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NK Cell Receptors of the Orangutan (*Pongo pygmaeus*): A Pivotal Species for Tracking the Coevolution of Killer Cell Ig-Like Receptors with MHC-C

Lisbeth A. Guethlein, Laura R. Flodin, Erin J. Adams, and Peter Parham

CD94, NKG2, Ly49, and killer cell Ig-like receptor (KIR) expressed by orangutan peripheral blood cells were examined by cloning and sequencing cDNA from a panel of individuals. Orthologs of human CD94, NKG2A, D, and F were defined. NKG2C and E are represented by one gene, *Popy-NKG2CE*, that is equidistant from the two human genes. Several *Popy-CD94*, NKG2A, and NKG2CE alleles were defined. *Popy-Ly49L* is expressed in cultured NK cells and has a sequence consistent with it encoding a functional receptor. Orangutan KIR corresponding to the three KIR lineages expressed in humans and chimpanzees were defined. *Popy-KIR2DL4* of lineage I is the only ortholog of a human or chimpanzee KIR, but in all individuals examined, the transcripts of this gene produced premature termination, either in the D2 domain or at the beginning of the cytoplasmic domain. Ten *Popy-KIR3DL* and one *Popy-KIR3DS* of lineage II are all closely related, but represent the products of at least two genes. The two *Popy-KIR2DL* and four *Popy-KIR2DS* of lineage III also represent two genes, both being more related to KIR2DS4 than to other human and chimpanzee KIR of lineage III. The *Popy-KIR2D* include ones predicted to be specific for the C1 epitope of MHC-C, but none specific for C2. This correlates with the observation that all orangutan MHC-C allotypes examined have the C1 motif. The *Journal of Immunology*, 2002; 169: 220–229.

Natural killer cells respond to infected and malignant cells using a variety of cell surface receptors that likely function in coordinated fashion. Among their number are receptors specific for MHC class I that are of two distinct molecular forms and encoded by genes either in the NK cell complex (NKC) or the leukocyte receptor complex (LRC) (reviewed in Ref. 1). As a group, the inhibitory receptors for MHC class I provide tolerance, preventing NK cells from attacking healthy autologous cells. Roles for the activating receptors for MHC class I remain poorly understood.

Analysis and comparison of NK cell receptors for MHC class I first involved human and mouse, species for which there appears no orthology between the MHC class I genes. The results revealed a remarkable difference in their NK cell receptors. The Ly49 lectin-like receptors that give mouse NK cells diverse receptors for polymorphic H-2 determinants (reviewed in Refs. 2 and 3) are represented by a single nonfunctional *Ly49L* gene in humans (4, 5). Conversely, the killer cell Ig-like receptors (KIR) that provide human NK cells with diverse receptors for polymorphic HLA class I determinants, are absent from mice. Common to human and mouse are the CD94:NKG2F2 form of lectin-like receptors that are specific for complexes of a nonclassical class I molecule (Qa1 in mouse, HLA-E in human) and hydrophobic peptides derived from the leader peptides of other MHC class I H chains (6–8).

To explore further this remarkable species diversity, we previously studied NKC and LRC receptors in the common chimpanzee (*Pan troglodytes*) (9, 10), a species which has orthologs for all the expressed human HLA class I genes (11). In comparison to human, the chimpanzee CD94 and NKG2 family members are structurally conserved as is the inhibitory class I specificity of the CD94:NKG2A receptor (9). Orthologs for each human CD94, NKG2 family member were identified, with the exception of NKG2C, for which there are two chimpanzee paralogs (10).

A quite different situation was observed for the KIR. A minority (three) of chimpanzee KIR are orthologous to human KIR, the majority having homologous relationships indicative of rapid evolution through gene duplication, deletion, and other mechanisms of recombination (9). Within the KIR specific for MHC-A and -B, species-specific divergence in structure correlated with differences in MHC class I specificity consistent with coevolution of MHC class I and KIR to maintain ligand-receptor interaction. In contrast, MHC-C-specific KIR with identical specificity in the two species had accumulated considerable structural divergence that could not simply be attributed to pressure for maintaining ligand-receptor interaction.

The study described here concentrates on another ape species, the orangutan (*Pongo pygmaeus*). Being more divergent from humans than the chimpanzee, the orangutan has an MHC in which the class I genes have several defined differences from their human counterparts (11). Whereas *Popy-E* and -G are orthologous (12, 13), the *Popy-A* and -B genes may not be orthologs of HLA-A and -B (14, 15). Most important is that the *Popy-C* locus is only present on ~50% of *MHC* haplotypes; and thus, a significant minority of orangutans lack an MHC-C locus (14). To see how these differences have impacted the evolution of NK cell receptors in the higher primates, we have characterized orangutan NK cell receptors.
Materials and Methods

Orangutans

Orangutan peripheral blood samples were obtained from the Yerkes Regional Primate Center (Atlanta, GA). Nine orangutans were used for this study comprising seven males and two animals of unknown gender. This number included two parent/offspring pairs, Lokan:Loklok and Ayer:Jantan. Results from Lokan:Loklok are consistent and show segregation of a haplotype with a single 3DL gene and a haplotype with two 3DL genes. Neither of Ayer’s 3DL alleles was recovered from Jantan; however, only four clones were available for sequencing. One of the alleles carried by Jantan is present in two other genotypes with three 3DL sequences, so it is possible that Jantan also has three 3DL sequences, one of which was not recovered in the course of this study.

RNA and cDNA preparation

PBMC were isolated on Ficoll-Hypaque gradients (Amersham Pharmacia Biotech, Piscatway, NJ). For one individual (Allen), cultured cells were used as the source of RNA. PBMC (5 × 10^6) were cultured with irradiated RPMI 8666 cells (1 × 10^6) in IMDM with 10% BCS, 2% human AB serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 50 U/ml of IL-2 for 10 days. RNA was prepared using RNeasy (Qiagen, Hilden, Germany) according to manufacturer’s instructions. Total RNA was used for cDNA preparation. Either avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) or Moloney murine leukemia virus reverse transcriptase (Life Technologies, Rockville, MD) was used for generation of cDNA according to the manufacturer’s protocols.

PCR and cloning of NK receptor genes

Lectin-like receptors.

A panel of five individuals was sampled in cloning the lectin-like receptors. Oligonucleotide primers based on conserved sequences of CD94, NK2 family members or Ly49L were used in PCR amplification of cDNA to obtain products corresponding to the lectin-like receptor genes. The primers for CD94 and NK2D were the same as described previously (10). For other members of the NK2 family and Ly49L, new primers were designed. The primer pairs are as follows: for NK2A, 5′-CTG CCG ACA GAA GAG TAC AGT-3′ and 5′-GCC CGA CAC AAA TGC TAG GAT-3′; for NK2D, 5′-AGA AGT GAC TCT GGC CCA GGA-3′ and 5′-TCA CCC ATG ATG GAT GAT GAC TGC-3′; for NK2F, 5′-ACC CAA AGA GCC AGC AAA GGA AAC T-3′ and 5′-ATG CCA ACC CAT GAG GGA ACT G-3′; for Ly49L, 5′-GGA TTT GAA TGC AAA ACT TA-3′ and 5′-TTC CAT TTTC CAC TTT TT-3′. Amplification conditions for Ly49L were 94°C for 1 min, 30 cycles of 94°C for 30 s, 47°C for 30 s, 72°C for 1 min, followed by a final extension at 72°C for 10 min. For the other primer pairs, the amplification conditions were 94°C for 1 min, 30 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 1 min, followed by a final extension at 72°C for 10 min. Amplification products were cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Clones were analyzed by restriction digest and/or partial nucleotide sequencing using T3 and T7 primers, followed by complete sequencing of selected clones using dye terminator automated sequencing on an ABI377 sequencer (PE Applied Biosystems, Foster City, CA).

Killer cell Ig-like receptor

KIR sequences were amplified initially using primers that had successfully amplified KIR in chimpanzee and human (9, 16). A panel of nine individuals was sampled. A single 3′ primer (NKR) was used in combination with one of two 5′ primers: the 2lgF primer amplifies both KIR2DL and KIR3DL, whereas the 3lg5′ primer was originally developed to be KIR3D specific. The priming site for 3lg5′ is situated 3′ of the site for the 2lgF primer. When sequence analysis of cDNA clones obtained with the 2lgF/NKR primers showed that orangutan KIR differ at several positions within the 3′-untranslated region. A 3′-lg5′ primer was made which incorporated the differences (5′-CAC ACA TGG GTG GTC AGG AGC-3′). The PopP-3lg5′/NKR primer combination also amplified Popy-KIR2DL. The following conditions were used for the amplification, 94°C for 5 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min, followed by a final extension at 72°C for 7 min. Amplification products were subjected to agarose gel electrophoresis and bands of the appropriate size were purified and cloned and sequenced as described above. A minimum of 96 clones was examined from each individual orangutan.

Phylogenetic analysis

Sequences were manually aligned using the GCG Wisconsin Package (Wisconsin Package Version 10.2; Genetics Computer Group, Madison, WI). Codon-aligned sequences were submitted to the SNAP server for dNdS analysis (hiv-web.lanl.gov/SNAP/SNAPSNAP.html) (17, 18). Neighbor joining trees were constructed using the PAUP (v4.0b8) program suite (19). Confidence in the branching of the tree was assessed by bootstrap analysis using 1000 replicates.

In this study, we use the term orthologous to describe genes derived from a common ancestor for which there is a single example in each of the daughter species. Paralogous refers to genes resulting from duplication within the common ancestor.

Popy-C motif analysis

Genomic DNA from a panel of 18 orangutans was analyzed for the presence of Popy-C as well as the identity of the KIR-binding motif present. First, a general amplification of a product corresponding to exons 2 and 3 of Popy-MHC class I loci was performed. The primers used were Exon2F (5′-AGG CTC CCA CAT CAT GAG GTA-3′) and Exon3R (5′-CTT CCC GTT CTC CAG GTA TC-3′) with the following conditions, 95°C for 5 min, 10 cycles of 95°C for 20 s, 62°C for 45 s, 72°C for 2 min, followed by a final extension at 72°C for 2 min. For a second set of amplifications, the product of the first PCR and typed for the presence of Popy-C as well as the identity of the motifs. Amplification conditions modified from the protocol described by Frohne et al. (20) were used. A common forward primer HLA-CF (5′-CCG GAC GAG TCC CAG AGG-3′) was used in combination with three different reverse primers. These primers were as follows: specific for Popy-C, Popy-CR (5′-GGT TAC GAG GAG AGA ATT CA-3′); specific for NK1, Popy-CT (5′-GGT GTA GCC GCG CAG T-3′), and specific for Lys88, NK2 (5′-GGT GTA GGA GCC GCG CAG-3′). A 1/10 dilution of the product resulting from the first amplification was used with the following amplification conditions: 95°C for 1 min, 10 cycles of 95°C for 10 s, 67°C for 30 s, 72°C for 30 s, 20 cycles of 95°C for 10 s, 62°C for 30 s, 72°C for 30 s followed by a final extension at 72°C for 10 min. Products were visualized by agarose gel electrophoresis. The Popy-C amplification product from individual not previously analyzed was cloned and sequenced as described above.

Accession numbers

GenBank accession numbers for the sequences described in this study are as follows: Popy-CD94 (AF740380-5), Popy-NKG2A (AF740391-7), Popy-NKG2CE (AF740398-402), Popy-NKG2D (AF740403-4), Popy-NKG2F (AF740405-6), Popy-Ly49L2 (AF740390), Popy-2DL (AF740388-9), Popy-3DL (AF740365-74), Popy-3DS (AF740375), Popy-2DL (AF740388-9), Popy-C (AF740376-9), and Popy-C intron 2 (AF740386-7).

Results

Complementary DNA clones encoding orangutan CD94, NKG2, Ly49L, and KIR were obtained by RT-PCR using primers based upon sequences conserved in humans and chimpanzees. In aggregate, the clones recovered from a panel of nine orangutans revealed that genes corresponding to all major lineages of C-type lectin receptors and KIRs are present in this species (Fig. 1A). All orangutan sequences have been given the prefix Popy (P. pygmaeus) followed by the name consistent with the family to which the sequence was assigned. Alleles of the CD94, NKG2 family members could be confidently assigned to single loci and were given numerical designations. In the KIR family, where locus assignment was ambiguous, sequences of each lineage were given letter designations. In comparison to humans and chimpanzee, overall sequence similarity was highest for the C-type lectin family and KIR lineage I. In contrast, KIR lineages II and III exhibited considerable variation (Fig. 1B).

Orangutan CD94 and NKG2 lectin-like receptors

Complementary DNA clones corresponding to CD94, NKG2D, NKG2F, and NKG2A were identified. Nucleotide differences from their human counterparts are 2.2, 3.4, 4.5, and 4.6%, respectively, consistent with an orthologous relationship (Fig. 1). Clones corresponding to a fourth member of the orangutan NKG2 family are closely related to human NKG2C and NKG2E, and on phylogenetic analysis was found to be equidistant from them (Fig. 2). These relationships indicate that the NKG2C and NKG2E genes were isolated in 1994.
are the products from duplication of an ancestor resembling the orangutan gene, which we have called Popy-NKG2CE.

Of the 24 aa substitutions that distinguish orangutan and human NKG2A, 19 cluster within the carbohydrate recognition domain (CRD) and make that region more like human NKG2E than NKG2A (Fig. 3). The differences are particularly evident in the region implicated in ligand binding (residues 221-241 in Fig. 3) (21-23). In the rhesus monkey, this region of MmNKG2A (24) is also more similar to human and chimpanzee NKG2E, indicating that this sequence may more closely represent the ancestral NKG2A sequence. Popy-NKG2A terminates at the same position as other NKG2A and NKG2C sequences (10, 25) and does not use the splice site (26) that results in the divergent end sequence of NKG2E in the human and chimpanzee sequences, indicating that this splice site usage has arisen in the lineage leading to the chimpanzee and human sequences.

Similar to the chimpanzee and human NKG2F sequences, Popy-NKG2F is also predicted to terminate early (27). However, while the human and chimpanzee sequences are predicted to terminate at residue 159 near the beginning of the CRD, the predicted terminus for orangutan NKG2F is at residue 88 within the transmembrane region of the molecule.

Polymorphism in orangutan CD94 and NKG2 genes was assessed by analysis of cDNA clones from five individuals. Multiple alleles for CD94, NKG2A, and NKG2CE were defined. For each locus, the alleles differ by one to five nucleotides. Unexpected was finding six alleles for CD94, a gene that appears monomorphic in humans and chimpanzees (10). By contrast, only two orangutan NKG2D alleles and two NKG2F alleles were defined (Fig. 1). No more than two variants for each lectin-like receptor were expressed by individual orangutans, consistent with each of the lectin-like receptors being encoded by a single locus.

**Popy-Ly49L, a potentially functional gene**

Although the Ly49L genes of human, chimpanzee, and gorilla appear nonfunctional, that is not the case for all primate species. Recently, expression of an intact Ly49L transcript in the baboon...
Lineage I KIR

Lineage I KIR have the D0 + D2 configuration of Ig-like domains and are represented by KIR2DL4 and KIR2DL5 in humans (29, 30). Seven of the nine orangutans gave clones corresponding to orangutan KIR2DL4A, which has 96.4% nucleotide sequence similarity to human KIR2DL4. No orangutan cDNA clone resembling human KIR2DL5 was obtained.

Two Popy-KIR2DL4 sequences that differ by nine nucleotide (2 aa) substitutions were defined, one individual giving both types of sequence. These sequences likely represent alleles of Popy-KIR2DL4. Of note is that neither Popy-KIR2DL4 allele encodes a protein corresponding to the full-length human KIR2DL4 (Fig. 5A). Popy-KIR2DL4A contains a point substitution that leads to premature termination in the D2 domain, at a position just before the end of the final β-strand. Therefore, this allele may encode a soluble protein having extracellular domains with normal conformation. Popy-KIR2DL4B contains a frameshift at the beginning of the region encoding the cytoplasmic tail that causes premature termination three codons downstream. As a consequence, the protein tail is predicted to be membrane bound but to have a highly foreshorteded cytoplasmic tail lacking any immunoreceptor tyrosine-based inhibition motifs (ITIM). The frameshift in the Popy-KIR2DL4B allele is due to deletion of a single A in a series of 11 As and is identical with that described in human KIR2DL4 alleles (30) (directly deposited by Selvakumar et al. in GenBank, accession no. AF276292).

Whereas human and chimpanzee KIR2DL4 have only one intact ITIM in the cytoplasmic tail, and it is a different ITIM in the two species, both Popy-2DL4 alleles have sequences that encode a pair of intact ITIMs, although they cannot be incorporated into the protein because of premature termination (Fig. 5B). Popy-KIR2DL4 combines the intact ITIMs from both the human and chimpanzee, as does the rhesus monkey ortholog, Mm-KIR2DL4 (32). However, the gene encoding the latter KIR has neither of the disabling mutations found in the orangutan sequence and is predicted to encode a full-length protein with two ITIMs in the cytoplasmic tail (Fig. 5B). These comparisons suggest that the Popy-KIR2DL4 gene has been subject to a process of modification or inactivation during evolution of orangutans.

Analysis of the frequencies of synonymous and nonsynonymous substitutions indicates that KIR2DL4 has been subject to purifying selection, as seen from dN/dS ratios that are much <1. This is true for both interspecies and intraspecies comparisons (Fig. 5C) with the exception of the intraspecific comparison for the rhesus monkey sequences where the value is 1.03, suggesting neutral evolution within this species. Inspection of the aligned sequences shows that substitutions are distributed relatively evenly throughout the coding region, with the exception of exon 3 encoding the D0 domain where substitutions are rare. Codon-by-codon analysis supports this conclusion providing no evidence for any mutational hotspot, although the region encoding the stem and the first part of the transmembrane region lacks nonsynonymous substitutions (data not shown).

Lineage II KIR

In humans and chimpanzees, the lineage II KIR have three Ig-like domains and include inhibitory receptors specific for MHC-A and -B (9, 33–35). KIR of this lineage were most abundant within the population of orangutan KIR cDNA clones sequenced. Clones encoding KIR3DL were obtained from all 9 individuals, and 10 different KIR3DL variants were defined (Fig. 6A). Of these, the D1/D2 and E1/E2 pairs of variants are only distinguished by single synonymous substitutions. Although the KIR3DL variants are
closely related (95–99.9% nucleotide sequence identity), they cannot all be alleles of a single locus as two individuals (JingJing and LokLok) expressed three KIR3DL variants. Indeed, comparison of the genotype of Lokan with his offspring Loklok confirms the inheritance of one haplotype containing the single KIR3DL, KIR3DLC from Lokan; and therefore, the inheritance of a haplotype containing the other two KIR3DL from the other parent. Of the remaining seven orangutans, six expressed two forms of KIR3DL while one individual (Allen) expressed a short-tailed KIR3DS form as well as two KIR3DL. Thus, there is evidence for two genes encoding KIR3DL, at least on some haplotypes.

Orangutan KIR3D differ from human and chimpanzee KIR3D of lineage II by \(/H110117\% of their nucleotide sequence. Phylogenetic analysis shows that they group separately from the human and chimpanzee sequences (Fig. 7). Thus, the orangutan sequences cannot be assigned as orthologous to any of the human or chimpanzee sequences on the basis of sequence comparison. This is consistent with the lack of orthology seen between human and common chimpanzee KIR of lineage II (9). The orangutan sequences of this lineage exhibit a patchwork pattern of substitution, as found in other KIR genes, which provides evidence for microrecombination having contributed to the diversity within Popy-KIR3D (Fig. 6A). Evidence for interlocus recombination is that Popy-KIR3DLE has a D2 domain more closely related to that of Popy-KIR2D of lineage III than to other KIR3D. This implicates a conversion between KIR3D and KIR2D genes, involving exon 5, in the formation of Popy-KIR3DLE. The Popy-KIR3DS sequence also appears to be a product of recombination. Although it is highly homologous to Popy-KIR3DL sequences in the extracellular domains, the transmembrane portion of the sequence is closely related to the KIR2DS sequences, having a termination codon that is, relative to KIR3D, introduced by point substitution. Popy-KIR3DS also has the characteristic lysine residue in the transmembrane segment that is associated with DAP12 interaction (36).

Most conserved of the extracellular part of Popy KIR3D is the D1 domain. Among the 11 variants, only one position of amino...
acid substitution is in D1. This conservation is comparable to that in human KIR3DL1 but greater than that in human KIR3DL2 (37).

Analysis of the substitutions in the extracellular domains reveals that in Popy-KIR3D, nonsynonymous substitutions are present in both loop and strand regions of the molecule, unlike either human 3DL1, where substitutions are found mainly in loop regions, or 3DL2, where substitutions are mainly in strands (37). However, inspection of the sequences does show that the nonsynonymous substitutions are clustered within discrete regions of the sequence. In addition, both nonsynonymous and synonymous substitutions are found, unlike the KIR3DL2 sequences in which the majority of the substitutions are nonsynonymous. The dN/dS ratios for KIR3D are ~1 (Fig. 6B), indicative of purifying selection, although the values are greater than seen for the lineage I KIR (Fig. 4C). This is true both within and between species.

Lineage III KIR

In humans, lineage III consists of eight KIR having two extracellular Ig-like domains in the D1 + D2 configuration (33, 38). By contrast, the six chimpanzee KIR of this lineage comprise two KIR2D and four KIR3D (9). In both species, lineage III includes the MHC-C-specific KIR. Only one lineage III KIR, KIR2DS4, has human and chimpanzee orthologs. All other loci appear to have arisen since the divergence of the human and chimpanzee lineages ~5 MYA (39). There is one exception to this, a pseudogene, KIR3DP1 (also called KIRX), that appears to be present on all human KIR haplotypes examined at the genomic level (40). In our phylogenetic analysis, KIR3DP1 groups with the chimpanzee KIR of lineage III (bootstrap value of 85%) indicative of its common origin with these KIR (Fig. 7).

In the orangutan, clones corresponding to lineage III KIR were relatively infrequent. Seven different KIR were characterized, all being KIR2D with a D1 + D2 configuration. These comprised five KIR2DS and two KIR2DL. Popy-KIR2D must be encoded by at least two loci, as individual orangutans expressed three of these KIR (Fig. 1A). As a group, the orangutan KIR2D form a cluster more closely related to human and chimpanzee KIR2DS4 than to any other KIR. In phylogenetic analysis, a 91% bootstrap value supports grouping of the orthologous human and chimpanzee KIR2DS4. When Popy-KIR2D are included, their grouping with human and chimpanzee KIR2DS4 is supported with 71% bootstrap value (Fig. 7). These data favor a model in which all Popy-KIR2D have evolved from a common KIR2DS4-like ancestor.

Within the Popy-KIR2D of lineage III group are good examples of recombinant KIR in which different extracellular domains are associated with the same stem, transmembrane, and cytoplasmic...
domains, and vice versa (Fig. 8). For example, in the extracellular domains, 2DLA is more like 2DSD than 2DLB. Similarly, 2DLB is more like 2DSC in this same region. For all Popy-KIR2D, the transmembrane and cytoplasmic domains contain the characteristic features associated with other long- and short-tailed KIR. The Popy-KIR2DS have a charged residue (Lys 233) in the transmembrane region that could facilitate interaction with the DAP12 adapter molecule and the sequences terminate in what would be the first ITIM sequence of a KIR2DL. The lineage III Popy-KIR2DL are all predicted to have two ITIMs.

The residue at position 44 in the D1 domain determines the HLA-C specificity of human KIR2D (41). Methionine 44 confers specificity for HLA-C allotypes having lysine at position 80, lysine 44 confers specificity for HLA-C allotypes having asparagine at position 80 (42, 43). Orangutan KIR with lysine 44 are included in both KIR2DL and KIR2DS (Fig. 8), but no Popy-KIR2D has methionine 44. Among the orangutan KIR2D, both KIR2DL and KIR2DS have glutamate 44, raising the possibility that this residue is associated with a distinct type of ligand specificity.

Unlike the lineage I and II KIR, an Ig domain with invariant sequence is not a feature of the lineage III KIR, regardless of whether the short- and long-tailed KIR are considered separately or together. The dN/dS ratios of the lineage III KIR are comparable to those for the lineage II KIR when compared within groups with a similar cytoplasmic tail (0.73 for the 2DL and 0.72 for the 2DS).

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Popy-C allotypes all have the C1 KIR-binding motif

Whereas MHC-C is fixed in the human and chimpanzee MHC, it is present only on ~50% of orangutan MHC haplotypes. Moreover, a preliminary analysis revealed only Popy-C allotypes having asparagine at position 80 and none having lysine at position 80 (11, 14). To explore further the apparent reduction in orangutan KIR epitopes compared with human and chimpanzee, we typed 18 orangutans, including 4 of those from whom KIR cDNA clones were isolated, for Popy-C and for presence of the C1 (Asn$^{80}$) and C2 (Lys$^{80}$) motifs. Eleven individuals had Popy-C and all of them typed positively for C1 and negatively for C2. These results indicate that Popy-C alleles encoding the C2 motif are either not present or are at low frequency in the orangutan. That this species also has KIR2D with potential specificity for C1, but lacks KIR2D with specificity for C2, suggests that MHC-C mediated regulation

FIGURE 6. Polymorphism in orangutan KIR3D of lineage II. A. The table shows positions of nucleotide substitution in Popy-KIR3D. Positions are numbered starting with the A of the ATG start codon of the human KIR of this lineage. Shaded boxes denote unique substitutions. N and S under the table indicate nonsynonymous and synonymous substitutions, respectively. For nonsynonymous substitutions in the extracellular Ig domains, their location in a loop or a strand (str) is indicated. Exon 5 of KIR3DLE was not included in the analysis as it more closely resembles exon 5 of a lineage III KIR. Exons 7–9 of KIR3DSA were not included, as they are the likely result of a recombination with a KIR2DS. B. The average dN/dS values for inter- and intraspecies comparisons.

FIGURE 7. Phylogenetic tree of KIR from five species of primate. The neighbor joining tree was constructed using an alignment of all KIR nucleotide sequences. Lineages II and III only contain KIR from hominoid. Lineage I contains KIR from five species. KIR2DP1 (also known as KIRZ or KIRY) and KIR3DP1 (also known as KIRX) are human pseudogenes. Branches with bootstrap support of <50 have been collapsed to polytomies. The lineage that includes Mm3DL, Mm3DH, and Mm1D comprises only KIR from rhesus monkey.

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of NK cells in the orangutan uses only one of the two ligand-receptor combinations available to humans.

**Discussion**

In a previous phylogenetic comparison of NK cell receptors, we chose for study the common chimpanzee, an African ape having orthologs for all the expressed human HLA class I genes (9). As counterpart, we have studied here the orangutan, a more distant Asian ape in which the MHC class I genes are not as congruent with their human counterparts. Orangutan “orthologs” have been reported for five of the six expressed human MHC class I genes, HLA-A, -B, -C, -E, and -G, and include all those presently implicated in NK cell regulation (11). Although Popy-E and -G may indeed represent orthologs of the human genes, the properties of Popy-A, -B, and -C imply less straightforward, paralogous relationships. Popy-A appears less related to HLA-A and Patr-A than to Patr-AL, a nonclassical class I gene present on some chimpanzee MHC haplotypes (15). Unlike HLA-A or Patr-A, which are fixed, Popy-C is present on ∼50% of Popy-MHC haplotypes (14); but in contrast, Popy-B appears duplicated such that individual orangutans express three or four Popy-B alleles (14, 44). We demonstrate in this study that the orangutan differs from human and chimpanzee to even greater extent in its NK cell receptors. The differences are greater for KIR than for the lectin-like receptors, consistent with previous phylogenetic comparison (9, 10, 45), but neither set of differences appears trivial.

The main points of difference in the lectin-like receptors are as follows. Orangutan NK cells express Ly49L, a gene that appears nonfunctional in humans and chimpanzee. The NKG2C and NKG2E genes are represented by a single Popy-NKG2C gene that has similarity to both of the human genes. A reverse trend occurs in the chimpanzee NKC receptor, which encodes a full-length protein with two ITIM motifs, the orthologs of human and chimpanzee are highlighted in gray. Only sequences of the six expressed human KIR are given.

Whereas five of six orangutan NKC genes identified have human orthologs (Popy-CD94, NKG2D, NKG2A, NKG2F, and Ly49L), only one of five expressed Popy-KIR has a human ortholog, Popy-KIR2DL4. Similarly, Mm-KIR2DL4 is the only ortholog of a human KIR among rhesus monkey KIR (32). Whereas Mm-KIR2DL4 encodes a full-length protein with two ITIM motifs, the orthologs in apes and humans all exhibit degradation either in one or other ITIM motif (chimpanzee and human) or change in the position of translational termination (orangutan and certain human alleles; Fig. 5). Thus, there has been a general trend toward inactivation of the ITIMs in this gene in hominoids. Indeed, the observation that anti-KIR2DL4 Abs can stimulate IFN-γ production by NK cells (47) and the presence in the KIR2DL4 transmembrane domain of an arginine residue absent from other KIR (48) point to KIR2DL4 having activating function. KIR2DL4 is proposed as a receptor for the MHC-G nonclassical class I molecule (49). Striking in this regard is that MHC-G is a pseudogene in rhesus macaque while being an active expressed gene in the hominoids (13). This difference may reflect functional differences in the KIR2DL4 receptors of hominoids and macaque.

The impression gained from our study is that the KIR system of the orangutan is markedly simpler than that encountered in either hominoids or macaque.

![FIGURE 8. Recombination has generated diversity in lineage III KIR. The table shows the amino acid substitutions found in the lineage III KIR. The dimorphism at position 44 is shown. At this position in human KIR, K determines C1 specificity, M determines C2 specificity.](http://www.jimmunol.org/)

![FIGURE 9. Comparison of MHC-E structure in four primate species. Shown are positions of difference relative to HLA-E as the consensus sequence. Common chimpanzee (Patr), orangutan (Popy), and rhesus macaque (Mamu). Residues common to orangutan and rhesus and absent from human and chimpanzee are highlighted in gray. Only sequences of the α1 and α2 domains are compared.](http://www.jimmunol.org/)
human or common chimpanzee. Major lineages are all represented, but within each lineage the number of loci is more restricted as is their divergence. At present our data can be accommodated minimally by five KIR genes: KIR2DL4, two KIR3D, KIR2DL, and KIR2DS. Of these the two Popy-KIR2D genes appear paralogs of KIR2DS4 and the two Popy-KIR3D are paralogs with KIR3DL1 and 2. Were these five genes to be organized into a single haplotype, it could resemble a hybrid consisting of the left hand (5') half of a human B haplotype (containing KIR2DL2) in combination with the right hand (3') half of a human A haplotype (having KIR2DL4, KIR3DL1/3DS1, KIR2DS4, and KIR3DL2) (50, 51).

A major implication of our data is that MHC-C-mediated regulation of NK cells is also simpler in orangutan than either human or chimpanzee. Several points suggest that HLA-C provides the more important inhibitory HLA class I ligand for KIR: all HLA-C allotypes are KIR ligands, all individuals can use at least one KIR2D as a self-receptor for HLA-C, the low expression of KIR2D as a self-receptor for HLA-C, the low expression of more important inhibitory HLA class I ligands for KIR: all HLA-C allotypes of the expressed potential for C1 specific and the two KIR2DS4 half of a human A haplotype (having KIR2DL4, KIR3DL1/3DS1, KIR2DS4, and KIR3DL2) (50, 51).

In human and chimpanzee the lineage II KIR have specific and the dominant ligand for KIR, we see radiation of lineage III KIR, which include the MHC-C receptors. We find two genes of this lineage in the orangutan, one KIR2DL4 and one KIR2DS. In the chimpanzee, there are six members of this lineage and in human eight (9). In addition to differences in number, the majority of the chimpanzee KIR are long-tailed, whereas in human five of the eight genes encode short-tailed KIR. The rapid emergence of new loci for g- and receptors and a stepwise increase in the complexity of the system is evidence for coevolution of HLA-C and certain types of lineage III KIR.

In human and chimpanzee the lineage II KIR have specificity for MHC-A and -B. Therefore, the parsimonious hypothesis is that Popy-KIR3D will include any receptors for polymorphic Popy-A and -B molecules. Striking is that neither KIR2D as a self-receptor for HLA-C, the low expression of more important inhibitory HLA class I ligands for KIR: all HLA-C allotypes of the expressed potential for C1 specific and the two KIR2DS4 half of a human A haplotype (having KIR2DL4, KIR3DL1/3DS1, KIR2DS4, and KIR3DL2) (50, 51).

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