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Marburg virus, the prototype of the family Filoviridae, differs genetically, serologically, and morphologically from Ebola viruses. To better define the genetic variation within the species, VP35 and glycoprotein (GP) genes of representative human isolates from four known episodes of Marburg virus hemorrhagic fever were analyzed. The percentage nucleotide differences in the GP gene coding regions of Marburg viruses (0.1±0.1%) was nearly equal to the percentage amino acid changes (0.2±0.3%), while the percentage nucleotide differences in VP35 coding regions (0.3±0.9%) were higher than the percentage amino acid changes (0.9±0.1%), indicating a greater number of nonsynonymous changes occurring in the GP gene. The higher variation in the GP gene and the corresponding protein, especially those changes in the variable middle region of the GP, suggests that the variability may be the result of responses to natural host pressures. Analysis of the GP gene open reading frame shows a nonrandom distribution of nonsynonymous mutations that may indicate positive Darwinian selection is operating within the variable region. A heptad repeat region and an adjoining predicted fusion peptide are found in the C-terminal third of Marburg virus GPs, as has been previously shown for Ebola virus, and are similar to those found in transmembrane glycoproteins of retroviruses, paramyxoviruses, coronaviruses, and influenza viruses. Comparative analyses showed that there are two lineages within the Marburg virus species of filoviruses. The most recent isolate from Kenya (1987) represents a separate genetic lineage within the Marburg virus species (21±23% amino acid difference). However, this lineage likely does not represent a separate Marburg subtype, as the extent of divergence is less than that separating Ebola virus subtypes.

INTRODUCTION

Marburg (MBG) viruses are nonsegmented, negative-strand RNA viruses, and together with Ebola (EBO) viruses constitute the family Filoviridae (Jahrling et al., 1995). Filoviruses can cause an extremely severe form of hemorrhagic fever in humans and/or nonhuman primates and are classified as Biosafety Level 4 agents (Centers for Disease Control and Prevention, 1993; Feldmann et al., 1996; Peters et al., 1996). The natural hosts have not been identified, and thus the factors that influence their evolution and ecology in the wild, and lead to human and nonhuman primate infections, are unknown.

Recent molecular studies have shown that these RNA viruses are similar in their genome organization and mechanisms of replication, but represent genetically distinct viruses (Feldmann et al., 1992, 1996; Sanchez et al., 1993, 1996; Peters et al., 1996; Georges-Courbot et al., 1997; Volchkov et al., 1997). These differences are in agreement with studies that have shown a complete lack of serologic cross-reactivity, contrasting structural protein profiles, and differences in virion morphology between MBG and EBO viruses (Kiley et al., 1988; Ksiazek et al., 1992; Peters et al., 1993, 1996; Feldmann et al., 1994, 1996; Geisbert et al., 1996). Isolates of MBG virus have tended to be fairly homogeneous, whereas EBO virus isolates appear more heterogeneous; four different EBO virus subtypes have been identified thus far (Sanchez et al., 1996). The heterogeneity of EBO viruses has also been demonstrated at the genetic level through sequence analysis of their glycoprotein (GP) genes (Sanchez et al., 1996). The nucleotide sequence differences between EBO subtypes vary from 37 to 41% (compared with a 55% difference between EBO and MBG viruses). For both MBG and EBO viruses, the GP genes are positioned fourth (3′ to 5′) in a series of seven linearly arranged genes and are the only genes to encode viral glycoproteins. The GP genes of EBO viruses were shown to have an unusual organization and to encode a nonstructural secreted glycoprotein (SGP) as the primary gene product in a single frame and the structural GP in two frames (expressed through transcriptional editing) (Volchkov et al., 1995; Sanchez et al., 1996). This unusual organization is absent in the GP genes of previously analyzed MBG viruses (Will et al., 1993; Bukreyev et al., 1995).

The VP35 genes of these viruses may also serve as a potential region of variability in MBG strains, since their gene products have been previously reported to differ in rates of migration in denaturing gels (Feldmann et al., 1994, 1996; Volchkov et al., 1995; Sanchez et al., 1996). These differences may be the result of responses to natural host pressures.
The filovirus VP35 gene product is a structural protein that is not membrane associated and probably functions as a cofactor in the transcription complex. Its position (second) in the genome makes it analogous to the P proteins of paramyxoviruses and rhabdoviruses, but its function has not been conclusively identified. Analysis of this gene may supply additional information about the evolution of MBG virus, since environmental constraints on the biology of the VP35 protein are likely to be distinct from those affecting the GP.

To better define the genetic variation within MBG viruses, we have analyzed representative strains from the four known episodes of MBG virus hemorrhagic fever in humans. We have examined the GP and VP35 genes of these MBG virus isolates to expand on earlier phylogenetic analyses, which primarily focused on EBO viruses (Sanchez et al., 1996; Georges-Courbot et al., 1997; Volchkov et al., 1997). We present here the results of these studies and a discussion of the evolution of filoviruses.

**MATERIAL AND METHODS**

Viruses and passage histories

The MBG virus isolates used to generate sequence information for this study are as follows: (1) an isolate from the original 1967 outbreak from Marburg, Germany, strain M/Germany/Marburg/1967/Ratayczak (RYC strain) (Siegert et al., 1967; Feldmann et al., 1994), passaged three times in Vero E6 cells (ATCC CRL 1586); (2) a 1975 isolate from a South African case, strain M/S. Africa/Johannesburg/1975/Ozolin (OZO strain) (Gear et al., 1975; Kiley et al., 1988), passaged three times in Vero 76 cells and four times in Vero E6 cells; (3) a 1987 isolate from Kenya, strain M/Kenya/Kitum Cave/1987/Ravn (RAV strain) (Johnson et al., 1997), passaged once in SW13 cells and three times in Vero E6 cells. Sequence information for the M/Germany/Marburg/1967/Popp (POP strain; passaged nine times in guinea pigs) and M/Kenya/Nairobi/1980/Musoke strains (MUS strains; isolated, plaque-purified three times, and passaged two to three times thereafter in Vero E6 cells) of MBG virus (Zaire species) were previously determined or obtained from GenBank (Bukreyev et al., 1992; Sanchez et al., 1993). Figure 1 shows the geographical locations where infections by the above viruses are believed to have originated.

Purification of viral RNA and RT-PCR

Virions were purified from clarified supernatant fluids by pelleting through a 20% sucrose cushion (Feldmann et al., 1994), and genomic RNA was extracted as described elsewhere (Sanchez and Kiley, 1987) or with a commercial kit (RNaid Kit, BIO 101, Inc.). For examination of VP35 gene sequences, first-strand DNA synthesis was performed using the primer 5'–GCCTAGATGATAACAGATAT (plus-sense within NP gene and near polyadenylation site) and random primers (hexamer) as previously described (Sanchez et al., 1989). DNA produced in reverse transcription (RT) reactions were diluted 1:50 in water and used as template in polymerase chain reaction (PCR) assays. For amplification of the entire VP35 genes, either the primer 5'–CTGCTCTCAGCTAAGGAGCCTCA or 5'–ATGATTCTATATTAAAGGTAAC (NP gene, plus-sense) and primer 5'–GACGCTACCCCTCCAGTAAAAAAGACAC-TAGCA were used with appropriate internal primers in PCR assays. Amplification was performed by mixing 10 μl of 10× reaction buffer (Boehringer Mannheim), 8 μl of 2.5 mM dNTPs (Promega), 3.5 μl of each primer (100 ng/ml), 2 μl of DNA template, 1 μl Taq DNA polymerase (Boehringer Mannheim), and water to a final volume of 25 μl.
amplify GP ORF regions. The primers were generated, specific primers were synthesized to products that spanned the coding region. As sequences primer sets were required to generate overlapping PCR and the variation seen in the MBG strains, multiple cycles of denaturing at 94°C for 1 min (except for the first cycle, where the time was increased to 5 min), annealing at 50°C for 1 min, and then extension at 72°C for 15 to 3 min, followed by a final extension at 72°C for 5 min and cooling to 4°C.

Due to the length of the GP open reading frame (ORF) and the variation seen in the MBG strains, multiple primer sets were required to generate overlapping PCR products that spanned the coding region. As sequences were generated, specific primers were synthesized to amplify GP ORF regions. The primers 5′-TGAAGAACAT-TAATTGCTGGGTAA and 5′-CCTAATCATACACATTTA-ACGTT correspond to plus-sense and minus-sense sequences flanking the MBG ORF (close to the N-terminal and C-terminal ends, respectively) and were used with internal primers to amplify the ends of the ORF. The primer 5′-CCGGTACC(T)35, which binds to poly(A), was used with plus-sense primers to amplify sequences within the ORF to the 3′-end of the GP mRNA. First-strand cDNA synthesis reactions, using genomic or mRNA preparations as template, were performed either as previously described (Sanchez et al., 1989), or using a commercial kit (SuperScript II, Life Technologies, GIBCO BRL). RT-PCR amplification assays used commercial kits and reagents (Boehringer Mannheim and Promega). Thermocycling was performed by denaturing at 94°C for 1 min (all cycles), and for the first 3 cycles annealing occurred at 37°C for 30 s with extension at 72°C for 2 min, followed by 30 cycles of annealing at 45, 50, or 55°C for 30 s and extension for 1 min at 72°C. Reactions were cooled to 4°C at the end of thermocycling.

Sequence analysis

Virus sequences were determined by direct sequencing of RT-PCR products, using an automated nonisotopic method (dye-terminator cycle sequencing; Perkin-Elmer). PCR products were isolated from TAE agarose gels, using a commercial extraction kit (QIAEX II, Qiagen Inc.), eluted into 50 μl of water, and used in sequencing reactions. ABI 373 and 377 automated sequencers were used to derive sequence information and generate sequence files. Computer-assisted nucleic acid and predicted amino acid sequence analyses were performed as previously described (Sanchez et al., 1996). Phylogenetic analyses of nucleotide alignments were performed using the PAUP software, Version 3.1 (developed by D. L. Swofford), run on a Power Macintosh 8100/110 (Apple).

RESULTS

The entire VP35 gene and the coding region of the GP gene sequences of the RYC, OZO, and RAV strains of MBG virus were successfully amplified and sequenced. Phylogenetic analyses were performed using the coding regions of these genes, and the corresponding ORFs of the MAY strain of EBO virus were included to provide an outgroup. Figure 2 shows phylogenetic trees based on these nucleotide sequences. Both trees show a similar topology in that the EBO taxon is well separated from the MBG taxa and that the most recent MBG virus isolate (RAV) appears distinct from the other four MBG strains and is extremely well supported by bootstrap values (Felsenstein, 1993). Relationships within the POP, RYC, MUS, and OZO clade in the VP35 tree are not fully resolved, as evidenced by poor bootstrap support for the node connecting MUS and OZO. A clearer relationship of these viruses is seen when GP ORF sequences are analyzed, with the 1980 MUS strain showing a greater similarity to the 1967 viruses than does the 1975 OZO strain. The same phylogenetic profile (as described above) is seen when amino acid sequences are used in maximum parsimony analysis (trees not shown), except that branch lengths are shorter.

To the right of the trees in Fig. 2 are shown matrices of percentage identity at the nucleotide (above diagonal) and amino acid level (below diagonal) for each gene ORF. For the VP35 gene, the nucleotide differences between the RAV and the other MBG virus strains is ~20–21%, while the differences within the POP/RYC/MUS/OZO grouping are at most only 6%. However, corresponding comparisons using amino acid sequences show a decrease to ~6 and 3%, respectively. Compared with EBO, both the nucleotide and the amino acid sequences of the MBG strains differed by ~52±53% and ~65%, respectively. Comparisons of GP ORF sequences showed a similar percentage nucleotide difference between RAV and the other MBG viruses (~21–22%), but the differences in amino acid sequences did not decrease and instead stayed at the same approximate level as nucleotide differences (~22±23%). Within the POP/RYC/MUS/OZO group, a difference of ~6±9% is seen at the amino acid level. In comparison with EBO, the MBG strains differed in the GP ORF nucleotide and predicted amino acid sequences by ~54±55% (comparable to VP35 ORF) and ~69% (4% greater than the VP35 amino acid sequence), respectively. These data indicate that within the MBG strains and between MBG and EBO, there is a greater variation in amino acid sequence for the GP than for the VP35.

Alignments of the predicted VP35 and GP amino acid sequences of the MBG and EBO viruses (used in phylogenetic analyses) were performed (Fig. 3). The VP35 alignment shows only three sites where there is variation in two or more MBG strains, and amino acid changes are primarily found from residues 27 to 184 (alignment numbers 46±204). In contrast, the middle of the GP alignment shows the greatest variation and is flanked by long conserved regions; the N-terminal region (signal sequence), however, does show a fair amount of divergence. The central variable region shows two marked
boxes (totaling \( \sim 200 \) residues) where most of the divergence in MBG sequences is located. The N-terminal-most box corresponds to a similar variable region found in the GPs of EBO virus species (EBO residues \( 300-500 \)) (Sanchez et al., 1996). Cysteine residues conserved in all the filoviruses are present in both proteins. Three are found in the VP35, while the GP alignment contains 5 in the N-terminal third and 7 in the C-terminal third. The highly conserved C-terminal \( \sim 180 \) residues contain a sequence very similar to an immunosuppressive motif that has been described for the P15 glycoprotein of oncogenic retroviruses (Volchkov et al., 1992; Will et al., 1993). This conserved sequence also partially overlaps an alpha-helical heptad repeat region (4-3 hydrophobic amino acids) (Chambers et al., 1990; Gallaher, 1996).

Immediately N-terminal to this helical region in all these viruses are predicted fusion peptides (Gallaher, 1996).

The GP alignment also shows that potential glycosylation sites are partly conserved. There are 14 predicted N-linked glycosylation sites that are found in all MBG strains, over half of which are located in the two variable boxes, and six sites are found in all MBG strains except for RAV, again located in the variable boxes. Nine sites are either unique to RAV or found in RAV and some of the other MBG strains, with all but one located in the variable boxes. These findings show that the central variable region is the area where N-linked glycosylation seems to be focused.

To more closely examine the evolutionary processes that are driving MBG GP gene evolution, the synonymous and nonsynonymous nucleotide substitutions in the GP alignment were mapped at each codon (Fig. 4). Stop codons were excluded from the analysis. Synonymous substitutions were found to be distributed throughout the GP gene though this pattern deviated from a random binomial (Poisson) distribution due to the presence of seven codons (numbers 107, 282, 358, 424, 448, 590, 611) exhibiting three or more synonymous substitutions.
A VP35

POP .............................. s h
RYC .............................. t p l i s r h
MUS .............................. t i s
OZG .............................. m s k d v k
RAV ................................

EBO mttrkgragtantgmd pgpelagw q r sd cd ... i ngplclyasmqmrpkp ktrmq p cnhsfew vq s atv q

|               |               |               |               |
|---------------|---------------|---------------|---------------|
| POP           | POP           | POP           | POP           |
| POP           | POP           | POP           | POP           |
| POP           | POP           | POP           | POP           |
| POP           | POP           | POP           | POP           |

DISCUSSION

A phylogeny profile of the family Filoviridae has been derived from GP gene coding sequences (Sanchez et al., 1996). This phylogeny showed that EBO and MBG viruses represent very divergent lineages of filoviruses. Within Ebola viruses there are four separate monophyletic lineages that differ from one another by approximately 37±40% at the nucleotide level. However, within individual lineages, the variation in nucleotide sequence has been shown to be less than 2%, indicating a remarkable degree of stability over a 20-year period (Sanchez et al., 1996; Georges-Courbot et al., 1997; Volchkov et al., 1997; A. Sanchez, unpublished data). Our analyses of MBG virus VP35 and GP gene sequences indicate a somewhat different pattern for these filoviruses. The most recent isolate, the RAV strain (1987), represents a separate lineage of MBG virus, while the strains that preceded it constitute another lineage. Phylogenetic analyses using either VP35 or GP sequences clearly demonstrate this distinction, but the phylogenetic relationship of the 1967 (POP & RYC), 1975 (OZO), and 1980 (MUS) isolates was resolved for GP sequences only. It is interesting to note that the Zimbabwe isolate (OZO) is genetically closer to the Uganda (RYC & POP) than to the most recent Kenyan isolate (RAV). This geographical discordance (~600-700 vs 100 km) may be explained if the natural host is a migratory species with a large range. Alternatively, this geographical inconsistency may merely reflect a divergence of the RAV strain following coevolution with a distinct natural host.

Differences in the nucleotide sequence of the GP gene (0.01 < P < 0.025, χ² = 7.47, df = 2). The distribution of nonsynonymous substitutions exhibited a much different pattern with a strong deviation from a random binomial distribution (P < 0.001, χ² = 79.52, df = 2). The majority of nonsynonymous distributions clustered at the 5' end of the GP gene ORF (codons 2 through 32) and in the middle of the GP gene between codons 201 and 501. The ratio of nonsynonymous to synonymous substitutions at 16 codons in this middle region exhibited a ratio $\approx 3$, with four exhibiting a ratio $\approx 4$ (codons 267, 364, 370, 414). All of these 16 codons were found to reside in the two most divergent regions of the GP identified by the amino acid alignment. Thus, the GP gene of MBG viruses exhibits a strongly nonrandom distribution of nonsynonymous changes and a number of sites are showing evidence of selection for amino acid changes over silent substitutions.
ORFs of MBG viruses (0.1±21%) were comparable to the percentage amino acid changes (0±23%). In contrast, the nucleotide differences in VP35 ORFs (0.3–20.9%) were higher than amino acid changes (0.9–6.1%), which indicates that the number of nonsynonymous changes is greater in the GP gene than in the VP35 gene. This finding may indicate that the variation in the GP genes of MBG viruses are changes that have been selected for and have not occurred as purely random events. The greater variation in the MBG GP gene versus the VP35 gene is not inconsistent with the variation seen between glycoprotein and P genes of other nonsegmented negative-strand RNA viruses. It was previously shown that there is more variation in the GP gene of the POP and MUS strains of MBG virus compared with other genes (Bukreyev et al., 1995). In addition, alignments of MBG or EBO GP amino acid sequences show that the middle portion of this molecule can be very divergent and is the primary site for N- and O-linked glycosylation (Sanchez et al., 1993). It is possible that this variable region reflects the adaptation of filoviruses as they responded to natural host selective pressures. During this time, GP may have been under greater selective pressure than other virus proteins, possibly from phenotypic changes in cell receptors or the immune system of the natural host.
tion, nonsynonymous substitutions that cause adaptive amino acid changes are preferred over silent synonymous substitutions (Endo et al., 1996). In the immunoexposed proteins of viral pathogens, the pressures of the host immune system are thought to drive positive selection of epitopes. The resulting antigenic polymorphism provides a mechanism for evading the immune response. Viral proteins thought to be evolving by positive Darwinian selection under immune pressure include the hemagglutinin gene of influenza and the \textit{env} gene of human immunodeficiency virus (Fitch et al., 1991; Yamaguchi and Gojobori, 1997). The strongly nonrandom distribution of nonsynonymous changes in the MBG GP gene and presence of codons exhibiting high ratios of nonsynonymous/synonymous substitutions strongly suggest that the GP is undergoing positive Darwinian selection. Mapping of epitopes within the MBG GP that are recognized by the vertebrate immune response will be necessary to confirm or refute this hypothesis.

Processes in tissue culture or experimentally infected animals that lead to changes in virus phenotypes, whether through pressures from immune responses or some other selective mechanism, may be different from those acting in the natural host. There are differences between the POP strain of MBG virus, which was derived from a virus stock that had been passaged nine times in guinea pigs (Bukreyev et al., 1995) and the RYC strain that we sequenced (both derived from the 1967 outbreak in Marburg, Germany). It is likely that passaging in guinea pigs has led to the selection of a modified strain, which is phenotypically altered in the VP35 protein (three amino acid changes) from the initial virus, but has not led to a change in the GP amino acid sequence.

Depending on the area of the predicted amino acid sequences of the GPs of different filoviruses compared, they can appear either very conserved or extremely variable. The C- and N-terminal regions tend to be generally hydrophilic and contain long regions of conserved residues, whose functions include a signal sequence for directing the protein into the endoplasmic reticulum, and a transmembrane sequence to help anchor the molecule in the virion envelope. These regions are also likely to function in the formation of virion spike structures, since they contain most of the highly conserved cysteine residues. The central, hydrophilic, and variable region of the GP is a platform for the addition of glycans and is presumed to project outward from the virion. The predicted amount of N- and O-linked carbohydrate is considerable, accounting for a third to half of the molecular weight, and likely has a role in the resistance of the virion to neutralization by specific antibody. The positioning of a predicted fusion peptide adjoining heptad repeats N-terminal to the transmembrane anchor in filovirus GPs, initially shown for Ebola virus by Gallaher (1996), is similar to motifs seen in the transmembrane glycoproteins of retroviruses, paramyxoviruses, coronaviruses, and influenza viruses (Chambers et al., 1990; Lambert et al., 1996; Chan et al., 1997). The structural similarity of MBG and EBO GPs argues for a common mechanism by which filovirus GPs form trimers (Feldmann et al., 1991) and mediate virus entry into host cells. This shared mechanism of glycoprotein structure and function, especially with respect to filoviruses and oncogenic retroviruses (Volchkov et al., 1992; Sanchez et al., 1996; Gallaher, 1996), points to either a common ancestral gene sequence or convergent evolution.

\begin{figure}
\centering
\includegraphics{fig4}
\caption{Distribution of synonymous (A) and nonsynonymous (B) substitutions in the codons of MBG GP genes (minus stop codons). Bars above plots indicate those sequences encoding the variable regions identified in the GP amino acid alignment (Fig. 3B).}
\end{figure}
The current taxonomy of the family Filoviridae places MBG and EBO viruses into a single genus, Filovirus. However, it is apparent from our phylogenetic analyses, together with prior studies that described important differences in the biology of these viruses (Kiley et al., 1988; Feldmann et al., 1992, 1994; Sanchez et al., 1993, 1996; Volchkov et al., 1995), that EBO and MBG viruses can readily be classified into separate genera. In our opinion, the four EBO subtypes should be considered as separate species, and MBG strains should all be placed in a single species. An earlier study of the RAV strain of MBG virus suggested that this virus represented a new subtype, comparable to that seen with EBO subtypes (Johnson et al., 1997). Our GP gene finding that RAV differed ~21±23% from that of the other MBG strains (at both nucleotide and amino acid level) is slightly more than half that found between EBO species. We believe that this level of difference is insufficient to consider RAV as a separate MBG species, but is sufficient to be regarded as a subspecies or variant. To better characterize the MBG lineage, it will be necessary to analyze additional novel isolates. However, as with EBO, identification of such viruses has relied on rare outbreaks in human and nonhuman primates, although this obstacle should be greatly reduced when the natural reservoir is eventually determined.

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