, in agreement with the results presented in Table I. Expression of all three forms of p62/E2 by the transformed cell lines was polar in filter-grown cells; that is, staining of the antigen was only observed when the antibodies were applied to the basolateral surface (Fig. 5 b). Thus it appears the E1 and an intact cytoplasmic domain are not essential for directed transport of p62/E2.

0.5-μm Sections. One of the limitations in the direct staining of MDCK cells grown on filters is that the apical and basolateral surface of one cell can not be observed simultaneously. To obtain a better view of the polar distribution of the mutant forms of p62/E2, semi-thin frozen sections of the cell monolayer grown on collagen-coated filters were cut perpendicular to the plane of the substratum. As a control, the distribution of two endogenous proteins, a 58-kD protein which in parental MDCK cells is confined to the basolateral plasma membrane domain (Balcarova-Ständer et al., 1984), and LAP, a marker for the apical surface (Louvard, 1980), were examined in the transformed cell line. The results obtained with the controls and two of the cell lines (d4 and dl) are seen in Fig. 6. The antibody to the basolateral antigen stains the basal and lateral surfaces (Fig. 6 a), while anti-LAP antibodies recognize an antigen at the apical surface as well as within an internal compartment (Fig. 6 b). In the MDCK-d4 cells (Fig. 6 c), the p62/E2 protein is concentrated at the basolateral surface, and staining of the apical surface was never observed. Occasionally with the d4 cells stained for either the basolateral antigen or p62/E2, it appeared that there was internal staining of a structure below (basal to) the nucleus. While we can not exclude that some of this staining may represent an internal pool of antigen, we attribute this pattern to the plane of the section examined since other data (see below) indicated that there was not a large pool of internal antigen. Similar results were obtained with the dl line (Fig. 6 e). Reproducible staining of the d9 line was not obtained since such a small percent of the cells in the monolayer was expressing p62/E2.

Quantitation of E2 Surface Distribution in the Established Cell Lines

The accumulation of the forms of p62/E2 on the basolateral surface of the cell lines grown on filters was quantitated by a surface radioimmunoassay (Table II and Fig. 7). As controls, the distribution of E2 in infected MDCK cells and the apical marker LAP were also examined. Of the p62/E2 pro-
Figure 5. Polarity of surface p62/E2 expressed by infected and transformed MDCK cells. Cells were grown for 4 d on 0.45-μm Millipore filters. Before fixation the electrical resistance was measured. All clones had resistances between 150–200 ohms/cm². After fixation, specific antibodies were applied to either the apical or basolateral side of the filter followed by a second rhodamine-conjugated antibody. (Upper left and middle panels: LAP) Antibodies to LAP were applied to the apical surface (LAP-A) or the basolateral surface (LAP-B) of control cells (MDCK-neo; MDCK cells that had been transformed with the selectable marker gene neo; see Table I). (Upper right panel: SFV) MDCK-neo cells infected with SFV 5 h before fixation, anti-E2 antibodies added to the basolateral surface. The established MDCK lines (d9, d4, and d1) were incubated with anti-E2 antibodies applied either to the apical (a) or basolateral (b) side of the filter followed by rhodamine-conjugated anti-rabbit second antibodies. (c) Hoechst patterns corresponding to column b. Bar, 20 μm.

Protein expressed at the cell surface of both infected and the transformed cell lines, 95–98% was on the basolateral side compared with 1–5% found in the apical membrane. In contrast, 96–99% of the LAP was concentrated at the apical surface while only 1–3% was localized to the basolateral domain. When the amount of E2 found at the basolateral surface was expressed as a function of the total amount of antigen present in the cells, 77–92% was present at that plasma membrane domain, and 7–23% was found to be internal (Fig. 7). This was quite different from the results obtained with LAP. For this marker, 40–50% was found to be internal. The presence of a relatively large internal pool of LAP and a small pool of E2 are corroborated by the immunofluorescent staining of the frozen semi-thin sections. The distribution of p62/E2 and LAP at the apical, basolateral, and internal sites is assessed by this binding assay. However, differences in the antibodies do not allow a direct comparison of the antigens. In addition, the low number of counts,
the high background, and the variability of the number of expressing cells in the different MDCK lines prevents an accurate assessment of the amount of antigen present. Despite these reservations, we feel that with the reproducibility of the relative distribution obtained that these data accurately reflect the distribution of p62/E2 and LAP at the apical and basolateral surfaces and at internal sites.

Discussion

Our results indicate that the expression of the gene encoding for wild-type and truncated forms of p62/E2 in the absence of E1 is polar in stably transformed lines of MDCK cells. Thus the formation of p62/E1 complexes characteristic of the viral spikes are not required for polar expression. The oligomeric state of the expressed proteins has not yet been established. As the structure of the cytoplasmic domains of the mutated and wild-type proteins differ substantially in length and amino acid sequence, it seems unlikely that this domain of the E2 protein carries the information for its directed transport to the basolateral domain of this polarized epithelial cell line. The only common structure of the various cytoplasmic domains is the basic Arg-Ser-Lys sequence adjacent to the membrane-spanning segment. The presence of basic amino acids on the cytoplasmic side of the membrane-spanning segment, however, is common to all single-span-

Table II. Surface Distribution of p62/E2 and LAP in Infected and Transformed MDCK Cells*

<table>
<thead>
<tr>
<th>Cells (n)</th>
<th>Antigen</th>
<th>Apical</th>
<th>Surface</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected† (4)</td>
<td>p62/E2</td>
<td>1,430 ± 240</td>
<td>42,500 ± 6,730</td>
<td>56,400 ± 5,680</td>
</tr>
<tr>
<td></td>
<td>LAP</td>
<td>3,200 ± 140</td>
<td>3,580 ± 200</td>
<td>7,400 ± 320</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>950 ± 30</td>
<td>1,460 ± 210</td>
<td>2,860 ± 100</td>
</tr>
<tr>
<td>d9 (11)</td>
<td>p62/E2</td>
<td>900 ± 130</td>
<td>6,460 ± 140</td>
<td>7,450 ± 220</td>
</tr>
<tr>
<td></td>
<td>LAP</td>
<td>5,100 ± 300</td>
<td>5,700 ± 410</td>
<td>10,600 ± 900</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>970 ± 150</td>
<td>1,620 ± 150</td>
<td>2,270 ± 150</td>
</tr>
<tr>
<td>d4** (3)</td>
<td>p62/E2</td>
<td>1,460 ± 60</td>
<td>20,300 ± 1,500</td>
<td>22,140 ± 2,000</td>
</tr>
<tr>
<td></td>
<td>LAP</td>
<td>5,150 ± 450</td>
<td>6,350 ± 430</td>
<td>9,400 ± 500</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1,290 ± 30</td>
<td>2,350 ± 40</td>
<td>2,793 ± 176</td>
</tr>
<tr>
<td>d1 (9)</td>
<td>p62/E2</td>
<td>1,120 ± 400</td>
<td>8,750 ± 1,250</td>
<td>10,600 ± 1,550</td>
</tr>
<tr>
<td></td>
<td>LAP</td>
<td>5,040 ± 270</td>
<td>5,500 ± 370</td>
<td>9,760 ± 530</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>830 ± 80</td>
<td>1,320 ± 200</td>
<td>2,460 ± 320</td>
</tr>
</tbody>
</table>

* MDCK cells were grown for 4 d on 0.45-μm nitrocellulose filters. Samples for surface and total labeling were treated with 2 mM EGTA for 5 min at 37°C before fixation. The distribution of antigen was measured by a 125I-protein A binding assay (see Materials and Methods) using 0.5–1 × 10⁶ cpm of 125I-protein A per filter wedge. The number of determinations is given in parentheses. The numbers given represent the average cpm ± the standard deviation. The control samples were the experimental cells probed with nonimmune antibodies.
† Antibodies were applied to the apical side of a closed monolayer.
‡ Antibodies were applied to both sides of EGTA- and Triton X-100–treated filters.
†† Monolayers of filter grown MDCK cells were infected with SFV (70 pfu/cell) 5 h before fixation.
** MDCK-d4 cells were incubated with 10 μM CdCl₂ for 3 h before fixation.
In this work we have used stable transformation as a way to introduce the wild-type and mutagenized cDNAs into MDCK cells. Recently Stephens et al. (1986) used vaccinia virus as a vector to examine the polar expression of several viral proteins in MDCK cells. These investigators obtained efficient transport of wild-type hemagglutinin to the apical surface and G-protein to the basolateral domain in the absence of other viral proteins. The major drawback of this system is that the cytopathic effects of viral infection disrupt epithelial cell polarity and eventually kill the cell. The major problem with our system was a relatively low level of protein expression. This is partly due to the fact that a fraction of the cells in each clone were expressing the p62/E2 protein. The mixed nature of the clones was apparent from the initial immunofluorescent screening of the colonies. It is possible that these colonies arise from aggregates of cells transformed with only the neo gene, and cells transformed with both the selectable and nonselectable plasmids. Alternatively, the expression of the p62/E2 protein or capsid could be toxic to the cells. However, the fact that the percent positive cells in each of the stable lines did not change significantly over passage suggests that the loss of the DNA encoding for p62/E2 could not account for the mixed populations. The highest number of p62/E2-positive cells were found in the d4 line where the selectable marker was present on the same plasmid as the DNA encoding for p62/E2. After incubation of MDCK-d4 with cadmium, a three- to fivefold induction of p62/E2 was observed. By immunofluorescence, it was determined that the induction reflected an increase in the amount of protein made per cell rather than an increase in the number of expressing cells. The fact that this construct has the selectable marker neo in the same plasmid and leads to a lower basal level of viral proteins does not allow us to distinguish between aforementioned alternatives. Heterogeneity of cloned populations of cells in terms of the number of expressing cells, the level of expression per cell, and the fate of the expressed protein have been described by others (Burgess et al., 1985; Florkiewicz et al., 1983; Moore et al., 1983; Zuniga and Hood, 1986). Since the proteins expressed by the stable lines were polar in their distribution, we feel that this heterogeneity does not affect the interpretation of our results.

Attempts to enrich the population of p62/E2-positive cells by dilution subcloning have only been marginally successful. The highly enriched colonies obtained by this approach often lost their epithelial morphology and ability to form monolayers with resistances >150 ohms/cm². Immuno-isolation techniques used to obtain MDCK transformed lines which stably express VSV G-protein at their basolateral surface (Roman et al., 1985) could not be used for the p62/E2 lines since the trypsin treatment needed to generate a cell suspension to present to the immunoadsorbent destroyed the surface p62/E2 molecules.

Possible Functions of the Cytoplasmic Domain

The results obtained in this and other studies indicate that there are no dominant signals within the cytoplasmic domain of transmembrane proteins that determines the efficiency, rate, or direction of their intracellular transport (for review see Discussion of Doyle et al., 1985). It is likely that this domain is functional in other recognition and targeting events. For example, it is thought that the viral nucleocapsid recognizes and binds to the cytoplasmic domain of the spike glycoproteins at the host plasma membrane and thereby surrounds
itself with a viral envelope containing only viral-encoded proteins (Simons and Warren, 1984). Similarly the cytoplasmic domain of transmembrane proteins may function in endocytic events. For the low density lipoprotein receptor, it has recently been shown that mutation of a single cysteine residue in the cytoplasmic tail of this membrane protein can prevent its internalization (Lehrman et al., 1985). Since a number of plasma membrane proteins share the same fate (clustering in coated pits → coated vesicles → endosomes → lysosomes), it will be of interest to determine whether any common feature(s) can be found in the cytoplasmic domain that will indicate the direct involvement of this protein domain in the endocytic process.

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