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Diversity of the Killer Cell Ig-Like Receptors of Rhesus Monkeys[1,2]

Karen L. Hershberger, Richa Shyam, Ayako Miura, and Norman L. Letvin[3]

Because the killer cell Ig-like receptors (KIRs) have only been characterized in humans and chimpanzees, we do not have a full understanding of their evolutionary history. Therefore, cDNAs encoding the KIR molecules of five rhesus monkeys were characterized, and were found to differ from the KIR molecules identified in humans and chimpanzees. Whereas only one KIR2DL4 molecule is detected in humans and chimpanzees, two distinct KIR2DL4 homologues were identified in the monkeys. Although the two human KIR3DL molecules are limited in their polymorphism, the KIR3DL homologues in the monkeys were highly polymorphic. Up to five KIR3DL homologues were identified in each monkey that was studied, and eleven distinct KIR3DL molecules were detected in the five rhesus monkeys. Two novel families of KIR molecules were identified in the rhesus monkeys, KIR3DH and KIR1D. The KIR3DH molecules have three Ig domains, transmembrane domains homologous to KIR2DL4 molecules that contain an arginine, and short cytoplasmic domains. With these features, the KIR3DH molecules resemble the activating forms of the human KIR molecules. The KIR1D molecule encodes only one complete Ig domain before a frame-shift in the second Ig domain occurs, leading to early termination of the molecule. Multiple splice variants of KIR1D exist that encode at least one Ig domain, as well as transmembrane and cytoplasmic domains. The extensive diversity of the rhesus monkey KIR3DL homologues and the novel KIR3DH and KIR1D molecules suggests that the KIR family of molecules has evolved rapidly during the evolution of primates. *The Journal of Immunology,* **2001, 166: 4380–4390.**

Killer cell Ig-like receptors (KIRs)[4] are members of the Ig superfamily of molecules that are expressed on NK cells and subsets of T lymphocytes in humans (1–4). These receptors interact with MHC class I molecules on target cells and mediate both inhibitory and activating signals (5–8). Human KIR molecules have been identified that have both two and three Ig domains. The three Ig domains are referred to as D0 (membrane-distal), D1 (middle), and D2 (membrane-proximal; Ref. 9). KIR molecules with two Ig domains can have either D1 and D2 (D1-D2) or D0 and D2 (D0-D2) domains. In addition, KIR molecules can have either long or short cytoplasmic tails. The long cytoplasmic tails have two immunoreceptor tyrosine-based inhibition motifs (ITIMs) that recruit tyrosine phosphatases to initiate the inhibition of NK and T cell cytolytic activity (10, 11). The KIR molecules with short cytoplasmic tails lack ITIMs and have a lysine in the transmembrane domain that interacts with DAP12, leading to NK cell activation (6, 12, 13).

Extensive characterization of cDNA from humans has revealed 13 forms of KIR molecules (1, 2, 9, 14, 15). The polymorphism, genomic organization, and alternative mRNA splicing of these molecules have been characterized. The number of KIR genes varies among individuals, and additional KIR genotypic diversity occurs due to allelic polymorphism (16–19). However, the sequence differences between these various alleles is minimal, usually fewer than five amino acid changes. KIR3D molecules and the KIR2D molecule with the D1-D2 Ig domains have nine exons, and KIR2D molecules with D0-D2 domains have eight exons (17, 20–22). Variations in mRNA splicing have been described for several human KIR molecules. Complete deletion of the exons encoding the Ig domains, the stem domain, and the transmembrane domain, as well as the first exon of the cytoplasmic domain have been reported (9, 14, 17, 23). In addition, deletions of only portions of the Ig domains have been detected (23, 24).

No KIR homologues have been identified in rodents, and the only species other than humans in which these molecules have been characterized is the chimpanzee (25). This recent analysis of cDNA from chimpanzees identified 10 forms of KIR molecules (Pt-KIR), four of which have at least 95% nucleotide sequence identity to their human homologues. The other six Pt-KIR molecules, although more divergent, have the same structural configuration as a human KIR molecule. Because humans and chimpanzees are very close phylogenetically, it is not surprising that all of the Pt-KIR molecules are similar to the human KIR molecules. Thus, we do not have a complete understanding of the evolutionary history of these molecules. In the present study, we have characterized the KIR molecules in rhesus monkeys. Surprisingly, we found two novel KIR molecules, as well as considerable diversity of the KIR3DL molecules in this species. The detection of this diversity of KIR molecules in rhesus monkeys indicates that extensive evolution has occurred in this receptor family in primates.

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[2] The sequences presented in this paper have been submitted to GenBank and assigned the following accession numbers: AF334616–AF334626 (Mm-KIR3DL), AF334627–AF334633 (Mm-KIR3DLsv), AF334634 (Mm-KIR1D), AF334635–AF334643 (Mm-KIR1Dsv), AF334644 and AF334645 (Mm-KIR2DL4), AF334646 and AF334647 (Mm-KIR2DL5), AF334678–AF334651 (Mm-KIR3DH), and AF334652–AF334658 (Mm-KIR3DHsv).

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[4] Abbreviations used in this paper: KIR, killer cell Ig-like receptor; ITIM, immunoreceptor tyrosine-based inhibition motif; RACE, rapid amplification of cDNA ends.

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Materials and Methods

Animals

EDTA-anticoagulated peripheral blood samples were obtained from five unrelated rhesus monkeys (*Macaca mulatta*). These animals were maintained in accordance with the guidelines of the Committee on Animals for the Harvard Medical School and the "Guide for the Care and Use of Laboratory Animals" (National Research Council, National Academic Press, Washington, DC, 1996).

RNA extraction and reverse transcription

PBMC were isolated from whole blood by centrifugation over Ficoll-Hypaque (Ficopaque; Pharmacia, Piscataway, NJ). Total RNA was extracted from 2–5 $\times$ 10^6 PBMC by using the RNeasy miniprep kit with a DNase treatment step (Qiagen, Chatsworth, CA). First-strand cDNA was generated by using avian myeloblastosis virus reverse transcriptase and an oligo(dT) primer (Promega, Madison, WI).

PCR amplification

Rhesus monkey KIR sequences were amplified by using the sense primer Ig3Up and the antisense primer Ig3Down, which are based on the human KIR molecules (26). Amplification of 2 μl of cDNA was performed in 50-μl reactions with 1$\times$ PCR Buffer II, 2 mM $MgCl_2$, 200 μM of each of the four dNTPs, 50 pmol of each primer, and 2.5 U of AmpliTaq Gold (Perkin-Elmer, Foster City, CA). PCR cycling conditions were as follows: initial denaturation and activation of AmpliTaq Gold for 10 min at 94°C, followed by 30–35 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s, extension at 72°C for 90s, and a final extension at 72°C for 10 min.

KIR molecules homologous to the human KIR3DL (Mm-KIR3DL) and KIR2DL4 (Mm-KIR2DL4.1 and Mm-KIR2DL4.2) molecules, as well as splice variants of these molecules, were amplified from the cDNA of rhesus monkeys by using the Ig3Up-Ig3Down primer combination. In addition, the novel rhesus monkey KIR molecule Mm-KIR3DH was amplified from one animal (577) by using these primers.

Additional KIR sequences were amplified from the cDNA of two rhesus monkeys (223 and 227) by using the human KIR-specific primers, sense primer F23, and the antisense primer R1441 (1). PCR conditions were the same as described above, but the annealing temperature was 57°C. Extensive analysis of the PCR products amplified by this primer combination from the cDNA of monkey 223 identified Mm-KIR3DL molecules, splice variants of Mm-KIR3DL, Mm-KIR2DL5.1, and the novel rhesus monkey KIR molecule Mm-KIR1D and its splice variants. A second Mm-KIR2DL5 sequence was identified in the F23-R1441 PCR products by using cDNA from monkey 227 as template.

The novel rhesus monkey KIR molecule Mm-KIR3DH and its splice variants were amplified from the cDNA of two rhesus monkeys (223 and 577) by using the Mm-KIR3DH-specific reverse primer (5′-CTGGGCT GGAGACAACGA-3′) in conjunction with Ig3Up. PCR conditions were the same as described above, but the annealing temperature was 55°C.

3′ Rapid amplification of cDNA ends (RACE)

For 3′ RACE, first-strand cDNA was synthesized with Superscript II reverse transcriptase by using the adapter primer (Life Technologies, Rockville, MD). PCR then was performed with Ig3Up and the abridged universal amplification primer (Life Technologies). Amplification of 2 μl of cDNA was performed in 50-μl reactions with 1$\times$ PCR Buffer II, 2.5 mM $MgCl_2$, 200 μM of each of the four dNTPs, 10 μM of each primer, and 2.5 U of AmpliTaq Gold (Perkin-Elmer). PCR cycling conditions were as described above, but the annealing temperature was 55°C. 3′ RACE with Ig3Up and abridged universal amplification primers amplified Mm-KIR3DL, Mm-KIR3DH, and Mm-KIR2DL4.1 molecules.

Cloning and sequencing

PCR products were analyzed on a 1% agarose gel and purified from the gel by using the Qiaex II gel extraction kit (Qiagen). Purified PCR products were cloned into the p-GEM-T Easy vector (Promega). Plasmids were isolated from bacterial clones with the Qiaprep spin miniprep kit (Qiagen). These plasmids then were sequenced by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and a 377 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Results

Mm-KIR3DL

We identified cDNA sequences that encode a family of rhesus monkey KIR molecules with three Ig domains and long cytoplasmic tails. Consistent with the nomenclature used to describe the homologous human KIR molecules, we refer to these molecules as Mm-KIR3DL with the prefix Mm denoting *M. mulatta*. Members of this family of rhesus monkey KIR3DL molecules (Mm-KIR3DL) were classified into distinct types based on predicted amino acid sequence homology. Members that differed by >2% were considered separate types. Although only two KIR3DL molecules have been defined in humans, eleven distinct molecules were identified by this criterion in PBMC of five unrelated rhesus monkeys (Figs. 1 and 3). The amino acid differences among these eleven types are scattered throughout the linear sequences of the molecules and do not cluster in any distinct regions. However, there are certain amino acid changes that are consistently seen in a number of these molecules. For example, Mm-KIR3DL1 through Mm-KIR3DL4 share a number of amino acid changes throughout the Ig domains but are divergent in their cytoplasmic domains (Fig. 1). Despite the sequence variation among the Mm-KIR3DL molecules, the two cysteines important for the Ig fold are conserved in all three Ig domains in all of these molecules. Also, like their human homologues, the cytoplasmic tails of the Mm-KIR3DL molecules contain two ITIMs.

The Mm-KIR3DL molecules have 74–77% amino acid identity to human KIR3DL1 and KIR3DL2. The amino acid sequences of the Mm-KIR3DL molecules are not significantly more similar to either KIR3DL1 or KIR3DL2. However, the cysteines in the D2 and stem domains that are thought to be important for homodimerization of KIR3DL2 are not present in the Mm-KIR3DL molecules (Figs. 1 and 2). In addition, the cytoplasmic tails of Mm-KIR3DL molecules are the same length as the cytoplasmic tail of human KIR3DL1. Therefore, these rhesus monkey molecules most closely resemble human KIR3DL1. Despite the similarity to the human KIR3DL forms, the Mm-KIR3DL molecules are distinguished from human KIR3DL1 and KIR3DL2 molecules by a 3-aa insertion in the D0 domain and a 1-aa deletion in the transmembrane domain (Fig. 2).

Either KIR3DL2 alone or both KIR3DL1 and KIR3DL2 have been detected in PBMC of every human who has been examined (16). In contrast, individual rhesus monkeys may express up to five distinct Mm-KIR3DL molecules (Fig. 3). In no rhesus monkey were fewer than three forms of Mm-KIR3DL detected, indicating that multiple Mm-KIR3DL gene loci exist in this species.

Alternatively spliced forms of Mm-KIR3DL

Seven alternatively spliced forms of the Mm-KIR3DL molecules were identified (Fig. 4). Splice variants Mm-KIR3DLsv1, Mm-KIR3DLsv5, and Mm-KIR3DLsv6 have deletions that follow exon boundaries and therefore are likely to occur as a result of exon skipping. The Mm-KIR3DLsv1 and Mm-KIR3DLsv5 variants lack an entire Ig domain and would form KIR molecules with only two Ig domains. The D0 domain deletion seen in Mm-KIR3DLsv1 has been reported previously for the human KIR3DL1 molecule (23). The combination of D0 and D2 domain deletions seen in Mm-KIR3DLsv6 would result in a KIR molecule with only a single Ig domain. Splice variants were also detected with only a portion of an Ig domain deleted. These variants (Mm-KIR3DLsv2, Mm-KIR3DLsv3, and Mm-KIR3DLsv4) remain in the same reading frame and have at least two complete Ig domains. Deletions of only a portion of an Ig domain have been described previously for the human KIR molecules (24). A splice variant of the human

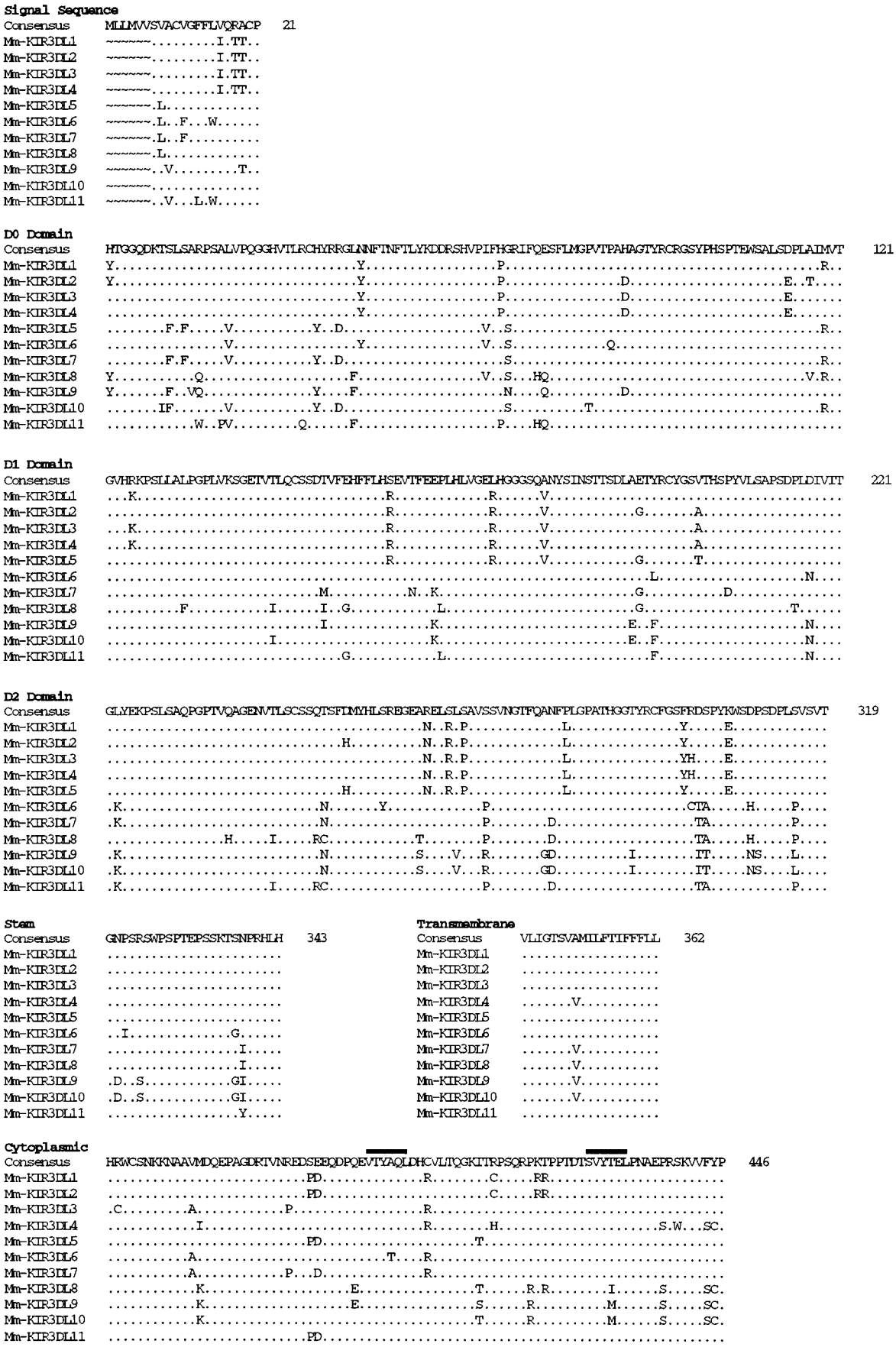

FIGURE 1. Predicted amino acid sequences and structural domains of rhesus monkey KIR3DL molecules. Periods (.) indicate identity with the consensus sequence and tildes (~) indicate amino acids encoded by the PCR primer used to amplify the cDNA. ITIMs are indicated by bars above the motifs.

FIGURE 2. Comparison of rhesus monkey KIR3DL7 with human KIR3DL1 and KIR3DL2. The predicted amino acid sequence of Mm-KIR3DL7 was aligned with those of human KIR3DL1 (NKAT3) and KIR3DL2 (NKAT4) (1). Periods (.) indicate identity with Mm-KIR3DL7, dashes (-) indicate absence of amino acids, and tildes (~) indicate amino acids encoded by the PCR primer used to amplify the cDNA. ITIMs are indicated by bars above the motifs.

KIR2DL3 molecule was identified previously with the same 50-aa deletion of the D2 domain as Mm-KIR3DLsv4. However, the deletions of Mm-KIR3DLsv2 and Mm-KIR3DLsv3 have not been reported previously for human KIR molecules. All variants resulting from these in-frame deletions have normal stem, transmembrane, and cytoplasmic domains. However, a deletion of D2 that resulted in a shift in the reading frame by one nucleotide was also identified (Mm-KIR3DLsv7). This frame-shifted molecule terminates early without an identifiable transmembrane or cytoplasmic domain. Because the nucleotide sequences of all of the splice variants identified in an individual monkey are in every case identical with a Mm-KIR3DL sequence detected in that monkey, these internally deleted cDNAs most likely represent splice variants rather than distinct genes.

Mm-KIR2DL4

We identified cDNA sequences that encode molecules with 84% amino acid identity to human KIR2DL4 that have only the D0 and D2 Ig domains and long cytoplasmic tails. These molecules also contain an arginine in their transmembrane domains. Because of these characteristic features, these molecules are likely to be rhesus monkey homologues of KIR2DL4 and therefore were designated Mm-KIR2DL4 (Fig. 5). Despite their obvious homology to human KIR2DL4, these molecules are distinguished by the two ITIMs in their cytoplasmic tails. Both the human and chimpanzee KIR2DL4 molecules have only one ITIM in their cytoplasmic domains (15, 25). The human KIR2DL4 has an ITIM at amino acids 298–303, the chimpanzee KIR2DL4 has an ITIM at amino acids 328–333, and the rhesus monkey KIR2DL4 molecules have ITIMs at both positions. Therefore, the positions of the two ITIMs in the Mm-KIR2DL4 molecules are the same as those of the ITIMs found individually in their human and chimpanzee homologues (Fig. 5).

Although all Mm-KIR2DL4 molecules share the features mentioned above, there are distinct sequence variations that differentiate two subtypes within this family of molecules. These subtypes are designated Mm-KIR2DL4.1 and Mm-KIR2DL4.2. The two subtypes differ by a single nucleotide deletion in the second exon encoding the cytoplasmic domain that results in a frame shift. This frame shift alters the predicted amino acids starting at amino acid 355 of Mm-KIR2DL4.2. Interestingly, this frame shift is always associated with two amino acid changes, a valine to threonine change (V238T) in the stem and an alanine to isoleucine change (A262I) in the transmembrane domain. Because of the position of the primer used to generate the clones, the end of the Mm-KIR2DL4.2 sequence is unknown.

In addition to subtype variation, allelic polymorphism was identified for both types of Mm-KIR2DL4 molecules. Every monkey

	Rhesus Monkey				
	227	223	577	576	173
Mm-KIR3DL1	X			X (2)	X
Mm-KIR3DL2	X				
Mm-KIR3DL3		X			
Mm-KIR3DL4		X			
Mm-KIR3DL5			X		
Mm-KIR3DL6			X		
Mm-KIR3DL7			X		
Mm-KIR3DL8	X	X			X (2)
Mm-KIR3DL9	X	X			
Mm-KIR3DL10				X	
Mm-KIR3DL11	X				

FIGURE 3. Mm-KIR3DL clones identified in five rhesus monkeys. An X indicates the detection of that Mm-KIR3DL molecule. Detection of two sequences in an individual rhesus monkey that differ by <2% when translated is indicated by (2).

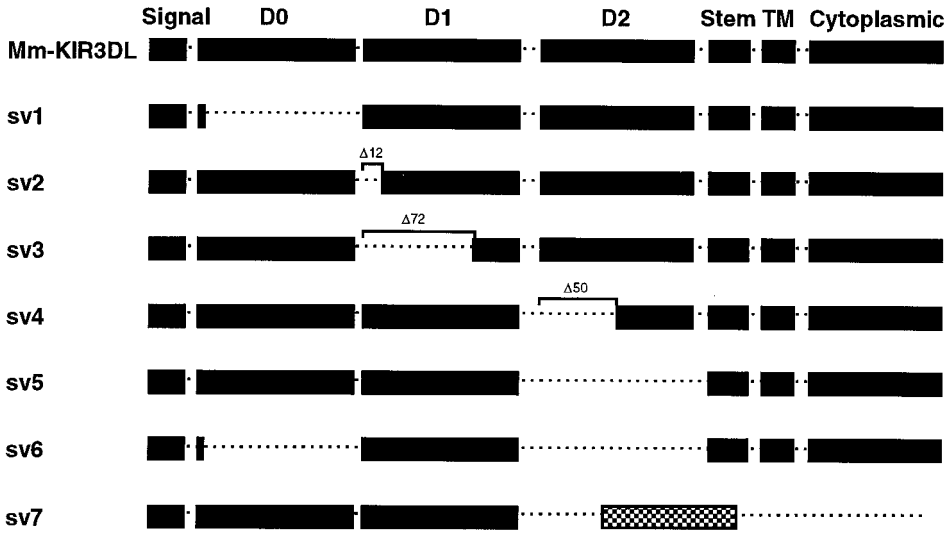

FIGURE 4. Alternatively spliced forms of rhesus monkey KIR3DL. Putative splice variants (sv) are shown schematically compared with full-length Mm-KIR3DL. Sv7 has a deletion in the D2 domain followed by a frame shift of 1 nt indicated by a checkered box.

characterized expressed mRNA for at least one subtype of the Mm-KIR2DL4 molecule. One monkey had molecules of both subtypes, and one monkey had two alleles of Mm-KIR2DL4.2 but no Mm-KIR2DL4.1. The remaining three animals expressed mRNA for a single allele of either subtype. Finally, a splice variant of an allele of Mm-KIR2DL4.2 lacking the D0 domain was detected (data not shown). Although alternatively spliced forms of the human KIR2DL4 molecule have been described previously, none involved an Ig domain (17).

Mm-KIR2DL5

We also identified cDNA sequences that encode a second KIR molecule with only D0 and D2 Ig domains. This molecule has ~80% amino acid identity to the human KIR2DL5 molecule. Although this rhesus monkey molecule also has similarities to human KIR2DL4 (79% amino acid identity), it lacks the arginine in the transmembrane domain that is characteristic of KIR2DL4 and contains two ITIM motifs in the cytoplasmic tail (Fig. 6). Therefore, we refer to this molecule as Mm-KIR2DL5. Distinct sequences of Mm-KIR2DL5 with >2% amino acid divergence were identified from two rhesus monkeys. Therefore, these molecules were designated Mm-KIR2DL5.1 and Mm-KIR2DL5.2.

Mm-KIR1D and alternatively spliced forms

A novel KIR cDNA was identified that has a nucleotide sequence that appears to encode a molecule with D1 and D2 Ig domains and

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Signal Sequence
Human KIR2DL4     MSMSPTVIILACLGFFLDQSVWA  23
Chimp KIR2DL4     ~~~~~~~~~~~~~~~~~.......
Mm-KIR2DL4.1      ~~~~~~~~~..........R.R.
Mm-KIR2DL4.2      ~~~~~~~~~..........R.R.

D0 Domain
Human KIR2DL4     HVGGQDKPFCSAWPSAVVPQGGHVTLRCHYRRGFNIFTLYKKDGVPVPELYNRIFWNSFLISPLTPAHAGTYRCRGFHPHSPTEWSAPSNPLVIMVT  120
Chimp KIR2DL4     ...........................................................V..............................
Mm-KIR2DL4.1      ........................W....P.........E.........K...........V.A.........V.....................
Mm-KIR2DL4.2      ........................W....P.........E.........K...........V.A.........V.....................

D2 Domain
Human KIR2DL4     GLYEKPSLTARPGPTVRAGENVTLSCSSQSSFDIYHLSREGEAHELRLPAVPSINGTFQADFPLGPATHGETYRCFGSFHGSPYEWSDPSDPLPVSVT  218
Chimp KIR2DL4     ..........R.....T.........R.........................V.........................E......NS....L....
Mm-KIR2DL4.1      ........S.Q.....PT...M......RR...M.................V...............GN......LRD.................
Mm-KIR2DL4.2      ........S.Q.....PT...M......RR...M.................V...............GN......LRD.................

Stem                                                                  Transmembrane
Human KIR2DL4     GNPSSSWPSPTEPSFKTGIARHLH  242                       Human KIR2DL4     AVIRYSVAIILFTILPFFLL  262
Chimp KIR2DL4     ........................                            Chimp KIR2DL4     ............IL.L....
Mm-KIR2DL4.1      ..................VT..P                              Mm-KIR2DL4.1      ........T.FL...L...H
Mm-KIR2DL4.2      ..................T...P                              Mm-KIR2DL4.2      I.......T.FL...L....
                                    *                                                   *

Cytoplasmic
Human KIR2DL4     HRWCSKKKNAAVMNQEPAGHRTVNREDSDEQDPQEVTYAQLDHCIFTQRKITGPSQRSKRPSTDTSVCIELPNAEPRALSPAHEHHSQALMGSSRETTALSQTQLASSHVPAAGI  377
Chimp KIR2DL4     ....................................C.....V............T.........Y..............K...........~~~~~~~~~~~~~~~~~~~
Mm-KIR2DL4.1      RC...D.......DP....D.......P..........T.....V..RG...R....P.T.P.....Y.........L........R..WK...M.......NR.H..N...V..
Mm-KIR2DL4.2      RC...D.......DP....D.......................V..RG...R....P.T.P.....Y.........S........R..WR.LLGRQQPCLK.~~~~~~~~~~~~
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FIGURE 5. Predicted amino acid sequences and structural domains of rhesus monkey KIR2DL4 molecules. The amino acid sequences of Mm-KIR2DL4.1 and Mm-KIR2DL4.2 were aligned with that of human KIR2DL4 (KIR103LP) and chimpanzee KIR2DL4 (Pt-KIR2DL4) (17, 25). Periods (.) indicate identity with human KIR2DL4 and tildes (~) indicate amino acids encoded by the PCR primers used to amplify the cDNA. Asterisks (*) mark the amino acids in the stem and transmembrane, as well as the frame shift in the cytoplasmic domain, all of which are characteristic changes for Mm-KIR2DL4.2. ITIMs are indicated by bars above the motifs. The human and chimpanzee KIR2DL4 have a single ITIM at amino acids 298–303 and amino acids 328–333 respectively, whereas the rhesus monkey KIR2DL4 molecules have an ITIM at both positions.

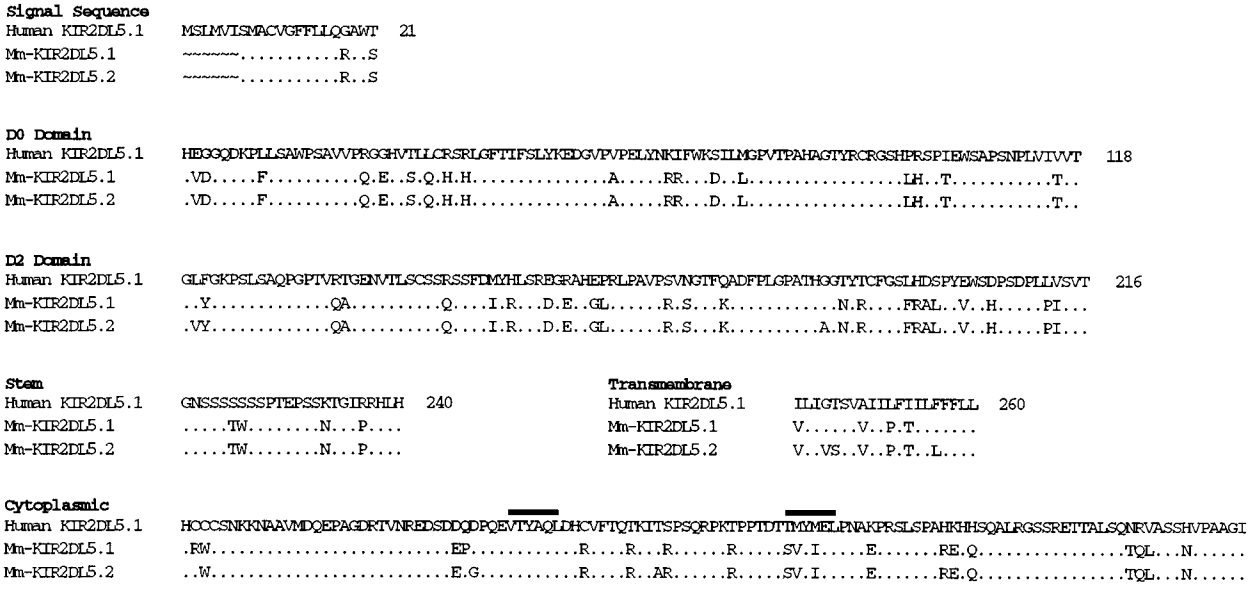

FIGURE 6. Predicted amino acid sequences and structural domains of rhesus monkey KIR2DL5 molecules. The amino acid sequences of Mm-KIR2DL5.1 and Mm-KIR2DL5.2 were aligned with that of human KIR2DL5 (KIR2DL5.1) (9). Periods (.) indicate identity with human KIR2DL5 and tildes (~) indicate amino acids encoded by the PCR primer used to amplify the cDNA. ITIMs are indicated by bars above the motifs.

a cytoplasmic tail that is similar in length to KIR2DL4. However, when translated, this molecule only aligns with the signal sequence, D1 domain, and the first two-thirds of the D2 domain of Mm-KIR3DL (Fig. 7). Because of a frame shift, the predicted amino acid sequence then changes to a novel domain of 55 aa, and the molecule terminates at a final length of 244 aa. Because this molecule has only one complete Ig domain and no recognizable cytoplasmic tail, we have designated it Mm-KIR1D.

We detected the cDNA for Mm-KIR1D in only one rhesus monkey and could amplify it with only a single primer pair, the F23-R1441 primer combination. To ensure that Mm-KIR1D was not a PCR artifact, a separate PCR was performed on cDNA from the same rhesus monkey; cDNA clones identical with Mm-KIR1D were identified.

Multiple splice variants of Mm-KIR1D were detected. In particular, three different deletions of the D2 domain were found (Fig.

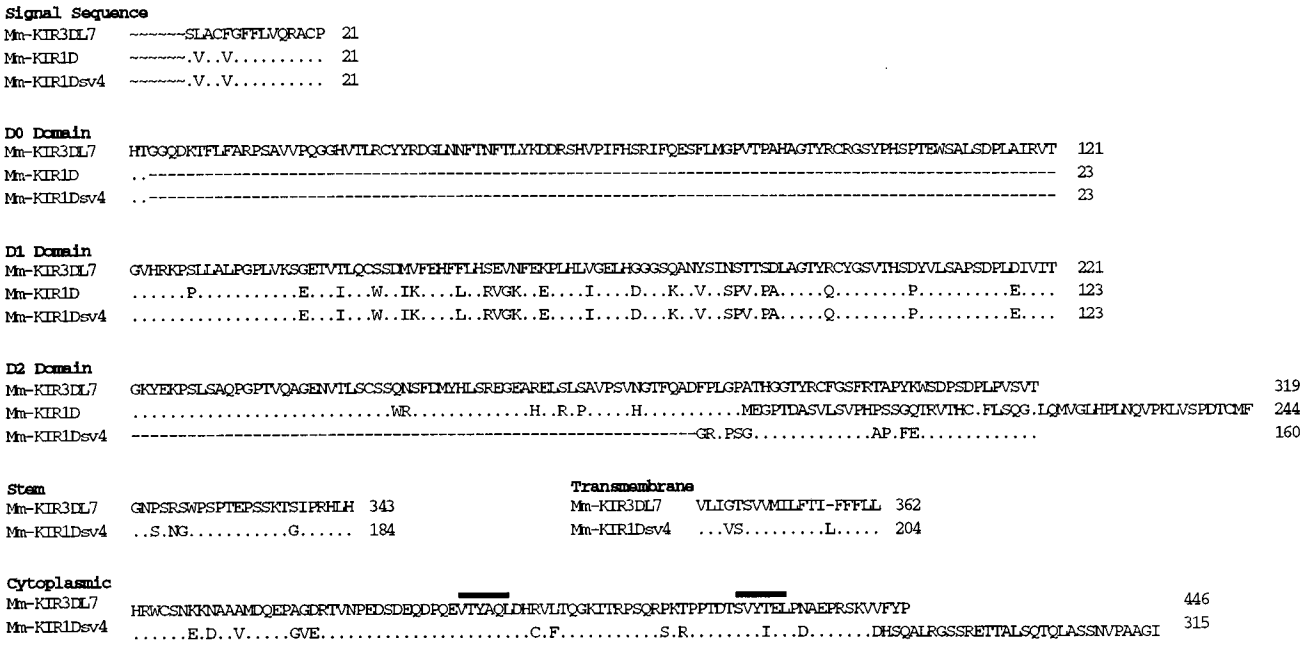

FIGURE 7. Predicted amino acid sequences and structural domains of the novel rhesus monkey KIR molecules Mm-KIR1D and Mm-KIR1Dsv4. The amino acid sequences of Mm-KIR1D and Mm-KIR1Dsv4 were aligned with that of Mm-KIR3DL7. Periods (.) indicate identity with Mm-KIR3DL7, dashes (-) indicate absence of amino acids, and tildes (~) indicate amino acids encoded by the PCR primer used to amplify the cDNA. ITIMs are indicated by bars above the motifs. The amino acid sequence for Mm-KIR1D does not have a recognizable stem, transmembrane, or cytoplasmic domain. Mm-KIR1Dsv4 is a splice variant of Mm-KIR1D with a deletion in the D2 domain.

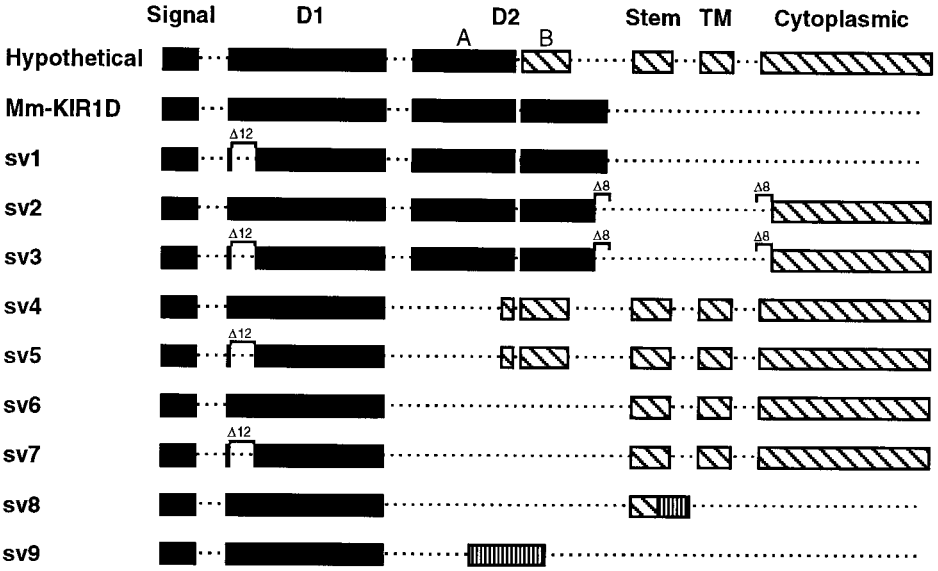

FIGURE 8. Alternatively spliced forms of the novel rhesus monkey KIR molecule Mm-KIR1D. Putative splice variants (sv) are shown schematically compared with a hypothetical molecule with two complete Ig domains. This hypothetical molecule would result from a frame shift by two nucleotides in the D2 domain (diagonal lines). The D2 domain of the hypothetical molecule is shown divided into A and B regions. The A region shown in black is similar (91% amino acid identity) to the corresponding region in Mm-KIR3DL7. The B region indicated with diagonal lines is similar (84% amino acid identity) to that region in Mm-KIR3DL7. The cDNA clone encoding Mm-KIR1D has no evidence of alternative splicing. Some splice variants of Mm-KIR1D contain a deletion followed by frame shifts by 2 nt (diagonal lines) or 1 nt (vertical lines).

8). Mm-KIR1Dsv4 has a deletion of 176 nt in D2 that results in a 2-nt frame shift. The predicted 32 aa of the D2 domain in this molecule align with Mm-KIR3DL. Its stem, transmembrane, and cytoplasmic domains are unique, but homologous to those of other KIR molecules. Mm-KIR1Dsv6 has a deletion encompassing the entire D2 domain, but has stem, transmembrane, and cytoplasmic domains that are identical with those of Mm-KIR1Dsv4. Mm-KIR1Dsv9 has a deletion of 115 nt in D2 and a resultant 1-nt frame shift. This frame-shifted molecule terminates early, before the stem domain.

The stem, transmembrane, and cytoplasmic domains of the Mm-KIR1D splice variants are distinct from those domains in Mm-KIR3DL molecules (Fig. 7). The transmembrane domains of the splice variants, like those of the human KIR3DL molecules, have one more amino acid than the homologous domains of Mm-KIR3DL molecules. The cytoplasmic tails of the splice variants have two ITIMs and are 27 aa longer than the cytoplasmic tails of Mm-KIR3DL molecules. These cytoplasmic tails are 4 aa shorter than the cytoplasmic tails of Mm-KIR2DL4 or Mm-KIR2DL5.

Mm-KIR1Dsv2 has a deletion of the exon encoding the transmembrane and portions of the stem and cytoplasmic domains (exon 7 in human KIR3DL1; Fig. 8). This 104-nt deletion results in a 2-nt frame shift. When translated, this molecule has most of the cytoplasmic tail, including the two ITIMs. The molecule Mm-KIR1Dsv8 has deleted both the D2 domain and exon 7. The net effect of these deletions is a 1-nt frame shift that, when translated, results in early termination. Finally, some of the Mm-KIR1D splice variants, such as Mm-KIR1Dsv1, have a 12-aa deletion at the beginning of the D1 domain.

Mm-KIR3DH and alternatively spliced forms

A second novel family of rhesus monkey KIR molecules was identified (Fig. 9). Like Mm-KIR3DL, the cDNA of these molecules encode three Ig domains. However, scattered nucleotide changes occur in all three of the Ig domains that are distinct from any Mm-KIR3DL molecule. Although these molecules have three Ig domains, portions of the stem, transmembrane, and cytoplasmic domains encoded by exon 7 are similar in sequence to the homologous region of Mm-KIR2DL4.2. In particular, the transmembrane domains of these novel rhesus monkey KIR molecules include an arginine as well as the threonine in the stem and isoleucine in the transmembrane domains that are characteristic of Mm-KIR2DL4.2. Nevertheless, these novel molecules have amino acid changes in the transmembrane domain that are distinct from Mm-KIR2DL4.2. The nucleotide sequence of exon 8 expected in a KIR molecule is not found in these rhesus monkey KIR molecules. This deletion of 53 nt leads to a 2-nt frame shift, and the novel molecules terminate after encoding only two further amino acids. This early termination occurs before the ITIMs seen in the Mm-KIR3DL and Mm-KIR2DL4 molecules.

Because these novel molecules have characteristics of both Mm-KIR3DL and Mm-KIR2DL4.2, we have designated them as Mm-KIR3DH, where H denotes the hybrid nature of the molecule. Although Mm-KIR3DH molecules have short cytoplasmic tails without ITIMs, we have not used the short (S) designation used in naming human KIR molecules because Mm-KIR3DH molecules have a large deletion that results in their early termination rather than the codon change seen in the human KIR2DS molecules or the 2-nt deletion seen in the human KIR3DS1 molecule (23).

In defining a type of Mm-KIR3DH molecule on the basis of >2% predicted amino acid sequence divergence between Mm-KIR3DH cDNA clones, three distinct types of Mm-KIR3DH were detected in a single rhesus monkey. In a second rhesus monkey, a fourth distinct Mm-KIR3DH molecule was identified (Fig. 9).

Alternatively spliced forms of Mm-KIR3DH were identified in both of these rhesus monkeys (Fig. 10). As seen in Mm-KIR3DL splice variant molecules, Mm-KIR3DH molecules with deletions of the D0 domain, 12 aa of D1, and 50 aa of D2 were detected. The deletion of a portion of the stem seen in Mm-KIR3DHsv4 is similar to the stem deletion of the human KIR3DL1 molecule NKB1B (14). However, Mm-KIR3DHsv4 also has two additional deletions in the Ig domains. A large deletion extending from the D2 domain to the end of the cytoplasmic domain is seen in Mm-KIR3DHsv5

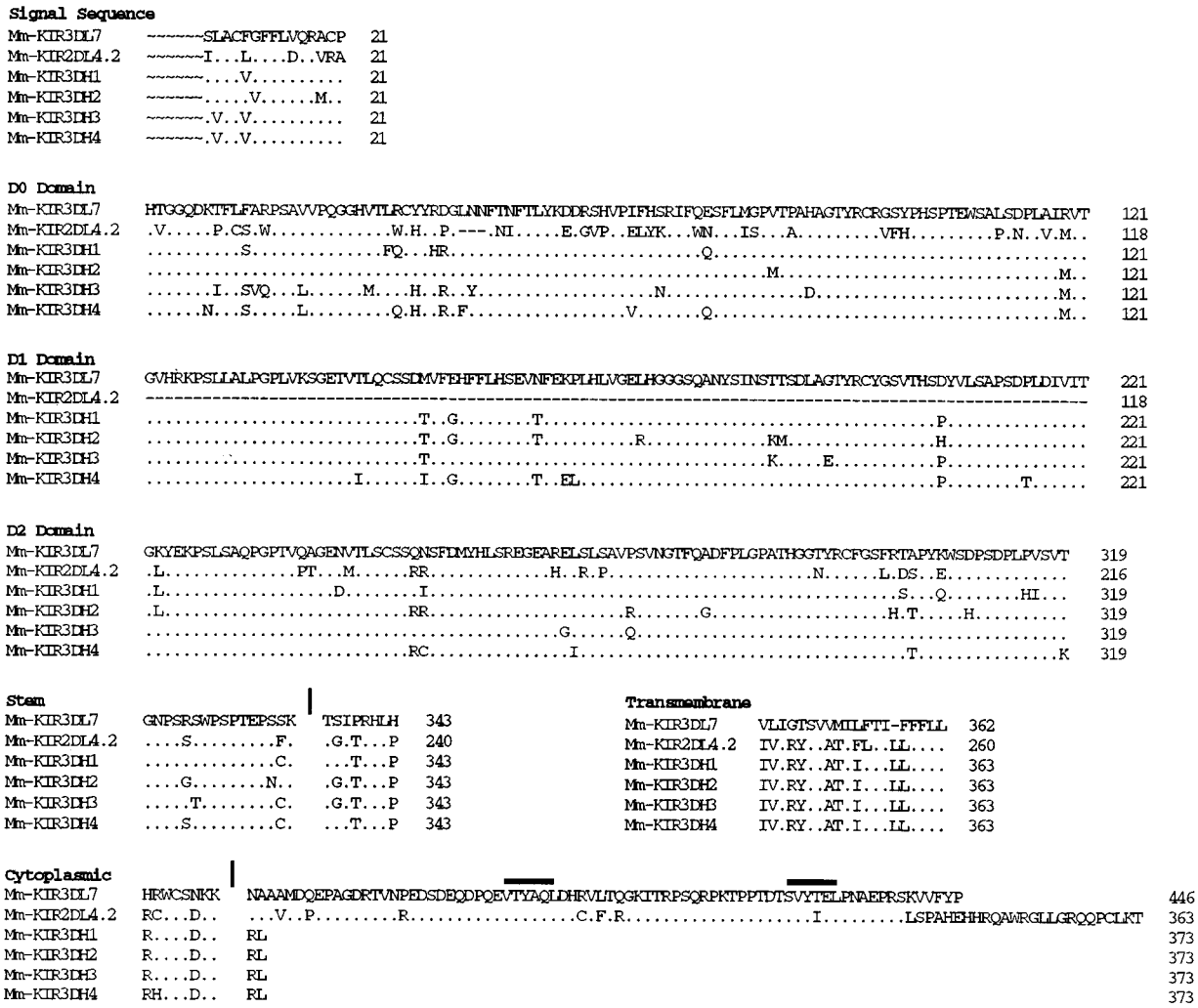

FIGURE 9. Predicted amino acid sequences and structural domains of the novel rhesus monkey KIR3DH molecules. The amino acid sequences of Mm-KIR3DH were aligned with those of Mm-KIR3DL7 and Mm-KIR2DL4.2 because it is a hybrid of Mm-KIR3DL and Mm-KIR2DL4.2 molecules. The predicted boundaries for the exon 6 of Mm-KIR2DL4.2 (which is predicted to be exon 7 for Mm-KIR3DL and Mm-KIR3DH) are indicated by vertical bars. Periods (.) indicate identity with Mm-KIR3DL7, dashes (-) indicate absence of amino acids, and tildes (~) indicate amino acids encoded by the PCR primer used to amplify the cDNA. ITIMs are indicated by bars above the motifs. The deletion of the sequence corresponding to exon 8 in the Mm-KIR3DH molecules leads to their early termination, before the ITIM motifs.

and Mm-KIR3DHsv6. This deletion, in combination with deletions in the D0 or D1 domain, results in molecules with only one complete Ig domain and no significant cytoplasmic tail. A further 27-aa deletion in the middle of D1, in combination with the 12-aa deletion at the beginning of D1, is unique to Mm-KIR3DHsv6. Mm-KIR3DHsv7 has a 176-nt deletion in the D2 domain that leads to a frame shift of 2 nt and subsequently to an early termination.

Discussion

In characterizing the rhesus monkey KIR molecules, we have identified five families of molecules: Mm-KIR3DL, Mm-KIR3DH, Mm-KIR2DL4, Mm-KIR2DL5, and Mm-KIR1D molecules (Fig. 11). The most striking aspect of the Mm-KIR3DL molecules in the monkey is their extreme polymorphism. Two KIR3DL molecules have been described in humans, and five distinct forms of KIR3DL have been identified in chimpanzees (14, 25, 26). The two human KIR3DL molecules are quite distinct from one another, differing in sequence by 75 aa. However, when sequences of KIR3DL1 molecules from different individuals are compared, fewer than five amino acid differences are detected (18, 19). Similarly, the nucleotide sequences of the same type of Pt-KIR3DL molecule are quite similar (<2% differences) when sequences from different chimpanzees are compared (25). In contrast to this, the sequences of the KIR3DL molecules identified in the rhesus monkey are strikingly variable when molecules from different animals are compared. Up to five forms of Mm-KIR3DL molecules were detected in a single rhesus monkey, and eleven distinct Mm-KIR3DL molecules were found in the five animals studied (Fig. 3). It is noteworthy that the five Mm-KIR3DL molecules detected in monkey 227 have predicted amino acid sequences that differed by >2% from all three of the KIR3DL molecules detected in monkey 577.

The amino acid substitutions in Mm-KIR3DL molecules are distributed throughout the molecule. Comparison of the D1 and D2 domains of Mm-KIR3DL molecules with the crystal structure of the homologous domains of the human KIR2DL2 molecule associated with its MHC ligand suggests that some of the amino acid differences observed between the different Mm-KIR3DL molecules occur in the six loops of the Ig-like domains that are likely to interact with their MHC ligands (27, 28). The portions of the

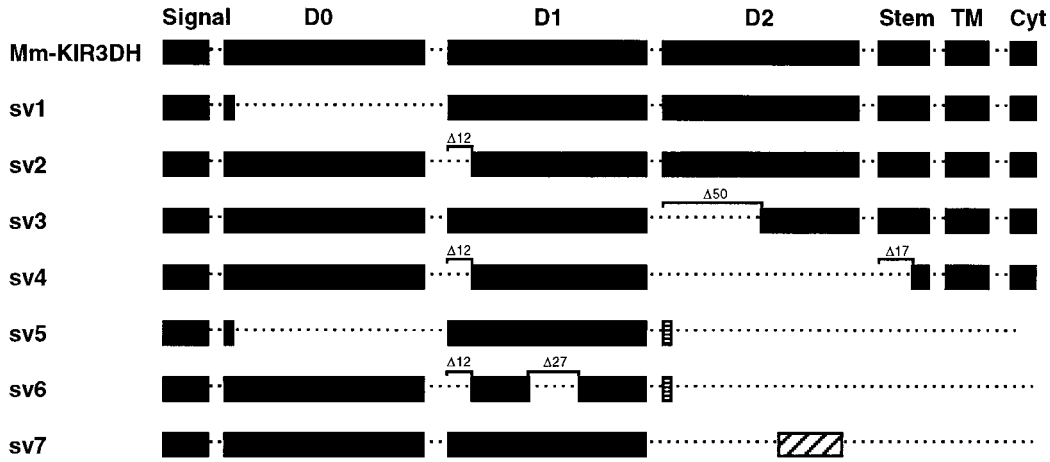

FIGURE 10. Alternatively spliced forms of rhesus monkey KIR3DH. Putative splice variants (sv) are shown schematically compared with full-length Mm-KIR3DH. Molecules sv5 and sv6 have a near complete deletion of the D2 through the cytoplasmic domain (horizontal bars). Sv7 has a deletion in the D2 domain followed by a frame shift of 2 nt indicated with diagonal lines.

Mm-KIR3DL molecules that are homologous with loops five and six in the D2 domain of the human KIR2DL2 structure are especially variable. In particular, the amino acid at position 251 in loop five can be the hydrophilic amino acids threonine or asparagine or the hydrophobic amino acid cysteine. The extra cysteine at this position could contribute to an additional disulfide bond. In loop six, the amino acid at position 302 can have very different chemical characteristics and is either the negatively charged aspartic acid, the hydrophilic amino acid threonine, or the hydrophobic amino acid isoleucine (Fig. 1).

Although a single form of KIR2DL4 has been defined in humans and chimpanzees, two forms of Mm-KIR2DL4 were identified. These two forms of Mm-KIR2DL4 have identical Ig domains, but the ends of their cytoplasmic tails differ as a result of a single nucleotide deletion. In contrast to both the human and chimpanzee KIR2DL4 molecules that have only one ITIM, these rhesus monkey molecules contain two ITIMs in their cytoplasmic domains (Fig. 5). It is possible that the function of the two Mm-KIR2DL4 molecules may differ because of differences in their cytoplasmic tails; however, no interaction with signaling proteins outside the ITIMs has been described for the cytoplasmic tails of the human KIR molecules.

The novel Mm-KIR3DH molecules have three Ig domains like the Mm-KIR3DL molecules, but lack ITIMs in their cytoplasmic tails because of an exon 8 deletion. Lacking ITIMs, they are not likely to act as inhibitory receptors. Rather, Mm-KIR3DH molecules may act as activating receptors for rhesus monkey NK cells. These receptors have a transmembrane domain that is most similar to the transmembrane domain of the Mm-KIR2DL4.2 molecule, which contains an arginine. The charged amino acid arginine in the transmembrane domains of the Mm-KIR3DH molecules may be capable of interacting with proteins that have an immunoreceptor tyrosine-based activation motif, such as DAP12 or FcRγ. In fact, the activating forms of the ILT and Ly49 receptor families both contain an arginine in their transmembrane domains that interacts with FcRγ and DAP12, respectively (29, 30). This suggests that an arginine may substitute in these monkey KIR molecules for the lysine seen in the activating form of the human KIR molecules. Nevertheless, an arginine is also seen in the transmembrane domains of the human and rhesus monkey KIR2DL4 molecules. These molecules have ITIMs, and are presumed to mediate inhibitory signaling.

Comparison of the D1 and D2 domains of the Mm-KIR3DH molecules with the human KIR2DL2 crystal structure revealed amino acid differences among the four types of Mm-KIR3DH in all but the first of the loops that are likely to interact with the MHC ligand (28). Loop 5 in the D2 domain is particularly variable, with four different amino acids at position 251 for the four different Mm-KIR3DH types (Fig. 9).

Multiple splice variants of Mm-KIR3DL and Mm-KIR3DH molecules were detected (Figs. 4 and 10). Most of these deletions remained in the same reading frame as the full-length KIR molecules. Many of these deletions correspond to exon boundaries and would occur as the result of exon skipping. Deletions of only portions of the Ig domains also were identified. These variants may be produced through the use of alternative splice sites, as has been

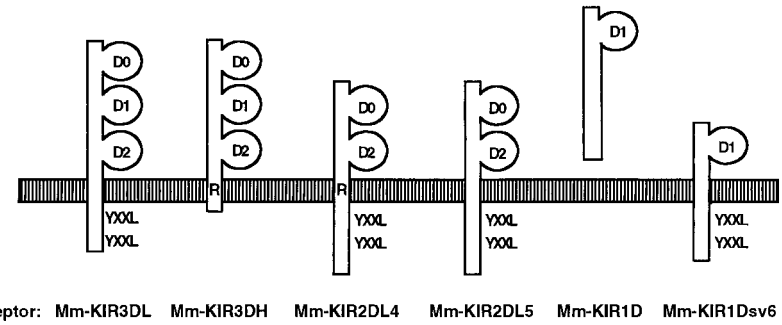

FIGURE 11. Schematic representation of the structures of the rhesus monkey KIR molecules. YXXL denotes an ITIM and R indicates an arginine in the transmembrane domain.

suggested for the human KIR splice variants (24). These molecules with deletions in the Ig domains may have altered binding to their MHC ligands. In addition, because these deletions result in the elimination of β sheets, the folding of these molecules is likely to be affected. Spliced forms also were identified that lack stem and transmembrane domains. These molecules may be soluble. However, it remains unclear whether any of the alternatively spliced Mm-KIR molecules have a biological function.

Because a PCR-based approach was used to characterize the KIR molecules of rhesus monkeys, rare mRNA forms could have been preferentially amplified, resulting in a distortion of the relative representation of these transcripts. Conversely, other KIR molecules may not have been amplified by the PCR primers used in this study. Indeed, certain KIR molecules seen in both humans and chimpanzees were not observed in rhesus monkeys. We did not identify rhesus monkey homologues for KIR2DL with the D1-D2 configuration. Because these KIR molecules interact with HLA-C alleles in humans, and rhesus monkeys have been shown to lack a homologue for HLA-C, it is possible that there was no selection for the D1-D2 form of KIR2DL in the rhesus monkey (31). Interestingly, the Mm-KIR1D molecule has a complete D1 Ig domain and a portion of a D2 domain preceding a frameshift. This molecule may in fact be a homologue of the human KIR2DL molecules.

We also did not identify rhesus monkey KIR molecules with short cytoplasmic tails and a lysine in the transmembrane domain homologous to the activating KIR molecules seen in humans and chimpanzees. It remains possible that the rhesus monkey homologues of KIR2DS and KIR3DS are too divergent from the human activating KIR molecules to be identified by using the human KIR-specific PCR primers. However, Mm-KIR3DH molecules may be activating KIR molecules in rhesus monkeys.

Finally, evaluation of the Mm-KIR3DL molecules revealed no homologues of the human KIR3DL2 molecule, which has two characteristic cysteines at amino acid positions 302 and 336. It has been suggested that one of these cysteines facilitates homodimerization of the KIR3DL2 molecule (26). Interestingly, cysteines in these positions were not found in any of the chimpanzee or rhesus monkey KIR molecules. These findings suggest that a homodimerized form of a KIR3DL molecule may not exist in primates other than humans.

In addition to the KIR molecules, the heterodimeric CD94/NKG2 family of molecules is a second type of MHC class I receptor expressed on human NK cells. The CD94/NKG2 receptor family recently has been characterized in rhesus monkeys (32). Both the inhibitory NKG2A and the activating NKG2C molecules were identified in this species, and these molecules have at least 85% amino acid identity to their human homologues. In humans, the ligand for these receptors is the nonclassical MHC class I HLA-E molecule (33). The homologue of HLA-E, Mamu-E, has also been identified in rhesus monkeys (34). Because HLA-E homologues in primates have limited polymorphism and are the most phylogenetically conserved of the MHC class I genes, it is not surprising that the NKG2 family is more conserved than the KIR molecules (35).

Although the extreme diversity of the rhesus monkey KIR molecules may be unexpected in view of our knowledge of their chimpanzee and human homologues, it may be readily appreciated in the context of our understanding of the classical MHC class I molecules of rhesus monkeys, the presumed ligand for most of the rhesus monkey KIR molecules. There appears to have been a duplication of the homologues of HLA-A and HLA-B, as well as the evolution of a novel MHC class I locus termed Mamu-I in this species (31, 36). This diversity of MHC class I molecules may have led to selection for a considerable number of KIR molecules, particularly Mm-KIR3DL molecules. Thus, the complexity of the rhesus monkey KIR molecules highlights their coevolution with their MHC ligands, as well as the rapidity of their evolution in primates.

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