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Syncytial Mutations in the Herpes Simplex Virus Type 1 gK (UL53) Gene Occur in Two Distinct Domains

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Syncytial (*syn*) mutants of herpes simplex virus cause cell fusion. Many *syn* mutations map to the *syn*1 locus, which has been identified with the gK (UL53) gene. In this work, the gK genes of eight *syn* mutants derived from the KOS strain were sequenced to identify residues and, possibly, domains important for the fusion activity of mutant gK. DNA sequencing showed that six mutants (*syn*30, *syn*31, *syn*32, *syn*102, *syn*103, and *syn*105) had single missense mutations in the gK gene. Two of these, *syn*31 and *syn*32, had identical mutations that caused the introduction of a potential site for N-linked glycosylation. *syn*31 gK was analyzed by in vitro translation and found to utilize the novel glycosylation site. Two other mutants, *syn*8 and *syn*33, had three mutations each, resulting in three amino acid substitutions in *syn*8 and two substitutions in *syn*33. Of the 10 gK *syn* mutant sequences known, 8 have mutations in the N-terminal domain of gK, suggesting that this domain, which is likely to be an ectodomain, is important for the function of the protein. The other two mutants, *syn*30 and *syn*103, have mutations near the C terminus of gK.

Cells infected with wild-type herpes simplex virus type 1 (HSV-1) normally become rounded and detach from the substrate, although a small percentage fuse with neighboring cells (21). However, mutants which cause extensive fusion of the cell monolayer have been isolated (2, 8, 14, 15, 21, 22, 26, 30, 31). These are known as syncytial (*syn*) mutants. The mechanism by which HSV-1 *syn* mutants induce cell fusion is not known. However, for some other viruses that induce cell fusion, the mechanism of fusion has been shown to be closely related to the mechanism of virus entry (10, 13). This may also be true for HSV-1. Syncytium formation has been shown to involve several of the glycoproteins required for virus penetration: gB, gD, gH, and gL (reviewed in reference 28).

Genetic studies have identified several genetic loci to which syncytial mutations map. The best characterized of these are the *syn*1 and *syn*3 loci, which were first identified by Ruyechan et al. (23). The *syn*2 locus was subsequently shown not to be the site of syncytial mutations but may influence cell fusion in some cell lines (17, 18, 23). Further characterization of the *syn*1 and *syn*3 loci has shown that they correspond to the gK and gB genes, respectively (1, 3, 6, 11, 15, 17, 23).

gK is the product of the UL53 open reading frame and is a highly hydrophobic glycoprotein that appears to be present in relatively small quantities in infected cells (11, 16, 20). Little is known about the role of gK in cell fusion or virus replication. However, identification of *syn* mutations in gK should contribute to the definition of important structural or functional domains. To pursue this objective, we obtained several *syn* mutants isolated from the KOS strain of HSV-1 by Person and coworkers (2, 21). Characterization of these mutants by those investigators indicated that their syncytial mutations are in the *syn*1 locus (2, 21). In the work described here, marker transfer experiments were done to confirm the locations of mutations that had not been physically mapped previously. The syncytial mutations were identified by DNA sequencing of mutant gK genes. These studies have shown that syncytial mutations can occur in two distinct domains of gK.

MATERIALS AND METHODS

Cells and viruses. Vero cells and HSV-1 (2, 21) were propagated as described previously (9). Mutant viruses *syn*8, *syn*20, *syn*30, *syn*31, *syn*32, *syn*33, *syn*102, *syn*103, and *syn*105 were obtained from S. Person (University of Pittsburgh).

Viral and plasmid DNAs. Viral DNA was isolated from lysates of infected cells (25). Briefly, cells were infected at a multiplicity of infection of 1. At 20 h postinfection, the cells were washed with phosphate-buffered saline and then wash buffer (10 mM Tris [pH 7.5], 10 mM EDTA). Cells were lysed in wash buffer containing 250 mg of proteinase K per ml and 0.6% sodium dodecyl sulfate (SDS) and incubated at 37°C for 4 to 16 h. Protein was removed by three phenol-chloroform extractions followed by chloroform extraction. Viral DNA was precipitated with 2 volumes of cold isopropanol and spun out on a Pasteur pipette. After drying, the DNA was resuspended in TE (10 mM Tris [pH 8.0], 1 mM EDTA).

pCR1000 (Invitrogen) was obtained in linearized form for cloning of PCR products. pBS− was obtained from Stratagene. Construction of pBS-KBLN and pBS-S20BLN was described previously (20). Lysis by SDS, followed by cesium chloride-ethidium bromide gradient centrifugation, was used for preparation of plasmid DNA (25).

PCR and cloning of amplified sequences. PCR (24) was performed with *Taq* DNA polymerase with approximately 200 ng each of two 20-mer primers and approximately 300 ng of viral DNA in a total reaction volume of 100 μl. The primers for amplifying the gK gene were CGCCAAATGCGACAGCAA CC and CAGTTTGCATATGCCGTTCC, starting at nucleotides 836 and 1954, respectively, of the DNA sequence (6). Oligonucleotides were synthesized by the Wayne State University Macromolecular Core Facility. The reactions were started

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at 94°C for 3 min followed by 35 cycles consisting of 2 min at 94°C, 1.5 min at 55°C, and 3 min at 72°C with a final incubation at 72°C for 10 min. The PCR fragments were cloned by ligating 1 μl of the PCR product with 2 μl (50 ng) of pCR1000 DNA.

Marker transfer. The fragments to be transfected were obtained either from the plasmid clones described above (linearized with *Eco*RI) or from 2.7-kb PCR products (see the diagram in Fig. 1). The primers used to generate the 2.7-kb marker transfer fragments were CTTTGACCTGTGCCACG AAGTC and TCCGTGGGGGTCTTCCATG, corresponding to nucleotides 111246 and 113898 of the HSV-1 strain 17 genome sequence (16), respectively. For electroporation, 4.2×10^6 Vero cells were suspended in a volume of 0.3 ml together with approximately 3 μg of wild-type KOS DNA, the individual transfer fragments (4.5 μg for plasmids and 155 to 310 ng for PCR products), and 10 μg of salmon sperm DNA as the carrier. DNA was introduced into cells by electroporation with a Bio-Rad Gene Pulser apparatus at 250 V with a capacitance of 960 μF. After pulsing, cells from each cuvette were distributed into two 60-mm-diameter dishes and incubated until a complete cytopathic effect was reached (4 to 5 days). At this time, the dishes were frozen and thawed. The supernatants were retained as virus stocks. These stocks were used to infect Vero cell monolayers in six-well trays and 100-mm-diameter dishes to titrate the recombination progeny.

DNA sequencing. Sanger dideoxynucleotide sequencing (27) of double-stranded plasmid or PCR product DNA was performed with Sequenase DNA polymerase (United States Biochemical) as described by the manufacturer for double-stranded DNA sequencing (7, 29). For sequencing of PCR products, mineral oil was extracted from the PCR samples with chloroform, and samples were run through Sephacryl S-400 spin columns (Pharmacia) to remove primers from the 1.1-kb gK gene fragment. At least three separate PCRs were pooled prior to sequencing to avoid errors in sequences which might have been introduced during early PCR cycles. Samples were run on 6% polyacrylamide wedge gels. Three primers (Wayne State University Macromolecular Core Facility) were used in each direction for sequencing of the entire gene (see Fig. 1).

In vitro transcription and translation and SDS-polyacrylamide gel electrophoresis. Plasmid pCR31 contains the *syn*31 gK gene cloned into pCR1000. The *syn*31 gK gene was excised from pCR31 with *Nae*I and ligated into the *Hinc*II site of pBS− to generate pBS31a for expression in vitro. The gK genes from pBS-KBLN (KOS, wild type), pBS-S20BLN (*syn*20), and pBS31a (*syn*31) were transcribed with T3 RNA polymerase as previously described (20). After isolation of in vitro-transcribed RNA, translation reactions were performed in both the presence and the absence of microsomal membranes (20). Proteins were electrophoresed on SDS–12.5% polyacrylamide gels and visualized by autoradiography as described previously (20).

Nucleotide sequence accession number. The nucleotide sequence data generated in this study have been submitted to the GenBank database and have been assigned accession number U14422.

RESULTS

Cloning and marker transfer of mutations. As mentioned above, recombination and marker rescue studies indicated that a number of syncytial mutations reside within the gK gene (1, 2, 21). KOS strain mutants *syn*20, *syn*102, *syn*103, and *syn*105 were previously mapped to the *syn*1 locus by marker rescue with the 2-kb *Nru*I-*Bam*HI restriction fragment containing the gK gene (1). *syn*8, *syn*30, *syn*31, *syn*32, and *syn*33, also derived from the KOS strain, were shown in virus recombination experiments to map close to *syn*20 (21), strongly suggesting that these mutations are also in the *syn*1 locus (gK gene). However, since this had never been confirmed by marker transfer or marker rescue, we performed marker transfer experiments to test whether the mutations conferring the *syn* phenotype on these viruses are within the gK gene.

TABLE 1. Frequency of *syn* plaques in marker transfer experiments

Source of transfer fragment	*syn* plaque frequency	
	Plasmids[a]	PCR[b]
KOS	2/3,548	0/6,507
*syn*8	7/6,800	49/10,059
*syn*30	21/3,097	130/9,717
*syn*31	18/8,286	10/11,943
*syn*32	12/3,762	39/11,417
*syn*33	11/6,645	153/9,491

[a] Number of *syn* plaques/total number of plaques from two experiments.
[b] Number of *syn* plaques/total number of plaques from three experiments.

DNA fragments (1.1 kb) containing the gK coding sequences of the wild-type virus, *syn*8, *syn*30, *syn*31, *syn*32, and *syn*33 were amplified by PCR and cloned. The cloned gK fragments from these viruses and intact wild-type virus DNA were cotransfected into Vero cells by electroporation. Progeny virus was allowed to form plaques on Vero cell monolayers to assay for the presence of syncytial recombinant viruses. These cotransfections showed that the plasmid clones of the mutant gK genes were able to transfer the syncytial phenotype to the wild-type virus (Table 1). However, in some cases, the marker transfer frequency was relatively low. To confirm these results, a 2.7-kb PCR fragment containing the gK gene was produced and used in additional marker transfer experiments. In addition to the gK gene, this fragment also contains the last 976 nucleotides of UL52 and the first 164 nucleotides of UL54. UL52 is a DNA synthesis gene, and UL54 is the gene for ICP27, an immediate-early regulatory protein. Syncytial mutations have not been reported in these genes. As shown in Table 1, the 2.7-kb PCR fragments from the five mutants transferred the syncytial phenotype to the wild-type virus. No syncytial plaques were isolated when the corresponding wild-type fragment was used. These experiments indicate that the syncytial mutations in these mutants map to the gK gene. This confirms the previous conclusions of Read et al. (21) and Bond et al. (2) that these mutations are in the *syn*1 locus.

Identification of mutations. The cloned mutant gK genes were analyzed by dideoxynucleotide sequencing of double-stranded DNA. Three primers in each direction were used so that both DNA strands could be sequenced completely (Fig. 1). Both strands were sequenced, with the exception of short stretches where the sequences were difficult to read in one direction. Mutations detected in the cloned DNA were confirmed by direct sequencing of PCR products. Point mutations were found within the gK genes of all of the mutants, as summarized in Table 2 and Fig. 2. For comparison, data for two previously sequenced mutants, *syn*20 and MP, are also included (6, 18). Of the eight mutants sequenced, six had single nucleotide substitutions, each of which resulted in an amino acid substitution. *syn*8 and *syn*33 each contained three mutations. In *syn*8, these resulted in three amino acid substitutions, whereas in *syn*33, only two substitutions occurred, since one of the mutations was silent. The fact that all of the mutants sequenced had mutations resulting in amino acid substitutions in the gK gene strengthens the identification of this gene with the *syn1* locus.

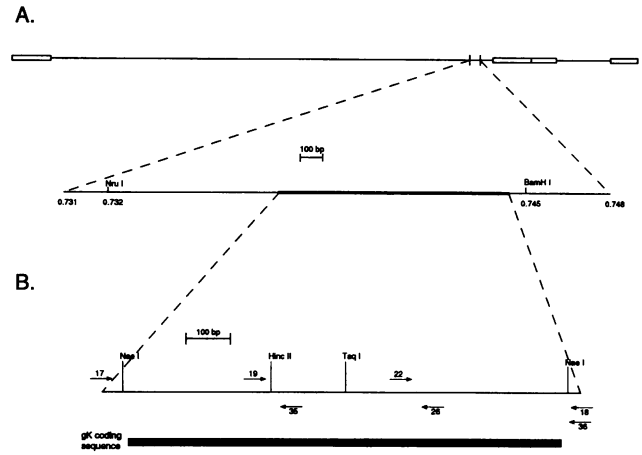

FIG. 1. (A) Location of the gK region in the HSV-1 genome. The endpoints of the 2.7-kb PCR fragment used in marker transfer are indicated (coordinates 0.731 to 0.748). The expanded diagram gives a detailed view of this fragment. The *Nru*I-*Bam*HI fragment used by Bond and Person for marker rescue (1) is labeled. The bold segment represents the 1.1-kb segment that was cloned, sequenced, and used for marker transfer in this study. (B) DNA sequencing strategy. Restriction enzyme sites and the binding sites of the sequencing primers (numbered arrows) are shown within the 1.1-kb PCR fragment. The position of the gK coding sequence within the PCR fragment is also shown.

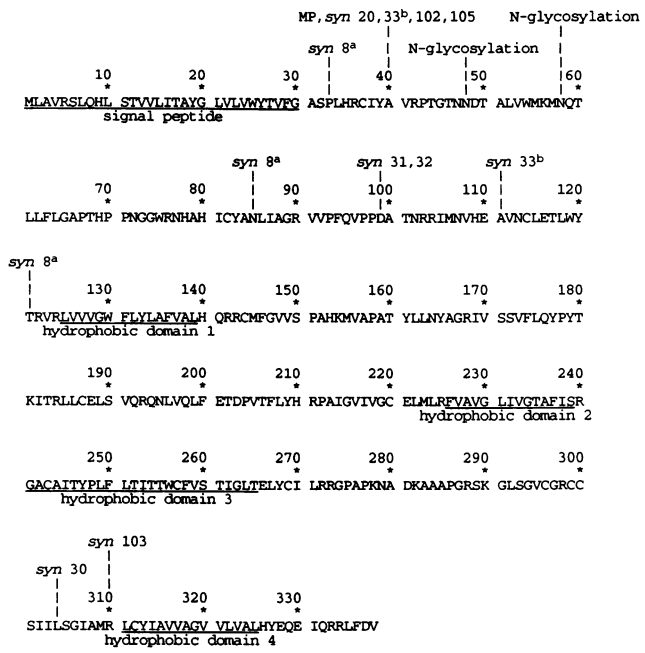

FIG. 2. Amino acid sequence of gK. The protein sequence of gK is shown with its signal sequence, glycosylation sites, and hydrophobic domains labeled (20). The locations of *syn* mutations are also indicated. *syn8*[a], *syn*8 has three amino acid changes; *syn33*[b], *syn*33 has two amino acid changes.

Significantly, of 10 independent mutants sequenced either here or previously, five had missense mutations in codon 40 of gK. These results identify this residue as having an important, although undetermined, role in gK structure or function. *syn*102 and *syn*105 both contained a change from Ala-40 to Val-40, the same mutation previously found in MP. MP is a syncytial mutant which arose spontaneously from the mP strain of HSV-1 (8). *syn*33 also contained this change as one of two amino acid substitutions, the other consisting of a change from Ala-111 to Val-111. *syn*20 also contains a mutation in codon 40, but this causes a change from Ala-40 to Thr-40. It may be significant that only two amino acid substitutions were identified at this position: Ala-40 to Val-40 (four of five mutants) and Ala-40 to Thr-40 (one of five mutants). Other amino acid substitutions are possible as a result of single base changes in codon 40 but were not found, possibly because they are lethal or do not result in the syncytial phenotype. Val-40 is a relatively conservative substitution in which a larger, more hydrophobic amino acid replaces Ala-40. Thr-40, found in *syn*20, is a nonconservative substitution which increases the hydrophilicity of the region. Three missense mutations were identified in *syn*8. Since none of these mutations occurred in any of the other mutants, the relative contribution of these mutations to the syncytial phenotype cannot be evaluated. Although *syn*31 and *syn*32 were isolated independently, they contained identical mutations which resulted in a change from Asp-99 to Asn-99. The mutations in *syn*30 and *syn*103 were different from the others in that they were located near the C terminus of the protein, rather than the N terminus. These were nonconservative missense mutations: in *syn*30, Leu-304 is changed to Pro-304, and in *syn*103, Arg-310 is changed to Leu-310.

TABLE 2. *syn* mutations in gK

Mutant	Nucleotide		Codon	
	Position[a]	Change	Position	Change
*syn*20[b] MP[c]	1010	G→A	40	Ala→Thr
*syn*102 *syn*105	1011	C→T	40	Ala→Val
	1011	C→T	40	Ala→Val
*syn*33	1224	C→T	111	Ala→Val
	1876	G→A	328	None
*syn*31	1187	G→A	99	Asp→Asn
*syn*32	989	C→T	33	Pro→Ser
*syn*8	1148	C→T	86	Leu→Phe
	1254	C→T	121	Thr→Ile
*syn*30	1803	T→C	304	Leu→Pro
*syn*103	1821	G→T	310	Arg→Leu

[a] Nucleotide numbering is based on reference 6.
[b] From reference 6.
[c] From reference 18.

Glycosylation of *syn*31 gK. The mutation in the *syn*31 and *syn*32 gK genes changed amino acid 99 from aspartate to asparagine. The presence of a threonine residue 2 amino acids downstream makes the asparagine at residue 99 a potential site for N-linked glycosylation. Glycosylation of this site would constitute a major alteration of the protein, which could contribute to a change in its function. Since we lack antibodies able to immunoprecipitate gK from infected cells, we examined the glycosylation of *syn*31 gK by using the same in vitro transcription-and-translation system we utilized previously to characterize the wild-type gK protein (19, 20).

As shown in Fig. 3, polyacrylamide gel electrophoresis indicated that without posttranslational processing, in vitro-translated wild-type, *syn*20, and *syn*31 gKs all had similar apparent molecular mass of approximately 28 kDa, as had

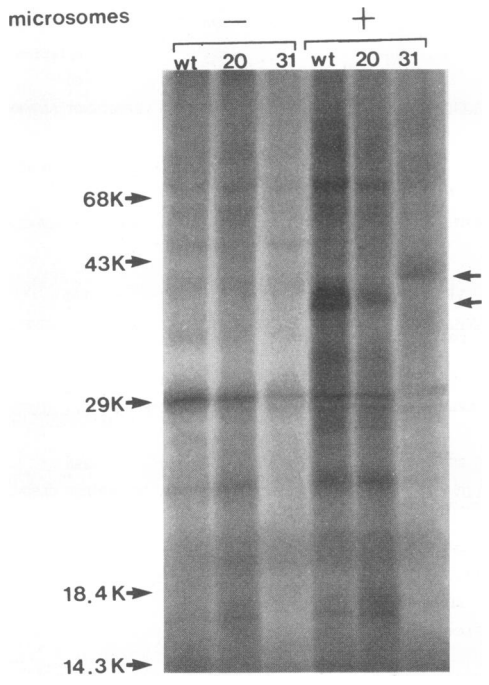

FIG. 3. Electrophoresis of in vitro-translated gK. RNA transcripts from pBS-KBLN (KOS, wild type [wt]), pBS-S20BLN (*syn*20), and pBS31a (*syn*31) were translated in the presence of [^{35}S]methionine with and without microsomes. The labeled proteins were electrophoresed on an SDS–12.5% polyacrylamide gel. K, kilodaltons. The arrows on the right indicate the positions of the 38-kDa band in KOS and *syn*20 and the 41-kDa band in *syn*31.

been observed previously for the wild-type protein (20). When synthesis was done in the presence of microsomes, both the 28 kDa gK precursor and bands with higher apparent molecular masses were observed. We have shown previously that the presence of microsomes in in vitro translation reactions increases the total amount of gK synthesized; however, not all of the gK synthesized in the presence of microsomes is processed to a higher-molecular-weight form (19, 20).

Microsomally processed wild-type and *syn*20 gKs migrated with an apparent molecular mass of 38 kDa. This size increase is largely, if not entirely, due to the addition of N-linked oligosaccharides (19, 20). However, the microsomally processed form of *syn*31 gK migrated with an apparent molecular mass of 41 kDa. Because the only amino acid difference between *syn*31 gK and wild-type gK is the substitution of Asn-99 for Asp-99, the difference in size between the processed proteins is apparently due to glycosylation of *syn*31 gK at Asn-99. The difference of approximately 3 kDa between the molecular masses of the two processed proteins is consistent with the addition of one additional N-linked glycosylation core oligosaccharide, which has a molecular weight of approximately 2,400 (12).

DISCUSSION

In this study, we identified the mutations which are responsible for the syncytial phenotype of a number of HSV-1 *syn*1 mutants that had previously been isolated and characterized by S. Person and coworkers. These mutations occurred in the gK (UL53) gene, which corresponds to the *syn*1 locus. Most of the mutant genes contained a single point mutation; however, *syn*33 and *syn*8 had three nucleotide substitutions, resulting in two and three amino acid substitutions, respectively.

Five of eight N-terminal domain mutants had mutations in codon 40, indicating that this region of gK may be especially critical. Since signal cleavage occurs after residue 30, residue 40 is very near the N terminus and also near the N-linked glycosylation sites, although mutations in codon 40 do not appear to affect glycosylation or signal cleavage (11, 20). Ala-40 of HSV-1 gK is part of a sequence, C(I/V)YA, that is conserved in the gK homologs of HSV-2, equine herpesvirus type 1, and varicella-zoster virus (4, 5, 32). Phenotypic differences have been noted between mutants containing mutations within the N-terminal domain of gK (2, 21). In mixed infections with wild-type virus, *syn*102 and *syn*105 exhibit a dominant phenotype, *syn*33 is classified as codominant, and *syn*20 is weakly codominant. These phenotypes correlate with the amino acid substitutions in the mutant gK molecules. *syn*102 and *syn*105 both substitute Val-40 for Ala-40. The same mutation is found in syncytial mutant MP, which also has an aggressive fusion phenotype (8, 17). Although *syn*33 also contains the Val-40 mutation, it contains a second mutation, Val-111. This second mutation may be responsible for its less dominant phenotype if it is able to compensate partially for the conformational change introduced into gK by the Val-40 substitution. The substitution of Thr-40 for Ala-40 in *syn*20 may be responsible for the less aggressive fusion phenotype of this virus in comparison with *syn*102 and *syn*105.

Examination of the locations of the mutations within the gK gene provides information about the functional domains of the gK glycoprotein. The UL53 gene encodes a 338-amino-acid protein in which several features can be identified, including an N-terminal signal sequence, N-linked glycosylation sites, and several highly hydrophobic domains (Fig. 2 and references 6 and 18). In a previous study (20), we showed by in vitro translation that gK is a membrane-bound glycoprotein with a cleavable amino-terminal signal sequence. Truncated gK molecules containing hydrophobic domain 1 (Fig. 2) remain membrane bound, suggesting that this domain is a transmembrane domain. Studies on the glycosylation of gK indicate that one or both of the N-linked glycosylation sites at residues 48 and 58 are utilized (11, 20), as was the novel site in *syn*31 (this study). Together, these facts indicate that the N-terminal domain of gK (residues 31 to 124) is an ectodomain. If gK is expressed on the plasma membranes of infected cells, as seems likely given its influence on cell fusion, this domain will be exposed on the cell surface. Thus, it is significant that 8 of 10 *syn*1 mutants had amino acid substitutions in the N-terminal domain of gK. This observation identifies this domain as critical for gK function and suggests that it interacts with the ectodomains of other membrane molecules.

Although the N-terminal domain of gK seems likely to be an ectodomain, the topology of the remainder of the molecule is difficult to predict. Hydrophobic domain 2 (Fig. 2) is shorter than most transmembrane domains, and hydrophobic domain 3 is less hydrophobic than most transmembrane domains (18, 20). In addition, these two domains are separated only by Arg-240. Thus, we cannot predict whether this region of gK contains two, one, or no transmembrane domains, and consequently, we cannot predict whether the domain where the *syn*30 and *syn*103 mutations occurred is an ectodomain or a cytoplasmic domain. As an ectodomain, this domain would have an opportunity to interact directly with the N-terminal domain. Alternatively, this domain could be cytoplasmic, in which case the mutations could affect the function of the protein in a more indirect manner. It should be noted that the syncytial mutations which have been identified in gB (the *syn*3

locus) are known to reside in the cytoplasmic domain of that glycoprotein (3).

The role of gK in cell fusion has yet to be established. It is uncertain whether wild-type gK acts to inhibit cell fusion or mutant gK acts to induce cell fusion. Dominant and codominant syncytial mutations could be indicative of gain-of-function mutations, which would indicate that mutant gK induces fusion. However, if gK forms homo- or hetero-oligomers, as many membrane proteins do, loss-of-function mutations could also result in dominant and codominant phenotypes. We note that none of the syncytial mutations in gK sequenced to date have been frameshift or nonsense mutations. This could be indicative of a requirement for gK in virus replication. However, it is also consistent with a requirement for a gain-of-function mutation in gK for induction of cell fusion.

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