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Role of Reticuloendotheliosis Virus Envelope Glycoprotein in Superinfection Interference

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Cells expressing specific proviruses are resistant to superinfection by viruses of the same subgroup. To investigate the role of the reticuloendotheliosis virus (REV) envelope glycoprotein (*env*-gp) in the establishment of resistance to superinfection, we constructed plasmids that express either the wild-type *env*-gp or an *env*-gp derivative that lacks part of the transmembrane (TM) protein. After transfection, transient expression of the wild-type *env* gene resulted in syncytium formation in a mammalian cell line permissive for virus replication, whereas synthesis of the TM-defective *env*-gp did not result in syncytium formation. Several stable cell lines expressing either the normal or TM-defective *env*-gp were isolated. Expression of the normal *env*-gp in the absence of expression of other viral genes induced resistance to infection by REV. Immunofluorescence analysis of cells expressing the TM-defective *env* derivative and an examination of the glycosylation pattern of this peptide indicated that it is not translocated to the cell surface but resides primarily in the rough endoplasmic reticulum. However, these cells were also resistant to REV infection. Thus, interaction between the *env* derivative and the cellular component that functions as a receptor for the virus can occur in the endoplasmic reticulum and renders the cell immune to superinfection.

Infection of chickens by members of the reticuloendotheliosis virus (REV) family results in a runting disease syndrome characterized by atrophy of the thymus and bursa of Fabricius, anemia, liver and spleen necrosis, and depression of the cellular and humoral immune responses (50). In cultured chicken embryo fibroblasts, REV infection induces acute cytopathic effects, as evidenced by extensive vacuolization, loss of normal cell shape, and extensive cell death (40).

One possible outcome of retrovirus infection is the formation of giant multinucleated cells resulting from cell fusion. This syncytium formation is mediated by the intercellular recognition by the membrane-anchored envelope glycoprotein (*env*-gp) of the viral receptor on the surface of a neighboring cell (21, 35). Such retrovirus-induced fusion is restricted to particular virus-cell-type combinations (36), and virus isolates (3) can differ in their ability to cause syncytia.

Retrovirus infection also leads to the eventual establishment of superinfection interference whereby chronically infected cells are resistant to further entry by the same retrovirus (37, 41). This process prevents reinfection of cells already harboring active proviruses. The inability to rapidly establish such interference may result in substantial superinfection, which may be responsible for the cytopathic effects observed in massively reinfected cells (13, 14, 19, 24, 30, 33, 39, 42, 46, 47). For the avian leukosis virus, a viral determinant of cytopathic effect appears to be localized within the *env* gene (6). The essential role of the *env*-gp in interference has been demonstrated in previous studies using cell lines carrying replication-defective proviruses (32, 38), by genetic analysis of resistance provided by germ line proviruses (29), and through the use of glycosylation inhibitors (17, 28). It has been hypothesized that superinfection interference is due to complex formation between the cellular protein that serves as the virus receptor and the endogenously synthesized *env*-gp (37, 38). This interaction could take place during translocation to the cell surface or be restricted to the cell surface after translocation of both ligands. This specific binding then results in the abrogation of superinfection due to the masking or reduction of functional virus receptor on the cell surface (5, 38).

We have investigated the consequence of REV *env* gene expression by using expression vectors containing either the wild-type *env* gene or an *env* gene that produces a protein lacking the C-terminal end of the transmembrane protein (designated *env* del-gp). In a transient transfection assay, expression of the wild-type but not the defective *env* gene resulted in syncytium formation. However, stable cell lines that harbored and expressed the wild-type *env* gene could be isolated, and these did not exhibit such cell fusion. These cell lines exhibited an approximately 100-fold increase in resistance to infection and were also resistant to cell fusion when tested in the syncytium assay. Stable cell lines expressing the *env* del gene produced an *env*-gp derivative that was not properly translocated to the cell membrane but remained in the rough endoplasmic reticulum (ER). However, as with the cell lines expressing the wild-type *env* gene, they were resistant to infection. Thus, immunity to superinfection can apparently result from interaction of the *env* gene product with its ligand in the ER.

MATERIALS AND METHODS

Plasmids and DNA sequence analysis. The eucaryotic expression vector into which spleen necrosis virus (SNV; an REV species) sequences were inserted is the retrovirus vector pJD214 (gift of J. Dougherty [7]). A *Sac*II-to-*Sac*I fragment of SNV provirus plasmid pPB101 (1) encoding the entire *env* open reading frame (48) was treated with S1 nuclease and inserted into the *Sma*I site of the polylinker in pJD214 to yield pENV. Plasmid pENVdel was constructed by cleaving pENV at an *Xba*I site, treating the DNA with DNA polymerase (Klenow fragment), and carrying out intramolecular ligation to the blunt *Xmn*I site in the TM region of the *env* gene. A frameshift mutation was introduced in pENV at the *Bst*EII site, which is located at a site in the *env* gene overlapping the junction between the leader peptide

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and the amino terminus of the processed *env*-gp. The *Bst*EII site was filled in with Klenow polymerase and religated to produce pENVX. This frameshift would be expected to result in premature translation termination.

For sequence analysis of the transmembrane region of pENV and pENVdel, a restriction fragment encoding the region was subcloned into the vector pTZ18R (Pharmacia, Uppsala, Sweden) and sequenced by the dideoxy-chain termination method.

Cells and viruses. The canine osteosarcoma cell line D17 (from the American Type Culture Collection, Rockville, Md.) and derived clones were grown in modified Eagle medium with 7% bovine calf serum (Hyclone). Chronically infected cells were prepared by transfection of D17 cells with plasmids encoding either replication-competent REV-A(pSW253)(2) or SNV(pPB101) (1). The cultures were then passaged for 6 weeks. REV-A and SNV are different REV strains (50) with approximately 98% nucleotide homology as determined by restriction mapping and by DNA sequence analysis of segments of both proviruses (7). Both viruses are members of the REV group. The helper cell line C3, which expresses all REV proteins required for replication, has been previously described (45). Helper cell line .2G was constructed in a manner similar to that used for construction of the C3 line except that it is methotrexate resistant (J. Dougherty, unpublished data). D17 cell lines expressing the *env* gene or *env* gene derivative were made by cotransfection with 5 μg of pENV, pENVdel, or pENVX and 0.5 μg of pFR400 (expressing a derivative of the dihydrofolate reductase gene) (34) by the polybrene-dimethyl sulfoxide method (18), followed by methotrexate selection (2×10^{-7} M). Cell lines were twice purified from isolated colonies and checked for uniform *env* expression by indirect immunofluorescence.

Assay for superinfection. REV particles carrying RNA with the *hyg*^{r} (hygromycin resistance) gene were generated by cotransfection of chicken embryo fibroblasts with 0.1 μg of pSW253 (helper virus) and 5 μg of pJD214Hy (a retrovirus vector carrying the *hyg*^{r} gene). Virus was collected 5 days later and stored at −70°C. Infections were done in triplicate with 4×10^5 D17 clone-derived or previously infected cells in the presence of polybrene (100 mg/ml) for 45 min. Medium was then added to give a polybrene concentration of 10 mg/ml. Hygromycin selection at 50 μg/ml was started 24 h after infection. Hygromycin-resistant colonies were quantified 10 days later. Violet blue staining of the resistant colonies was used to facilitate quantification. In the cell-mixing experiment, half the usual number of cells from two different cell clones were plated and grown together 24 h before infection. The medium was neutralized with a 200-fold dilution of rabbit anti-*env* serum in normal medium for 24 h before infection.

Transient assay for syncytium formation. A total of 10^6 D17 cells on 60-mm plates were transfected with 5 μg of pENV and 0.5 μg of pJD214Hy by the polybrene-dimethyl sulfoxide technique (18). Hygromycin selection at 50 μg/ml was started 24 h later. Cells were monitored frequently after transfection. Syncytium formation was observed 6 days posttransfection. It should be noted that syncytium formation is cell density dependent; when 5×10^5 cells or fewer are used in the assay, no syncytium formation is observed. Cotransfection of pENV and pJD214Hy followed by hygromycin selection was found to be required for visualization of syncytia. Without selection, we observed continued growth and division of cells that were not transfected or not involved in syncytium formation. This continued proliferation obscures syncytia that may be present on the plates.

Antisera and virus neutralization. For generation of *env* antigen, the entire extracellular region of the SNV *env* gene was inserted into the *Escherichia coli* expression vector pIC20R (22). The *Bst*EII-to-*Xmn*I fragment of pPB101 (encoding the *env* gene from the leader processing site to the transmembrane region [1]) was made blunt ended by treatment with the Klenow fragment of DNA polymerase I and was inserted in the correct reading frame at the *Sma*I site of pIC20R. This plasmid was introduced into *E. coli* JM83, and the expressed protein was isolated by preparation of inclusion bodies (25). The *env* peptide isolated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the inclusion body preparation was injected into rabbits. Rabbit antiserum against *gag* antigen expressed in *E. coli* was provided by Tom Weaver.

A 200-fold dilution of the immune serum was able to neutralize REV. A 1-h incubation on ice was sufficient to neutralize 10^6 *hyg*-transforming virus particles in a stock containing such particles and associated helper virus.

Immunofluorescence and immunoprecipitation. Indirect immunofluorescence staining of D17-derived cells was achieved by growing the cells overnight on a glass cover slide and fixing the cells in −20°C methanol for 10 min. Rabbit anti-*env* antiserum was used at a 1:100 dilution, followed by treatment with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G. The subcellular location of the Golgi was determined by using rhodamine-labeled wheat germ agglutinin.

Cell lysis, immunoprecipitation, and washes were performed in phospholysis buffer, which contained 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10 mM NaH_2PO_4, 0.01% bovine serum albumin, and 1% Trasylol (FBA Pharmaceuticals, New York, N.Y.). Labeled cell lysate was precleared with preimmune sera and protein A-Sepharose. After the first immunoprecipitation, the pellet was resuspended in phospholysis buffer and boiled for 4 min. The supernatant was again immunoprecipitated. The sample was then boiled in loading buffer (10% [wt/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 2.3% [wt/vol] SDS, 0.0625 M Tris hydrochloride [pH 6.8], 0.001% bromophenol blue) and run on an SDS-polyacrylamide gel.

Protein labeling was done by using 125 mCi of [^{35}S]methionine with 10^6 cells in methionine-free medium. Label chase was performed with complete modified Eagle medium. Sugar labeling of 10^6 cells was done with 100 mCi of [^{3}H]mannose or -fucose for 8 h in modified Eagle medium.

RESULTS

Induction of cell fusion by transient expression of the SNV *env*-gp. To study the effect of *env*-gp expression on the cell and its role in superinfection interference, the SNV *env* coding region was subcloned from a plasmid containing an infectious SNV provirus (pPB101) (1) into a eucaryotic expression vector (JD214) (7) to yield a plasmid designated pENV (Fig. 1A). This plasmid would be expected to express the *env* gene from an unspliced message, since construction of the plasmid results in deletion of the splice acceptor for the *env* gene. This construct was then used to make two mutations in the *env* gene (48). The first alteration was a deletion mutant (pENVdel) in which most of the transmembrane region and the entire cytoplasmic region were replaced with 14 amino acids encoded by vector sequences (Fig. 1B). The second mutation generated a frameshift after codon 36 in the *env* gene (pENVX). Because of premature translation termination, pENVX would be expected to produce a trun-

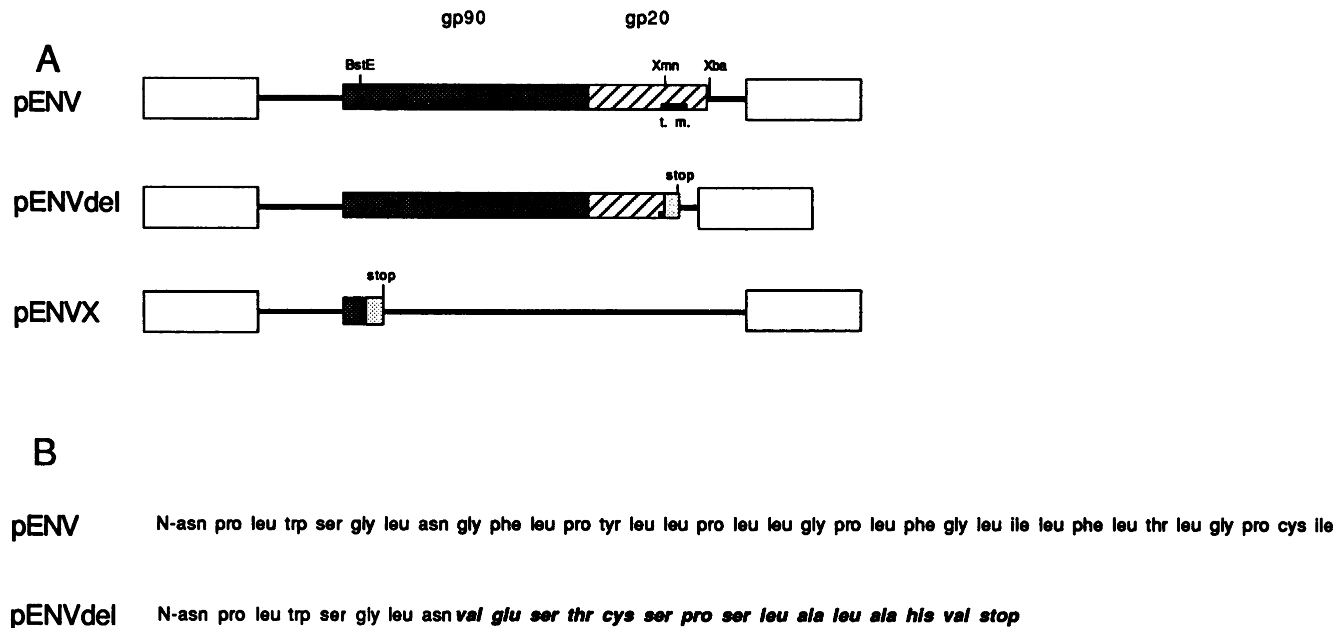

FIG. 1. Envelope expression constructs. (A) Symbols: ▭, SNV long terminal repeats; ——, nontranslated SNV sequences; ▬, region of the SNV *env* gene encoding gp90 (43); ▨, SNV *env* gene segment encoding gp20; ▤, open reading frames derived from vector sequences. t.m., The putative transmembrane domain of gp20, so designated on the basis of comparison with the transmembrane domains of other viruses as well as a hydrophobicity plot of the entire *env*-gp (not shown). (B) Amino acid sequence of the wild-type transmembrane domain of SNV aligned with the sequence of pENVdel that results from deletion of the segment of the *env* gene encoding the transmembrane domain (see Materials and Methods). The amino acid sequence was determined by DNA sequence analysis of pENV and pENVdel. Italicized amino acids indicate those amino acid residues expected from out-of-frame translation before the occurrence of a stop codon.

cated *env* gene product containing only the amino-terminal signal sequence.

To determine the effect of transient expression of the *env* gene, cells of a dog osteosarcoma line permissive for REV replication (D17) were cotransfected with each of the *env* expression plasmids and a plasmid that permits growth in the presence of hygromycin. Six days after hygromycin selection, 200 to 300 syncytia were observed on plates transfected with pENV but not on plates transfected with pENVX or pENVdel (Fig. 2E and F). However, the syncytia were not stable, and none remained after 12 days of selection. Transfection would be expected to result in the introduction and expression of the plasmids in only a small fraction of the cells on the plate. Thus, it appears that expression of the wild-type *env* gene in a minority of the cell population is sufficient to induce cell fusion with neighboring cells that do not contain or express the *env* gene. Such cell fusion may be attained through the interaction of the anchored *env*-gp on the surface of cells successfully transfected cells with the virus receptor on adjacent nontransfected cells. Since pENVX and pENVdel did not induce similar cell fusion after transfection, the *env* protein derivatives encoded by these plasmids must be defective in posttranslational modification or processing, in proper translocation to the cell surface, or in recognition of the virus receptor.

Close examination of the plates transfected with pENV by indirect immunofluorescence, using antiserum to the *env* protein (data not shown), indicated that some hygromycin-resistant cells expressed the *env* gene but had not served as the nucleus for syncytium formation. Thus, it appeared that it might be possible to isolate individual stable cell lines that expressed either the wild-type *env*-gp or *env* del-gp in order to determine the functional defect of the altered glycoprotein.

Cellular localization of wild-type and mutant glycoproteins in stable cell lines. To study the subcellular localization of expressed *env* proteins, the three plasmids (pENV, pENVX, and pENVdel) were individually cotransfected into D17 cells with a dihydrofolate reductase gene containing plasmid pFR400 (34). Selection was applied, and several methotrexate-resistant cell clones were isolated (Table 1). *env*-gp expression was assessed by immunofluorescent staining, using rabbit serum raised against the *env* protein produced in *E. coli* (Fig. 2). Cell lines expressing the normal *env* gene showed a fluorescence pattern identical to that of cells chronically infected with SNV (Fig. 2B and C). In both cases, much of the translocated product appeared to be localized on the cell membrane and in the Golgi. In contrast, cell clones expressing pENVdel showed no apparent membrane or Golgi staining, but *env*-cross-reactive material was distributed in a reticular fashion throughout the cytoplasm (Fig. 2D) (49). Such a pattern is consistent with the possibility that the protein lacking most of the transmembrane domain is not translocated out of the ER. Cells transfected with pENVX produced no detectable *env* protein (Fig. 2A).

Proteolytic processing and glycosylation. To characterize further the cellular localization of the wild-type *env*-gp and the *env* del-gp, chronically infected cells and D17 cell clones were pulse-labeled with methionine and chased, and the *env* products were immunoprecipitated (Fig. 3A). In the case of both infected cells and cells expressing pENV, the primary *env* gene product (Pr75) was modified by further glycosylation to a product of 115-kilodalton molecular mass (gPr115) (43). This molecule was then rapidly cleaved to a transmembrane protein (gp20) and a surface protein (gp90) (Fig. 3A). In contrast, *env* del-gp was synthesized as a molecule shorter than the wild-type precursor and exhibited a constant size throughout the 4-h chase (Fig. 3A). This finding

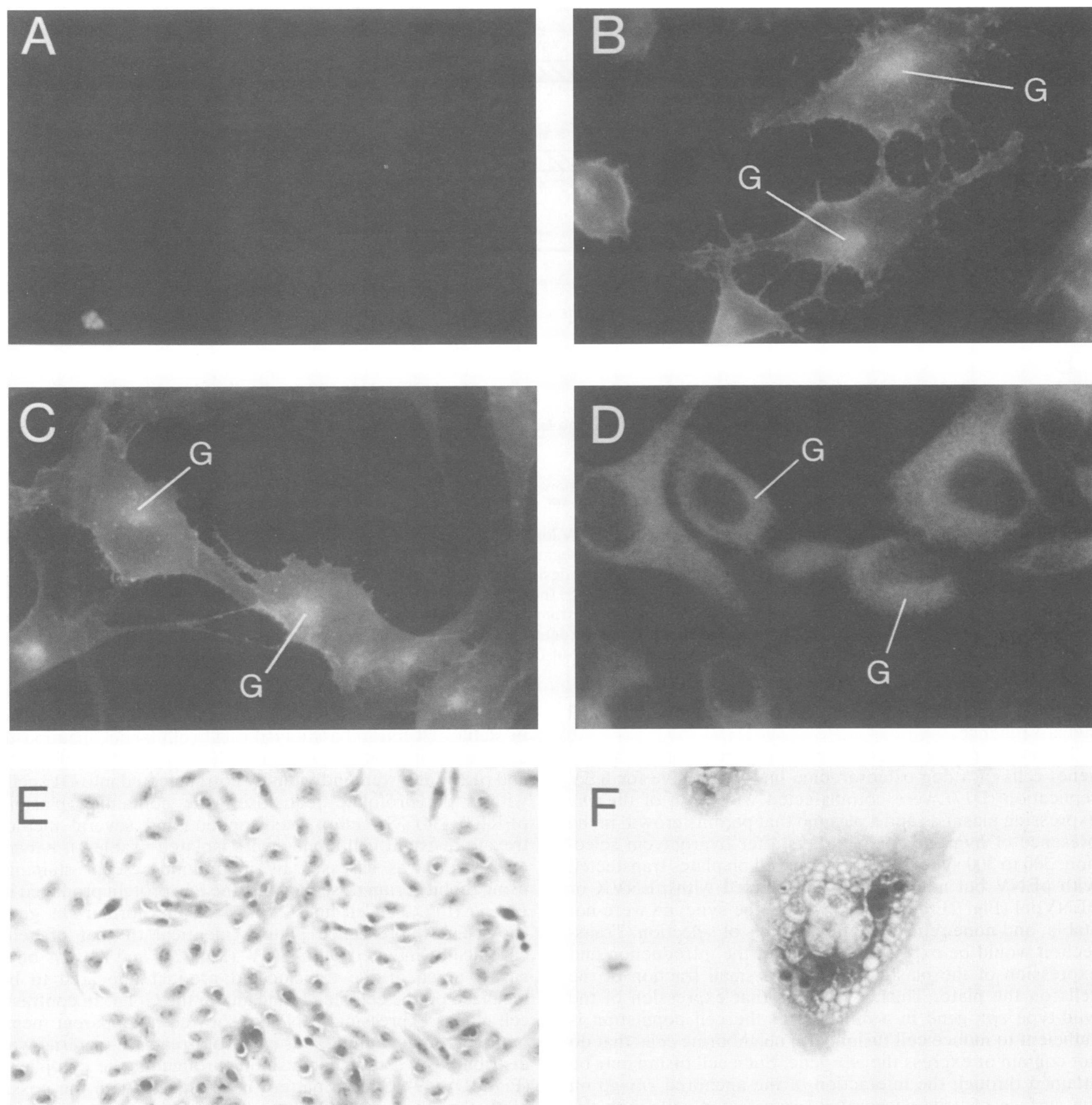

FIG. 2. *env*-gp expression in stable transfectants. Shown are immunofluorescent staining of D17 clones and syncytium formation. (A through D [magnification, ×586]) Intracellular distribution of *env*-gp assayed by immunofluorescent staining. (A) Cells transfected with pENVX (clone 1.1); (B) cells chronically infected with SNV; (C) cells expressing pENV (clone 2.2); (D) cells expressing pENVdel (clone 3.1) (for clone designations, see Table 1). G, Location of the Golgi as determined by double staining with wheat germ agglutinin conjugated to rhodamine (data not shown). (E and F [magnification, ×186]) Violet blue-stained D17 cells and a syncytium formed by transfection with pENV, respectively.

suggests that no Golgi-dependent glycosylation (49) or proteolytic cleavage (8, 26) took place.

The extent of glycosylation of the translocation-deficient polypeptide of pENVdel was analyzed by labeling with mannose or fucose followed by immunoprecipitation. Mannose is normally added to glycoproteins cotranslationally in the ER, whereas fucose is added as the protein travels through the Golgi (16). As expected, the wild-type *env* protein was labeled efficiently with both sugars (Fig. 3B). In contrast, the *env* derivative encoded by pENVdel was labeled with mannose but not with fucose (a 2-month exposure showed only a minute amount of fucose incorporation) (Fig. 3B). To confirm the state of glycosylation of the mutant and wild-type *env* proteins, methionine-labeled immunoprecipitates were digested with endoglycosidase H. Endoglycosidase H removes high-mannose sugar chains, whereas

TABLE 1. Resistance to REV superinfection and syncytium formation after transient transfection[a]

Cell line	Virus protein expression			Relative superinfection[b]	Cell fusion[c]
	gag	*pol*	*env*		
D17	−	−	−	1.0	+
1.1(pENVX)	−	−	−	1.0	+
1.2(pENVX)	−	−	−	1.1	ND
1.3(pENVX)	−	−	−	1.0	ND
1.4(pENVX)	−	−	−	1.1	ND
2.1(pENV)	−	−	+	3.5×10^{-3}	−
2.2(pENV)	−	−	+	2.1×10^{-3}	−
3.1(pENVdel)	−	−	+[d]	6.2×10^{-3}	−
3.2(pENVdel)	−	−	+[d]	1.1×10^{-2}	ND
3.3(pENVdel)	−	−	+[d]	1.5×10^{-2}	−
C3	+	+	+	1.0×10^{-2}	ND
.2G	+	+	+	1.2×10^{-2}	ND
D17(SNV)[e]	+	+	+	1.4×10^{-5}	−
D17(REV-A)[e]	+	+	+	1.2×10^{-5}	ND
1.2 + 2.2[f]	−	−	+	5.6×10^{-1}	ND
1.2 + 3.3[f]	−	−	+[d]	1.0	ND
1.1(Ab)[g]	−	−	−	1.0	ND
2.1(Ab)[g]	−	−	+	7.9×10^{-3}	ND
3.1(Ab)[g]	−	−	+[d]	3.0×10^{-3}	ND

[a] Cell lines were tested for expression of virus proteins by using immunofluorescence staining (see Fig. 2 and Materials and Methods) with anti-*env* or anti-*gag* antiserum. Cell clones expressing either the wild-type *env*-gp or *env* del-gp were tested for resistance to superinfection by REV virion particles carrying the *hyg*^{r} gene (see text).

[b] Arbitrarily normalized to the level of superinfection exhibited by one of the cell lines (1.1) that did not show resistance to superinfection. A value of 1.0 indicates that 2.9×10^5 Hyg^r colonies arose as a result of infection with 1 ml of the virus stock. Except for cells tested in the presence of antiserum (see footnote *g*), relative superinfection levels were determined in two independent experiments, in duplicate, using parallel infection of all plates. Relative superinfection in the presence of antiserum was determined by a single experiment carried out with triplicate plates.

[c] Some of the cell lines were also tested for the presence of syncytia after cotransfection with pENV and pJD214Hy in the fusion assay (see Materials and Methods). +, Cell fusion observed after transfection; −, no syncytium formation observed; ND, not determined.

[d] Cell lines 3.1, 3.2, and 3.3 were transfected with pENVdel and expressed the transmembrane-defective *env*-gp.

[e] Cells were chronically infected with the virus shown in parentheses before challenge with the virus carrying the *hyg*^{r} gene.

[f] The two cell lines were mixed 1:1 24 h before challenge with the virus bearing the *hyg*^{r} gene.

[g] Cells were treated with SNV-neutralizing antiserum for 24 h. The fluid was changed to remove the antiserum, and the cells were immediately infected with virus containing the *hyg*^{r} gene.

complex sugar branches acquired in the Golgi are resistant to digestion (31). Most of the label from the wild-type *env* protein was endoglycosidase resistant, with the exception of a small fraction composed of the unprocessed precursor from the ER. This latter fraction yielded an extra labeled band of the molecular weight expected of the unprocessed wild-type protein backbone (Fig. 3C). In contrast, after digestion of *env* del-gp, a single new labeled protein of the molecular weight expected for the unprocessed mutant protein backbone was observed (Fig. 3C). These results confirm that the unprocessed, partially glycosylated mutant *env* del-gp is blocked in the ER.

Superinfection interference. To examine the effect of SNV *env*-gp expression on superinfection by exogenous REV, we tested the susceptibility of cell lines expressing the *env* genes to exogenous virus infection. First, an REV stock containing the *hyg*^{r} gene was generated by cotransfecting chicken embryo fibroblasts with an REV-A provirus plasmid that encodes a replication-competent virus (pSW253) and a retrovirus vector carrying hygromycin resistance (JD214Hy). Successful infection by virus particles containing the JD214Hy RNA resulted in the generation of hygromycin-resistant cells that could be detected by hygromycin selection. Virus was harvested from chicken embryo fibroblasts and used to infect the various D17 clones expressing pENV or pENVdel. To quantify virus infection, hygromycin selection was applied 24 h later, and resistant colonies were scored 10 days later. Cell lines expressing pENV were resistant to infection, as evidenced by an apparent reduction in the virus titer to 0.3% of that for infection of cells that did not express viral proteins (Table 1). Helper cell lines (derived by transfection of D17 cells with DNA encoding the viral genome and expressing all retrovirus proteins but producing no packagable virus RNA) exhibited a similar level of interference (Table 1). Surprisingly, the cell lines expressing the *env* del-gp also showed resistance to REV infection (Table 1). This finding was unexpected, since the *env* del-gp is not translocated to the cell surface where the receptor is active in virus binding. This result suggests that the *env* del-gp may interact with its receptor in the ER, reducing the availability of the receptor to exogenous virus. Concurrent with resistance to infection, cell lines expressing either pENV or pENVdel were refractory to the induction of syncytia after transfection of pENV (Table 1). This result probably reflects a decreased receptor density on the cell surface, also detected as interference to infection (Table 1).

Interestingly, cells chronically infected with either SNV or REV-A (two closely related REV species) exhibited a higher level of resistance to infection than did either the cell clones expressing only the *env* gene or the helper cell lines that expressed all of the viral components required for replication (Table 1). This difference was apparently not due to higher levels of *env*-gp expression in chronically infected cells, since *env*-gp synthesis appeared to be similar in both chronically infected cells and cell clones derived from transfection (Fig. 3A and C). Endogenous synthesis of *gag* and *pol* proteins is also unlikely to account for the increased interference seen in chronically infected cells, since the helper cell lines which expressed all of the viral proteins did not show such high levels of interference. Moreover, a cell line that expressed the *gag* and *pol* genes but not the *env* gene exhibited no detectable resistance to superinfection (data not shown). It is possible that continual exposure to exogenous virus particles during infection resulted in selection of cells that were highly immune to superinfection. Such selection could not have taken place in the cell clones isolated by transfection, since they were not continuously exposed to virus particles.

As shown previously, a minute amount of the pENVdel gene product is labeled with fucose (Fig. 3B), which may indicate that a very small amount of this protein is translocated through the Golgi. It is unlikely that this protein was responsible for the observed interference to superinfection, given that the cell lines expressing the pENVdel gene exhibited interference levels similar to those of cell lines expressing the wild-type gene, whereas there was profound effect on translocation due to deletion of the carboxy-terminal region of the transmembrane domain of the protein (Fig. 3B). However, we performed additional tests to see whether the exceedingly small amount of *env* del-gp contain-

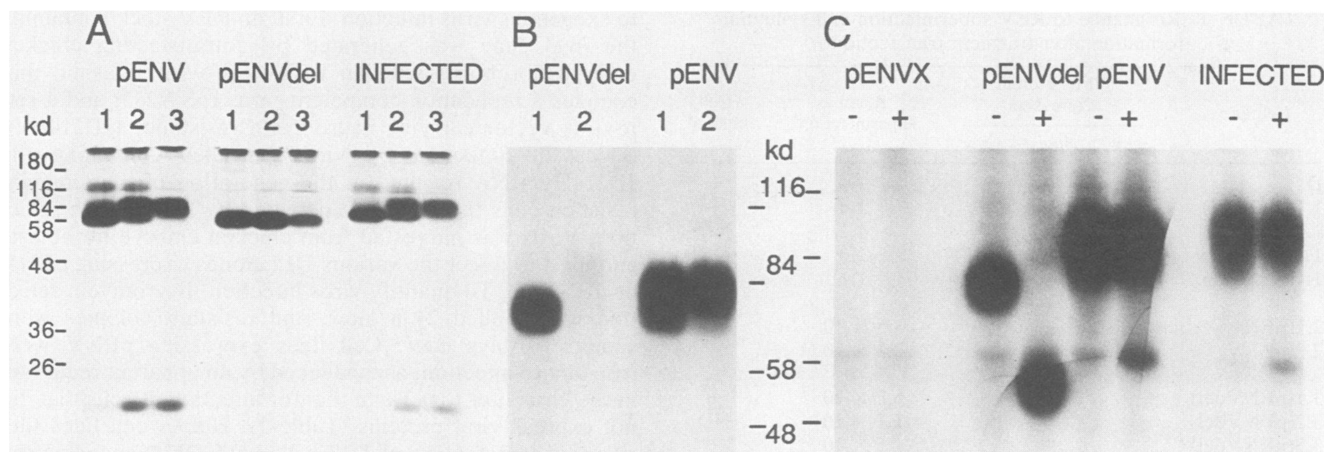

FIG. 3. Immunoprecipitation of methionine- or sugar-labeled wild-type and mutant *env*-gp. Lanes: pENV, transfected D17 cell clone 2.1, expressing *env*-gp; pENVdel, transfected clone 3.1, expressing *env* del-gp; pENVX, transfected clone 1.1, expressing pENVX (Table 1); infected, D17 cells chronically infected with SNV. (A) Pulse-chase analysis of the SNV *env*-gp. Cells were labeled with [^{35}S]methionine for 1 h, immediately immunoprecipitated (lane 1), and chased for 1 h (lane 2) or 4 h (lane 3). Samples derived from equal cell equivalents were run on a 10% SDS-polyacrylamide gel and autoradiographed for 24 h. (B) Sugar labeling of the SNV *env*-gp. Cells were labeled with [^{3}H]mannose (lane 1) or [^{3}H]fucose (lane 2); proteins from equal cell equivalents were subjected to electrophoresis on a 7% polyacrylamide gel and autoradiographed for 8 weeks. (C) Acquisition of complex sugar branches. Cells were labeled for 6 h with [^{35}S]methionine. The immunoprecipitates from equal cell equivalents were suspended in loading buffer, and half of the sample was digested overnight with 0.01 U of endoglycosidase H. A 7% SDS-polyacrylamide gel was used for analysis of the proteins and autoradiographed for 7 days. −, Undigested; +, digested.

ing fucose might partially contribute to establishment of interference.

Since the *env* del-gp derivative lacks much of the wild-type transmembrane and cytoplasmic domains, it might be expected that any protein translocated to the surface would be secreted from the cell. Hypothetically, this could result in binding to the receptor at the external cell surface and result in superinfection interference. Immunoprecipitation of [^{35}S]methionine-labeled supernatant from a pENVdel transfectant indicated that there was no secreted *env* at a detection level of 1% of endogenously labeled envelope (not shown). If interference is due to an undetected amount of secreted protein, then interference may be transferred to susceptible cells that do not express the *env* gene. Cells expressing *env* del-gp were cocultivated overnight with cells that produced no *env* protein. These cells were subsequently challenged with exogenous virus as before. The results of this mixing experiment indicate that interference was not transmitted to the susceptible cells (Table 1). Likewise, growing pENVdel-transfected cells in medium containing REV-neutralizing antibodies (anti-*env* antisera) before infection did not decrease resistance of the cells to infection (Table 1). Thus, we could detect no contribution of secreted protein to superinfection interference. These results support previous data indicating that the *env* del-gp binds its receptor intracellularly and that potentially fusogenic *env*-gp need not be present on the cell surface for interference to take place.

DISCUSSION

We have shown that transfection of D17 cells with an SNV *env*-gp expression plasmid (pENV) can induce syncytium formation. However, SNV infection of D17 cells does not result in obvious syncytium formation. There are several possibilities that may account for this difference between transfection and infection. Transfection-induced cell fusion might be caused by a level of viral *env*-gp synthesis not initially reached during infection. Transfection is likely to introduce into cells many copies of a plasmid that expresses the *env*-gp from an unspliced message. In contrast, *env*-gp synthesis after infection is directed from a spliced message that constitutes only a subset of the viral mRNA. Moreover, at a low multiplicity of infection, only a single provirus would be expected to form in infected cells. Thus, according to this hypothesis, after transfection only a small fraction of the cells might express high levels of *env*-gp, and interaction between this *env*-gp and receptors on nontransfected neighboring cells could result in cell fusion. It has been previously postulated that the cell fusion seen after transfection with plasmids that express the human immunodeficiency virus (HIV) *env*-gp results from fusion with such nontransfected cells (35). It is also possible that during SNV infection, rapid spread of virus results in infection of most or all of the cells on the plate before syncytium formation can take place. Endogenous interaction of the *env*-gp and the receptor might result in the loss of available receptor molecules on the cell surface, which would render the cells incapable of fusion.

Transient expression of pENVdel does not result in syncytium formation. This fact is almost certainly due to the lack of translocation of the expressed protein to the cell surface. Similar C-terminal deletions in the *env*-gp of the vesicular stomatitis and Rous sarcoma viruses also result in arrest of the protein in the ER (31, 49). The ability of the pENVdel gene product to induce resistance to infection indicates that this protein remains capable of recognizing its receptor. It is likely that membrane anchorage is not required for receptor interaction (20).

The ability of the *env* del-gp blocked in the ER to provide interference to superinfection raises the possibility that wild-type *env*-gp also initially binds the receptor in that compartment. The rapid folding of the globular domain of the influenza hemagglutinin in the ER before trimerization supports that possibility (4, 10, 51). The *env*-receptor complex might then travel to the cell surface or, alternatively, be

unable to translocate and be degraded in the ER. Intracellular *env*-receptor binding is supported by coprecipitation studies which have shown that complex formation occurs between an unprocessed form of HIV *env*-gp and the CD4 receptor (15, 38).

The SNV *env* del-gp blocked in the ER is in a high-mannose, endoglycosidase H-sensitive state. In the ER, mannose and glucose residues are cotranslationally added en bloc to glycoproteins and then processed by enzyme-mediated trimming during translocation (9). After trimming, complex oligosaccharides are formed by the addition of other sugar residues (16). Inhibitors of trimming glycosidases appear to prevent complex glycosylation of HIV *env* protein without interfering with its receptor-binding function (12, 44). A partially deglycosylated HIV *env*-gp also retains receptor-binding ability (23). The ability of the partially glycosylated SNV *env* del-gp to provide immunity to superinfection also seems to indicate that complex glycosylation is not essential for receptor binding. However, the abrogation of interference shown by using the N-glycosylation inhibitors tunicamycin and 2-deoxy-D-glucose (16, 28) and the inability of unglycosylated *E. coli*-synthesized HIV *env* to bind the CD4 receptor (27) suggest that cotranslational glycosylation (11) is required for the proper conformation to be adopted by these retrovirus proteins for receptor binding.

Studies on superinfection interference have indicated that synthesis of the *env*-gp is required for resistance to infection but have not ruled out the involvement of other virus proteins in that process (17, 28, 29, 32). We have shown that expression of REV *env*-gp is sufficient to induce resistance to infection by REV. A similar finding has been made for the HIV *env*-gp (38). The level of resistance to REV infection was similar to that shown by helper cell lines which express all retrovirus proteins required for replication. It appears, therefore, that expression of *env*-gp alone confers resistance to infection and that endogenous synthesis of other virus proteins does not enhance that process. Chronically infected D17 cells exhibit significantly higher resistance to REV infection than do helper cells or cell clones expressing only *env*-gp. Such levels of interference may be the result of selection for increased resistance to superinfection in the presence of exogenous virus particles. In this way, interference may limit retrovirus superinfection, thereby allowing the survival of chronically infected cells.

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