Short *KIR* Haplotypes in Pygmy Chimpanzee (Bonobo) Resemble the Conserved Framework of Diverse Human *KIR* Haplotypes

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Abstract

Some pygmy chimpanzees (also called Bonobos) give much simpler patterns of hybridization on Southern blotting with killer cell immunoglobulin-like receptor (*KIR*) cDNA probes than do either humans or common chimpanzees. Characterization of *KIRs* from pygmy chimpanzees having simple and complex banding patterns identified nine different *KIRs*, representing seven genes. Five of these genes have orthologs in the common chimpanzee, and three of them (*KIRCI*, *KIR2DL4*, and *KIR2DL5*) also have human orthologs. The remaining two genes are *KIR3D* paralogous to the human and common chimpanzee major histocompatibility complex A– and/or -B–specific KIRs. Within a pygmy chimpanzee family, *KIR* haplotypes were defined. Simple patterns on Southern blot were due to inheritance of "short" *KIR* haplotypes containing only three *KIR* genes, *KIRCI*, *KIR2DL4,* and *KIR3D*, each of which represents one of the three major *KIR* lineages. These three genes in pygmy chimpanzees or their corresponding genes in humans and common chimpanzees form the conserved "framework" common to all *KIR* haplotypes in these species and upon which haplotypic diversity is built. The fecundity and health of individual pygmy chimpanzees who are homozygotes for short *KIR* haplotypes attest to the viability of short KIR haplotypes, indicating that they can provide minimal, essential KIRs for the natural killer and T cells of the hominoid immune system.

Key words: natural killer cells • killer cell immunoglobulin-like receptors • evolution • recombination • polymorphism

Introduction

Killer cell Ig-like receptors (KIRs)[1] are expressed on NK cells and subsets of T cells, mostly $CD8^+$, having activation or memory phenotype (1–6). *KIR* genes have been detected in several primate species but appear to be absent from rodents, including mice (7). In humans, the KIRs are encoded by a family of genes in the leukocyte receptor complex on chromosome 19 (8–13). The products of these genes differ in having either two or three extracellular Ig domains and also in having either long cytoplasmic tails, associated with inhibitory signal transduction, or short tails associated with activating function (14–16). *KIR* haplotypes differ in the total number of *KIR* genes they contain (~6–12) and in the relative number of genes encoding inhibitory versus activating KIRs (8, 17, 18). Some genes appear to be conserved features of *KIR* haplotypes, for example *KIRCI* (also called *KIR3DL3*), *KIR2DL4*, and *KIR3DL2* (8, 17), whereas others are restricted to a subset of haplotypes, for example *KIR2DL5* (19). Certain human KIRs have specificity for polymorphic determinants of HLA-A, -B, or -C molecules. KIR2DL1, KIR2DL2, and KIR2DL3 are inhibitory, and KIR2DS1 and KIR2DS2 are activating receptors with HLA-C specificity (1, 20, 21); KIR3DL1 and KIR3DS1 are receptors with HLA-B specificity having inhibitory and activating function, respectively (22, 23), and KIR3DL2 is an inhibitory receptor with HLA-A specificity (24, 25). In addition, KIR2DL4 is reported to have specificity for HLA-G (26).

Population analysis and phylogenetic comparison have shown that *MHC-A*, *-B*, and *-C* genes evolve rapidly compared with most other genes (27–31). *KIR* genes can also evolve rapidly as shown by comparison of human and common chimpanzee *KIR* (32). A minority of *KIR* genes are conserved, whereas the majority have undergone substantial "species-specific" divergence in the ~5 million

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[1]*Abbreviation used in this paper:* KIR, killer cell Ig-like receptor.

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years since chimpanzees and humans shared a common ancestor. In terms of the type and number of genes, the *MHC class I* gene family appears conserved in comparison to the *KIR* gene family: all the functional *HLA class I* genes have chimpanzee orthologs (31) whereas only three human *KIR* genes are in this category (32). Thus, from comparison of these two species, the *KIR* gene family is seen to have evolved faster than the *MHC class I* gene family. Whereas receptors of innate immunity have often been considered as being highly conserved (33, 34), KIRs may provide an example where the opposite is true.

To investigate further this unusual phenomenon, we have now studied the *KIR* gene family of the pygmy chimpanzee (*Pan paniscus*), also called bonobo, a species that is estimated to have last shared an ancestor with the common chimpanzee (*Pan troglodytes*) some ~2.3 million years ago (35). This study has therefore allowed an assessment of *KIR* divergence over a time period that is about half of that which separates humans and chimpanzees. The results highlight the evolutionary instability of the *KIR* gene family and have revealed a simple form of *KIR* haplotype that provides new insight into the basic requirement of the KIR system of NK cell receptors.

Materials and Methods

Chimpanzees. Peripheral blood was obtained from healthy chimpanzees housed at Yerkes Regional Primate Center at Emory University School of Medicine (Atlanta, GA) and at the Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP) at New York University Medical Center (Tuxedo, NY). PBMCs were isolated on Ficoll-Hypaque gradients and used for the isolation of total RNA as well as for establishing EBV-transformed B lymphoblastoid cell lines.

Mitochondrial DNA typing indicated that 43 of the 48 common chimpanzees studied were of the subspecies *P. troglodytes verus*, 3 were of subspecies *P. troglodytes troglodytes*, and 2 were of subspecies *P. troglodytes schweinfurthii* (31, 36). The individuals in this panel were chosen because they were either wild-born, or unrelated to other chimpanzees in the panel as documented by breeding records and supported by the analysis of *MHC class I* alleles (31, 36). Within this panel, 30 different *KIR* genotypes are represented at relatively even frequency, of which the highest was 0.11 (32). Attesting to the genetic heterogeneity within the common chimpanzee panel was that a similar sized panel of unrelated humans had 18 different genotypes, for which the most common had a frequency of 0.33 (17).

Southern Blot Hybridization. Genomic DNA was isolated from B lymphoblastoid cell lines using standard methods as described by us previously (17). Genomic DNA from pygmy chimpanzees, common chimpanzees, and humans were digested with HindIII (Boehringer) and Southern blots were made using the same protocol we described previously for humans (17). The blots were hybridized with a ^{32}P-labeled cDNA probe encoding either a common chimpanzee KIR, Pt-KIR3DL6, or human KIR3DL1, and autoradiographed using standard protocols (37).

Isolation and Analysis of Pygmy Chimpanzee KIR cDNA Clones. Total RNA isolated from PBMCs was used to synthesize first strand cDNA using previously described methods (17). *KIR* transcripts were PCR amplified from pygmy chimpanzee cDNA using methods that had worked for common chimpanzees and humans (32, 38). The oligonucleotide primers are based upon conserved segments of human *KIR* sequences. The PCR products were purified from the reaction mixture using a QIAquick PCR purification kit (QIAGEN) and cloned into pCR4-TOPO vectors (Invitrogen) according to manufacturer's instructions. Partial sequences were determined on randomly picked clones using standard T7 or M13R primers and the BigDye terminator cycle sequencing kit (Applied Biosystems) in a 377 automated DNA sequencer (Applied Biosystems). Six different *KIR* sequences were distinguished and have been submitted to EMBL/GenBank/DDBJ under accession nos. AF258798 (*Pt-KIR3DL1/2-v1*), AF266729 (*Pt-KIR3DL1/2-v2*), AF266730 (*Pt-KIR3DL1/2-v3*), AF266731 (*Pp-KIR3DL4*), AF266732 (*Pp-KIR3DLa*), AF266733 (*Pp-KIR3DLb*), AF266734 (*Pp-KIR3DLc*), AF266735 (*Pp-KIR3DSa*), and AF266736 (*Pp-KIR2DL4*).

DNA Analysis of Genes Related to KIRCI and KIR2DL5 in Chimpanzees. Using human *KIRCI*-specific primers (sense 5′-GCACTGTGGTGTCTGAAGGAC-3′, anti-sense 5′-GTAGCTCCCTCCGTGGGTCA-3′), fragments that cover exon-3 (D0 domain) through to exon-5 (D2 domain) were PCR amplified from pygmy chimpanzee Matata and common chimpanzee Alex. Primer sets designed to amplify the transmembrane region through the cytoplasmic tail of *KIRCI* failed in both pygmy and common chimpanzees. Using *KIR2DL5*-specific primers, based on human and common chimpanzee sequences, fragments covering exon-3 (D0 domain) through exon-4 (D2 domain; sense 5′-GGTGGTCAGGACAAGCCCTTG-3′, anti-sense 5′-GGTCTGACCACTCATAGGGT-3′), and exon-6 (transmembrane region) through exon-8 (cytoplasmic tail; sense 5′-TTTCTCCTTCATCGCTGC-3′, anti-sense 5′-ACCTCCTGAGGGTCTTGA-3′) were amplified from DNA of pygmy chimpanzee Matata who was typed positive by human *KIR2DL5*-specific primers. All PCR were carried out using the Expand Long Template PCR System (Boehringer) according to the manufacturer's instructions. The PCR conditions included 2 min initial denaturation at 94°C, 30 cycles of 20 s at 92°C, 30 s at 62°C, 8 min at 68°C, and final extension at 68°C for 10 min. PCR products were purified using the QIAEX II Gel extraction kit (QIAGEN) and the exon sequences were determined by direct sequencing.

Sequence Analysis. Sequence alignments and pairwise comparisons were performed using the AutoAssembler, v2.1 (Applied Biosystems) and the Wisconsin sequence analysis software, v10.1 (Genetics Computer Group). Phylogenetic trees were constructed with PAUP 4.0b2a software (Sinauer Associates; available at http://www.sinauer.com/) using the maximum parsimony analysis (39) and neighbor-joining method (40). The level of confidence in each node of the tree was assessed from 1,000 replications by the bootstrap method (41).

PCR Typing of KIR Variants. Pygmy chimpanzee genomic and/or cDNA were PCR typed for 14 human *KIRs* and 10 common chimpanzee *KIRs* using the typing systems we developed previously (17, 19, 32). To type for *KIRCI* (42), which was not included in the previous typing system, an additional set of primers was included. A typing system was also developed for the nine pygmy chimpanzee *KIR* sequences defined in this study. Typing for common chimpanzee *KIR* was refined to type for *Pt-KIRCI*, and to distinguish the variants of *Pt-KIR3DL1/2*. The oligonucleotide primers and the size of the products expected in the DNA typing are as follows: *Pt-KIR3DL1/2-v1*: sense 5′-GTGATCCCCTGGACATCA-3′, anti-sense 5′-TGCAGGACAAGGTCACGC-3′, 1,700 bp; *Pt-KIR3DL1/2-v2* and *v3*:

sense 5′-GTGATCCCCTGGACATCA-3′, anti-sense 5′-TGACCTTGCGCACTGCAC-3′, 1,800 bp; *Pp-KIR3DLa*: sense 5′-ACATGCAGGGAACTACAC-3′, anti-sense 5′-GCGCAAAGTGTCCTCAAC-3′, 1735 bp; *Pp-KIR3DLb*: sense 5′-ATCCTCTTCTTTCTCCTTCATCA-3′, anti-sense 5′-GCTGCTGGTGCATTGGAT-3′, 945 bp; *Pp-KIR3DLc*: sense 5′-AACCCCAGACACCTACAT-3′, anti-sense 5′-TTCCGTGTACACGCTGGTG-3′, 865 bp; *Pp-KIR3DSa*: sense 5′-GTCAGTGGTCAAAATCCCTTTCAC-3′, anti-sense 5′-TCATGGTGTGAGGAAGAGCA-3′, 715 bp; *Pp-KIR2DL4*: sense 5′-TACAGATGTCGAGGTTTTCACCCG-3′, anti-sense 5′-TGTGGGGCCCCGCCGGGCTGTGAGT-3′, 900 bp; *Pp-KIR3DL4*: sense 5′-TAACGACACTTTGCGCCA-3′, anti-sense 5′-GAGCCTACGTTCATGAGA-3′, 1,575 bp; *KIRCI*: sense 5′-GGACCTACAGATGTTGC-3′, anti-sense 5′-TAGTTGACCTGGGAACCCG-3′, 1,575 bp; *Pt-KIRCI* and *Pp-KIRCI*: sense 5′-GGAACCTACAGATGTTGC-3′, anti-sense 5′-TAGTTGACCTGGGAACCCG-3′, 1,575 bp. *Pp-KIR2DL5*- and *Pp-KIR3DL5*-specific typing were performed using the primer sets developed for typing their common chimpanzee othologs (32). The PCR reaction mixture and the temperature conditions were the same as used for human and common chimpanzee *KIR* typing, with minor modification in the annealing temperature (61°C) used for the second set of cycles for primer sets specific to *Pt-KIR3DL1/2* variants, *Pp-KIR3DLa*, *Lb*, and *Lc* (17, 32). The amplified products from all pygmy chimpanzees and some common chimpanzee typing reactions were directly sequenced, confirming the fidelity of the typing reactions.

Results

Genomic DNA from B cell lines derived from pygmy chimpanzees, common chimpanzees, and human controls were digested with HindIII and compared in Southern blotting using a common chimpanzee *KIR* cDNA probe (Fig. 1). All three species exhibit polymorphism in the *KIR* banding pattern, and in humans one such difference (the presence or absence of the ~24-kb band numbered 1 in Fig. 1) has been correlated with differences in the number and type of *KIR* genes (17, 19). However, the overall number of bands in the Southern blots of different humans and common chimpanzees is similar. Distinguishing the pygmy chimpanzee is the much larger extent of the differences between individuals in the Southern blot banding patterns. The number of bands varied from three (Bosondjo and Jill) through seven (Matata), with the latter pattern being of a complexity approaching that seen in humans and common chimpanzees.

Typing systems developed previously for the analysis of human and common chimpanzee *KIR* (17, 19, 32), and including an additional set of primers for human *KIRCI* (42), were used to type genomic DNA from 11 pygmy chimpanzee B cell lines, including the 6 analyzed by Southern blotting. The results are summarized in Fig. 2. 13 (8.4%) of the 154 typing reactions targeted at human *KIR* (Fig. 2 A), and 22 (20%) of the 110 typing reactions targeted at common chimpanzee *KIR* (Fig. 2 B), were positive. The relatively low frequency of positive reactions suggested that pygmy chimpanzee *KIRs* are considerably diverged from both human and common chimpanzee *KIRs*, although they are closer to the latter. All 11 pygmy chimpanzees typed with primers specific for *Pt-KIR2DL4* and human *KIRCI,* whereas *Pt-KIR3DL4* and *Pt-KIR3DL5* were scored by four and five individuals, respectively. For each individual, the number of positive typing reactions roughly correlated with the complexity of the banding pattern in Southern blot (Fig. 1). Thus, Bosondjo and Jill, who had the simplest banding pattern, typed only for *Pt-KIR2DL4* and *KIRCI,* whereas Matata, who had the most complicated banding pattern, typed for four different *Pt-KIRs* and two human *KIRs*: *KIR2DL5* and *KIRCI* (Fig. 2, A and B).

Definition of Six Transcribed Pygmy Chimpanzee KIRs. Our next goal was to characterize cDNA encoding pygmy chimpanzee KIRs. Because of the restricted quantities of pygmy chimpanzee blood available, cDNA was made from RNA isolated from the PBMCs of Lisala, a pygmy chimpanzee for which a B cell line had not been made and frozen PBMCs were available. Analysis of Lisala's cDNA with the typing reactions targeted towards common chimpanzee or human KIR gave results similar to those obtained for

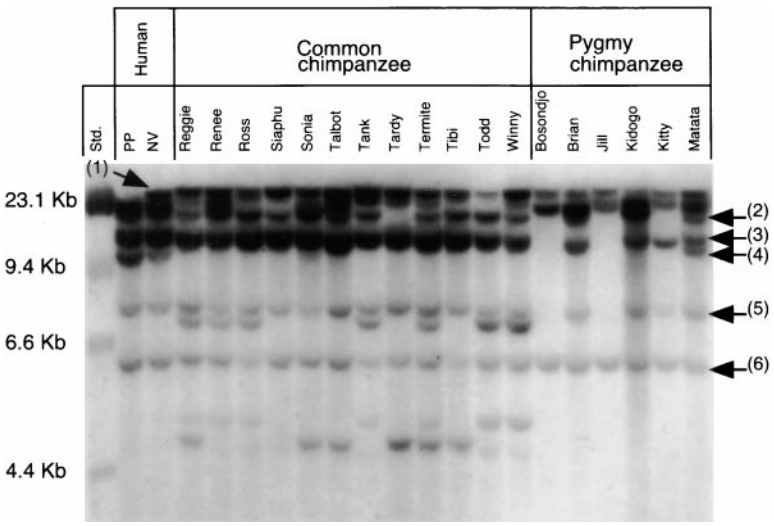


Figure 1. Comparison by Southern blotting of *KIR* gene families in pygmy chimpanzees, common chimpanzees, and humans. Genomic DNA was digested with HindIII and a full-length common chimpanzee *KIR* cDNA (*Pt-KIR3DL6*) was used as the probe. Bands referred to in the text are numbered in order of decreasing size. Similar results were obtained when human *KIR3DL1* cDNA was used as the probe (not shown). Std., standard fragments of HindIII-digested λ-DNA.

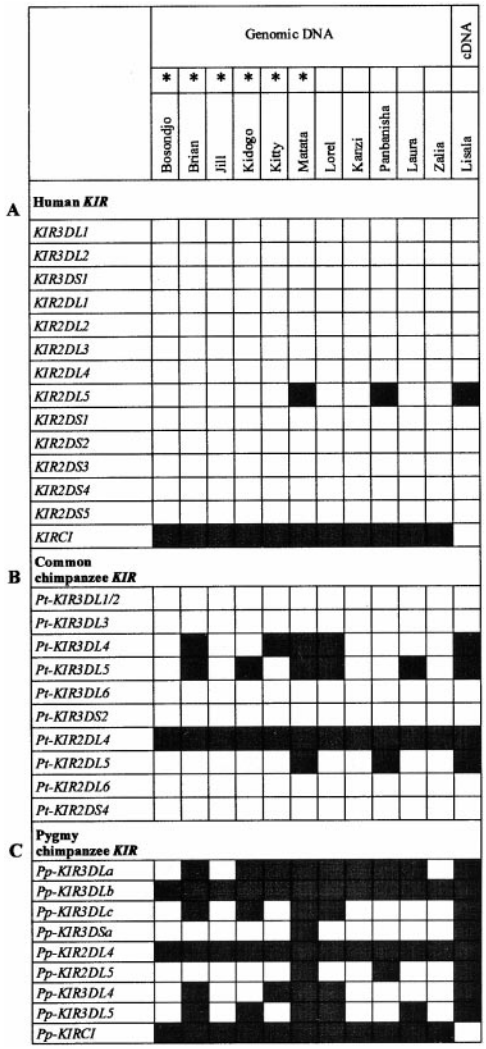


Figure 2. Pygmy chimpanzees have diverse *KIR* genotypes. Genomic DNA samples from 11 pygmy chimpanzees were typed using sequence-specific PCR. Three sets of oligonucleotide pairs were used for typing: the first based on the sequences of human *KIR* (A), the second on common chimpanzee *KIR* cDNA (B), and the third based on the sequences of pygmy chimpanzee *KIR* (C). Positive typing reactions are indicated by a filled box, and negative typing reactions by an open box. Individuals analyzed by Southern blotting in Fig. 1 are marked by an asterisk (*). For Lisala, typing was performed on cDNA isolated from PBMCs.

Matata's genomic DNA (Fig. 2, A and B). The one difference was the absence of *KIRCI* in Lisala's cDNA. A similar lack of *KIRCI* transcription was obtained in all humans (42) and most common chimpanzees tested (Rajalingam, R., unpublished observations). Complementary DNA clones encoding pygmy chimpanzee KIR were obtained after PCR using primers based upon the conserved sequences of human *KIR*, an approach successfully used to isolate cDNA clones encoding common chimpanzee *KIR* (32). Partial nucleotide sequences were determined for 186 individual cDNA clones permitting them to be sorted into six different groups.

The most abundant *KIR* cDNA (42 clones) corresponds to the pygmy chimpanzee ortholog of *KIR2DL4*, which has 96.9 and 98.7% sequence similarity with human *KIR2DL4* and common chimpanzee *Pt-KIR2DL4*, respectively. These relationships are apparent in a phylogenetic tree of chimpanzee and human *KIR* (Fig. 3). Consequently, this pygmy chimpanzee *KIR* has been named *Pp-KIR2DL4*, where Pp signifies *P. paniscus*. Pp-KIR2DL4 and Pt-KIR2DL4 have identical amino acid sequence in the extracellular domains and differ by just four amino acid substitutions elsewhere: two in the transmembrane region and two in the cytoplasmic tail.

The least abundant pygmy chimpanzee *KIR* cDNA was represented by a single clone from the initial screen. Three more clones corresponding to this *KIR* were obtained on screening a further 384 clones. This *KIR* is closely related to common chimpanzee *Pt-KIR3DL4*, with which it has 98% sequence homology, and is likely an ortholog (Fig. 3). The gene for this pygmy chimpanzee *KIR*, named *Pp-KIR3DL4*, was probably the target for the primers specific for *Pt-KIR3DL4* and which were positive with Lisala and four other pygmy chimpanzees (Fig. 2 B). Pp-KIR3DL4 differs from Pt-KIR3DL4 by 19 amino acid substitutions. Of these, only three are in the extracellular Ig-like domains; seven being present in the transmembrane region, five in the cytoplasmic domain, two in the stem, and two in the leader. In the common chimpanzee, Pt-KIR3DL4 is an inhibitory receptor for C2 type of MHC-C allotypes having the asparagine 77, lysine 80 motif, and in human KIR this C2 specificity is correlated with the presence of methionine at position 44 of the D1 domain (43, 44). Pp-KIR3DL4, like Pt-KIR3DL4, has methionine at this position, raising the possibility that it too is an inhibitory C2 receptor. Excepting Lisala, all the pygmy chimpanzees studied here have been characterized previously for alleles of *Papa-C*, the ortholog of the human *HLA-C* locus (36). All the *Papa-C* alleles in this cohort of animals encode heavy chains having the asparagine 77, lysine 80 motif, C2-type motif.

Most of the cDNA clones (143 clones) from the initial screen were shown to represent four *KIR3D,* which differ from one another by 2–6% of the nucleotide sequence. They belong to the lineage of 3Ig KIR that in humans and common chimpanzees embraces the inhibitory receptors for MHC-A (KIR3DL2, Pt-KIR3DL1/2) and MHC-B (KIR3DL1, Pt-KIR3DL1/2) allotypes (Figs. 3 and 4). In comparison with complete coding region sequences, the four pygmy chimpanzee *KIR3D* have 91–98% sequence similarity with *KIR3D* of the corresponding human and common chimpanzee lineage. However, none of the four pygmy chimpanzee *KIRs* appears orthologous to either a common chimpanzee or human *KIR* (Figs. 3 and 4), and for this reason we have provisionally designated them as *Pp-KIR3DLa*, *Pp-KIR3DLb*, *Pp-KIR3DLc*, and *Pp-KIR3DSa*.

To considerable extent the pygmy chimpanzee KIR3D consists of sequence elements present in human and common chimpanzee KIR, but in novel combination (Fig. 5 A). In the region encoding the extracellular part of the molecule (Ig domains and stem), the four *Pp-KIR3D* form a clade with the common chimpanzee *Pt-KIR3DL3* (Fig. 5

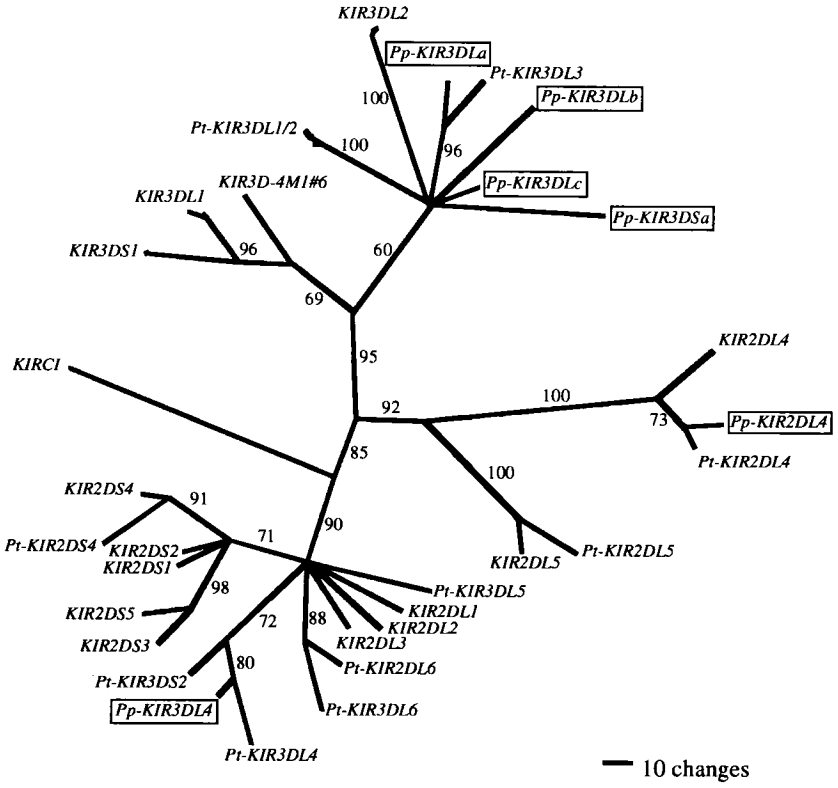


Figure 3. Four of six pygmy chimpanzee *KIR* cDNA are not orthologous to human or common chimpanzee *KIR*. The phylogenetic tree was constructed from full-length coding region sequences from cDNA with the exception of human *KIRCI* for which the coding region sequence was derived from the genomic sequences (reference 42). The tree is unrooted and was derived using the maximum parsimony method (reference 39). Bootstrap values determined by 1,000 replications are given for pairs of branch points (reference 41). Trees with similar topology were obtained by the neighbor-joining method (reference 40) and also when constructed from the amino acid sequences (not shown). The names of the pygmy chimpanzee *KIR* are boxed. Several variants of *Pt-KIR3DL1/2* (*Pt-KIR3DL1/2-v1*, *Pt-KIR3DL1/2v2*, and *Pt-KIR3DL1/2v3*), *KIR3DL1* (*Nkb1* and *Nkat3*), *KIR3DS1* (*3DS1v* and *Nkat10*), and *KIR3DL2* (*Nkat4*, *AMC5*, and *17.1c*) are included in the tree but because of the proximity of the branches they are not individually named (reference 15).

B). Comparison of the sequences encoding individual Ig-like domains revealed the D0 domains of Pp-KIR3DLa and Pp-KIR3DSa to be identical and to share sequence segments with both human KIR3DL1 and KIR3DL2. The D0 domains of Pp-KIR3DLb and Pp-KIR3DLc are also identical and have 97.2% sequence similarity with the corresponding domain of human KIR3DL2. In the D1 domain, Pp-KIR3DSa groups with Pp-KIR3DLb and Pp-KIR3DLc, and these closely related sequences have 96% sequence similarity with the D1 domain of KIR3DL2. In the D1 domain, Pp-KIR3DLa diverges from the other Pp-KIR3D but has 98% sequence similarity with the D1 domain of common chimpanzee Pt-KIR3DL3. In the D2 domain, the four Pp-KIR3D are very similar to each other and to Pt-KIR3DL3 (98.5% sequence similarity). Unique to the carboxyl terminal half of the D2 domain of these five KIRs is an insertion of two amino acids followed by a motif of five amino acid substitutions (Fig. 4).

In the transmembrane region and cytoplasmic tail, the relationships between the four Pp-KIR3D (Fig. 5, A and C) differ from those in the extracellular domains (Fig. 5, A and B). Here, Pp-KIR3DLa and Pp-KIR3DLb are like each other, and similar to the common chimpanzee Pt-KIR3DL1/2 and Pt-KIR3DL3 (and to a lesser extent with human KIR3DL2). In contrast, *Pp-KIR3DLc* is most closely related to KIR3DL-4M1#6, a divergent human KIR3DL sequence (sequence data are available from GenBank/EMBL/DDBJ under accession no. X97230), whereas *Pp-KIR3DSa* is closest to human KIR3DS1. The latter two short-tailed KIR3D share the KxPxT transmembrane motif (amino acids 330–334) which is characteristic of all human short tailed KIR. The lysine residue in this motif binds to cosignaling molecules such as DAP-12 (45). In the long-tailed KIR3D, the sequence and spacing of the immunoreceptor tyrosine-based inhibitory motifs are conserved in Pp-KIR3DLa, Pp-KIR3DLb, Pp-KIR3DLc, Pt-KIR3DL1/2, Pt-KIR3DL3, and human KIR3DL2 (Fig. 4)

To search for additional pygmy chimpanzee *KIR*, cDNA clones were derived from the PBMCs of Bosondjo and Matata. These two animals are those with the simplest (Bosondjo) and most complicated (Matata) *KIR* types as assessed by Southern blot (Fig. 1). No novel *KIR* was identified. From Bosondjo, only clones corresponding to *Pp-KIR2DL4* (47 clones) and *Pp-KIR3DLb* (13 clones) were obtained, consistent with the typing of this individual (Fig. 2). From Matata, clones corresponding to *Pp-KIR2DL4* (33 clones), *Pp-KIR3DLa* (7 clones), *Pp-KIR3DLb* (28 clones), *Pp-KIR3DLc* (1 clone), and *Pp-KIR3DSa* (6 clones) were obtained, again consistent with the typing.

Characterization of Pp-KIR2DL5, Pp-KIRCI, and Pp-KIR3DL5 Genes in Pygmy Chimpanzee. The positive typing reactions of pygmy chimpanzee genomic DNA for *Pt-KIR3DL4* and *Pt-KIR2DL4* (Fig. 2 B) were explained by the identification of pygmy chimpanzee KIR cDNA corresponding to these genes. In contrast, the genomic typing reactions with primers specific for *KIR2DL5*, *Pt-KIR2DL5*, *KIRCI*, and *Pt-KIR3DL5* could not be matched to any of the pygmy chimpanzee cDNA sequences.

We therefore performed further analysis of genomic DNA from Matata, who typed positively with primers for

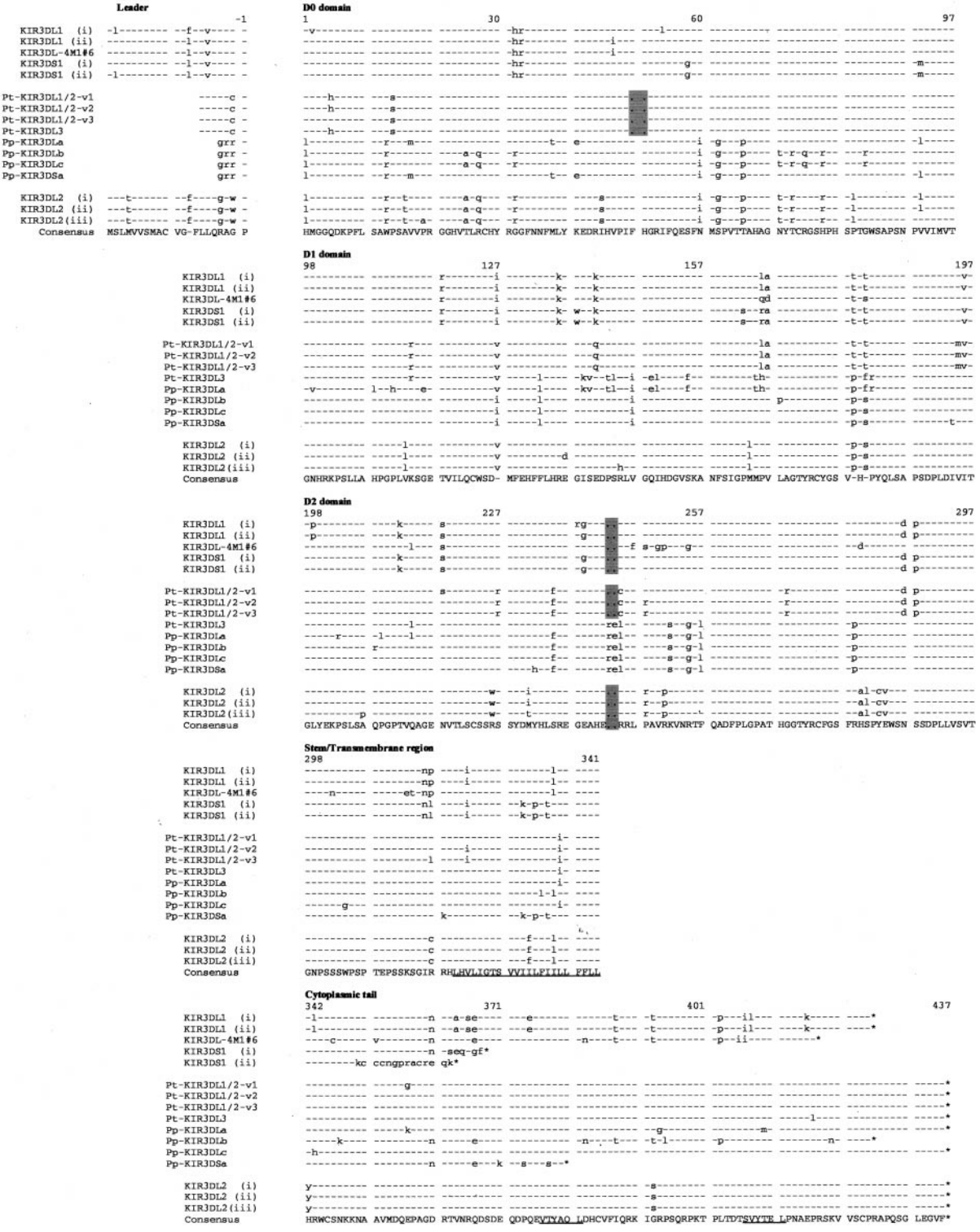


Figure 4. Predominant in pygmy chimpanzees are KIR3D of the lineage that in humans and common chimpanzees recognize MHC-A and -B. Amino acid sequences of these KIRs were aligned using the Wisconsin package version 10.1 (Genetics Computer Group). Pygmy chimpanzee KIRs are prefixed by Pp, common chimpanzee KIRs by Pt. Different allotypes of human KIR are indicated by i (KIR3DL1:Nkb1, KIR3DS1:Nkat10, KIR3DL2: Nkat4), ii (KIR3DL1:Nkat3, KIR3DS1:3DS1v, KIR3DL2:AMC5), or iii (KIR3DL2:17.1c) in parentheses after name (reference 15). Positions identical to the consensus are indicated by dashes (−). Numbering starts from the first residue of the mature protein. The immunoreceptor tyrosine-based inhibitory motif sequence consensus in the cytoplasmic tail and putative transmembrane segment are underlined. Gray boxes indicate deletions. The residues in the D2 domain that uniquely group Pp-KIR3D with Pt-KIR3DL3 are the insertion of "RE" at positions 243 and 244, and substitutions L245, S252, G255, L257, and P279.

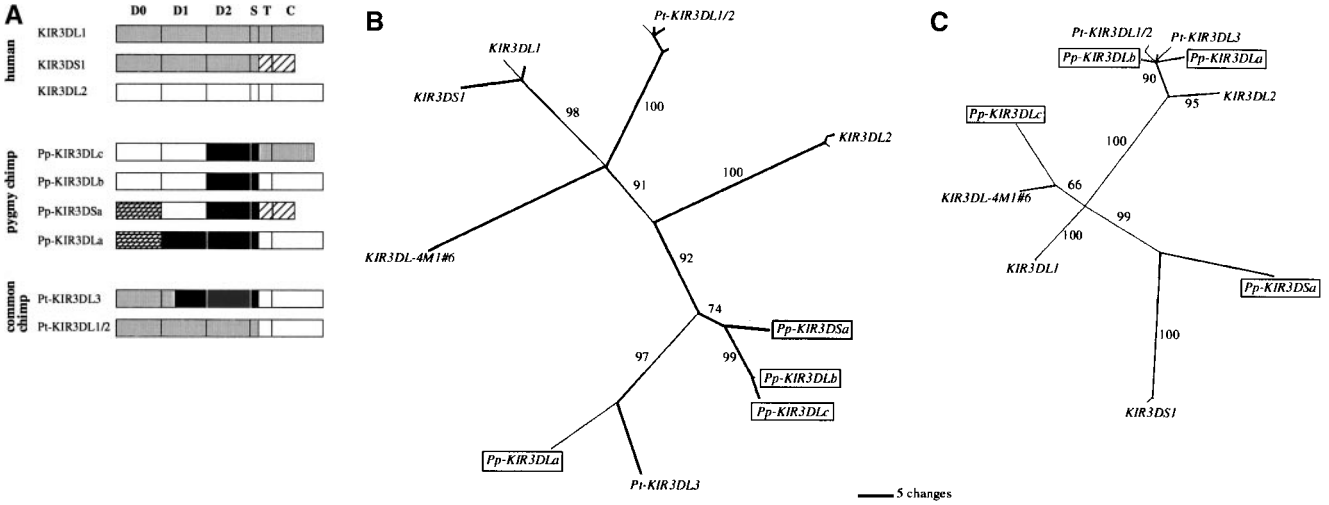


Figure 5. Pygmy chimpanzee KIR3D share sequence elements with common chimpanzee and human KIR3D. (A) The scheme in A depicts relationships between the primary structure of KIR3D in the three species. The individual Ig domains (D0, D1, and D2), stem region (S), transmembrane region (T), and cytoplasmic tail (C) are marked. Regions with >96% nucleotide sequence similarity are denoted by common patterns of shading. Relationships in the nucleotide sequences encoding the extracellular domains (panel B) and the transmembrane region plus cytoplasmic domain (panel C) are compared in unrooted phylogenetic trees obtained by maximum parsimony analysis. The names of the pygmy chimpanzee *KIRs* are boxed. Bootstrap values determined by 1,000 replications are given for pairs of branch points. Trees with similar topology were obtained by the neighbor-joining method and also when constructed from the amino acid sequences (not shown).

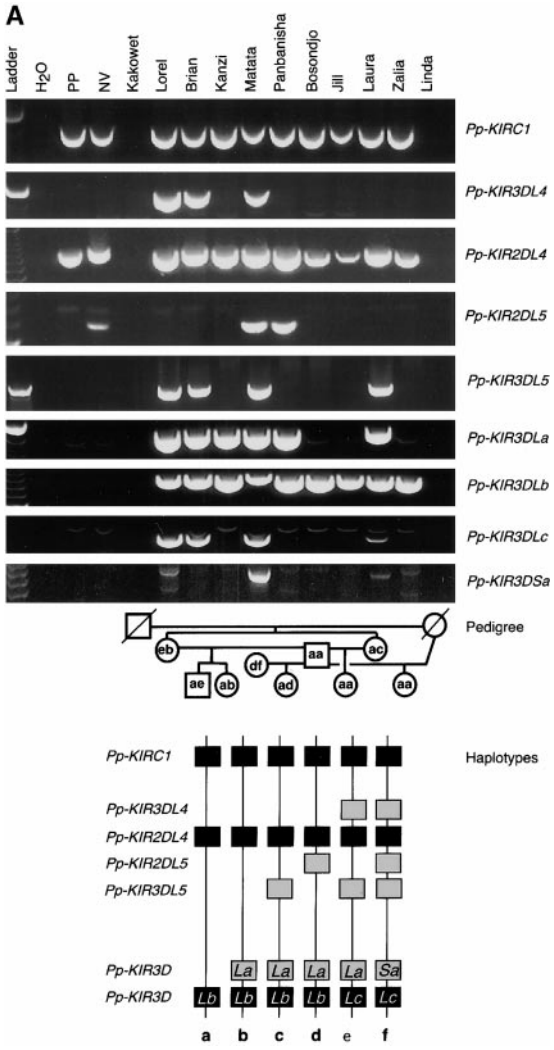
KIR2DL5 plus *Pt-KIR2DL5*, *KIRCI*, and *Pt-KIR3DL5*, to identify these genes. Contiguous sequences for the coding regions of pygmy chimpanzee orthologs of the *KIR2DL5* and *KIRCI* genes were obtained from PCR-generated fragments. *Pp-KIR2DL5* exhibits 97.0 and 99.2% sequence similarity with human and common chimpanzee *KIR2DL5*, respectively. *Pp-KIRCI* shows comparable levels of sequence similarity with human (97.6%) and common chimpanzee (98.5%) *KIRCI* in the sequence encoding the extracellular region. Attempts to amplify sequences corresponding to the transmembrane and cytoplasmic regions of *Pp-KIRCI* with primers based on either the human or common chimpanzee *KIRCI* sequences were unsuccessful. In this 3′ part of the gene, *Pp-KIRCI* may be more divergent from the human and common chimpanzee genes than the 5′ part encoding the extracellular domains.

Although various sets of primers were designed to amplify pygmy chimpanzee sequences related to *Pt-KIR3DL5*, only those used in the initial typing analysis (Fig. 2 B) gave a pygmy chimpanzee product. This gene fragment gave 423 nucleotides of sequence encoding the D1 and D2 domains. In this sequence, *Pp-KIR3DL5* differs by three nucleotide substitutions from the corresponding *Pt-KIR3DL5*. The 99.3% sequence similarity of these sequences is consistent with them being derived from orthologous genes.

A Pygmy Chimpanzee KIR Haplotype with Few Genes. From the pygmy chimpanzee *KIR* sequences, a PCR typing system was developed and the genomic DNA of the 11 pygmy chimpanzees were analyzed (Fig. 2 C). They all typed positively for three genes, *Pp-KIRCI*, *Pp-KIR2DL4*, and *Pp-KIR3DLb*. In the Southern blot, the only HindIII band present in all individuals of the three species is the one numbered 6 in Fig. 1. Thus, this ~6-kb band is a candidate for containing the *KIR2DL4* and/or the *KIRCI* gene. Typing for *Pp-KIR3DL4* revealed that only 5 of 12 pygmy chimpanzees have this *KIR* (Fig. 2 C). Bosondjo and Jill, who both have the simplest Southern blot pattern, were among those lacking *Pp-KIR3DL4* (Figs. 1 and 2 C). Typing for the presence of the four *Pp-KIR3D* related to *KIR3DL2* revealed three individuals having one *Pp-KIR3D*, four having two, three having three, and two having all four of them (Fig. 2 C).

Bosondjo, Jill, and Zalia have only *Pp-KIRCI*, *Pp-KIR2DL4*, and *Pp-KIR3DLb*, consistent with Bosondjo and Jill having the simplest banding pattern in Southern blot (Fig. 1; Zalia was not analyzed by blot). Kitty has *Pp-KIR3DLa* and *Pp-KIR3DL4* in addition to *Pp-KIRCI*, *Pp-KIR2DL4*, and *Pp-KIR3DLb*, correlating with an additional ~15-kb band in the blot (band 3 in Fig. 1). Brian has *Pp-KIR3DLc* and *Pp-KIR3DL5*, in addition to *Pp-KIR3DLa*, *Pp-KIR3DL4*, *Pp-KIRCI*, *Pp-KIR2DL4*, and *Pp-KIR3DLb*, correlating with additional bands of ~20 and ~7 kb in the blot (bands 2 and 5 in Fig. 1, respectively). Finally, the additional presence of *Pp-KIR3DSa* and *Pp-KIR2DL5* in Matata is associated with an additional band of ~11 kb in the blot (band 4 in Fig. 1).

Nine of the pygmy chimpanzees are members of a family within which we could trace the segregation of KIR and infer possible haplotypic associations (Fig. 6 A). A minimum of six different haplotypes is required to explain the observed genotypes. The most common haplotype in the family (labeled "a" in Fig. 6 A) appears to contain just three genes: *Pp-KIRCI*, *Pp-KIR2DL4*, and *Pp-KIR3DLb* with Bosondjo, Jill, and Zalia being homozygous for this haplotype (Fig. 6 A). Less frequent are five haplotypes containing two *Pp-KIR3D*: *Lb* plus *La*, *Lc* plus *La*, and *Lc* plus *Sa*; data



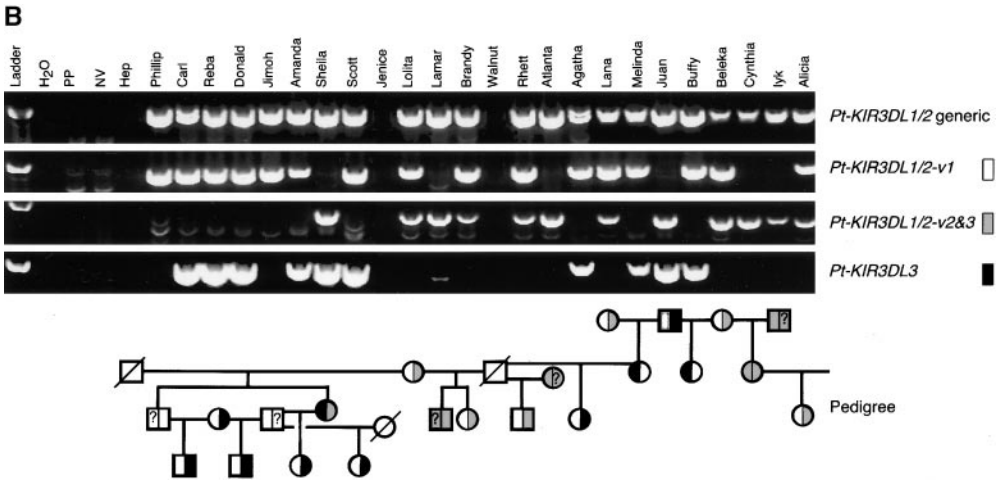


Figure 6. (A) Segregation of short *KIR* haplotypes in a pygmy chimpanzee family. The top shows agarose gels of the PCR products obtained from typing genomic DNA from a family of nine pygmy chimpanzees for nine *Pp-KIRs*. The middle shows the pedigree for this family and two additional members for whom samples were not available. The segregation of six *KIR* haplotypes (a–f) within the pedigree is shown. The genes present on each haplotype are shown in the bottom of A. The order of the genes is arbitrary. Three genes shown in dark boxes are the common features of *KIR* haplotypes that are conserved in all humans and both species of chimpanzees (references 8 and 32). (B) Analysis of the segregation of alleles of the *KIR3D* gene encoding MHC-A and -B receptors in an extended pedigree of common chimpanzees. Different shadings in the pedigree symbols give the assigned *KIR3D* variants. Question marks indicate apparent homozygotes where the inheritance of an allele has not been independently confirmed by typing of both parents. Distilled water and DNA from two human donors were used as negative controls in the typing reactions (lanes 2–4 in both A and B). For individuals marked by symbols ⍁ or ∅ in the pedigree and blank lanes in the typing gels, DNA were not available for analysis. For both pedigrees, the *KIR* data are consistent with the family relationship determined by the analysis of *MHC class I* and breeding records (references 31 and 36).

raising the possibility that *Lb* and *Lc* are alleles of one locus, with *La* and *Sa* being alleles of a second locus. The results demonstrate the existence of two genes encoding Pp-KIR3D with some haplotypes having both genes and others only one. This is analogous to the human situation, where some haplotypes have *KIR3DL1* and *KIR3DL2* and others have only *KIR3DL2* (8).

In the common chimpanzee, four *Pt-KIR3D* have also been found to be part of the *KIR3D* lineage that includes all genes encoding KIR specific for MHC-A and -B (32). Three of these (Pt-KIR3DL1/2v1, Pt-KIR3DL1/2v2, and Pt-KIR3DL1/2v3) are closely related variants having extracellular regions similar to human KIR3DL1 and transmembrane and cytoplasmic regions similar to human KIR3DL2 (Fig. 4). The fourth common chimpanzee KIR3D (Pt-KIR3DL3) diverges from Pt-KIR3DL1/2 in the D1 and D2 domains, where we now see that it has sequence similarity with the pygmy chimpanzee KIR3DL (Figs. 3–5). DNA typing of a panel of 48 unrelated common chimpanzees shows that the three variants of *Pt-KIR3DL1/2* and *Pt-KIR3DL3* segregate as alleles of one locus (Table I, and Fig. 6 B). Thus, no chimpanzee with *Pt-KIR3DL3* carries two variants of *Pt-KIR3DL1/2*, whereas many individuals who lack *Pt-KIR3DL3* are heterozygous for *Pt-KIR3DL1/2* variants. Thus, in common chimpanzee there is a single gene encoding this lineage of KIR3DL, a situation contrasting with that in the pygmy chimpanzees and humans, where *KIR* haplotypes having two genes in this lineage are both present and frequent.

Discussion

This study has examined the *KIR* gene family of the Pygmy chimpanzee or Bonobo (*P. paniscus*) and compared it to that of its closest relative, the common chimpanzee (*P. troglodytes*), and its second most close relative, the human species (*Homo sapiens*). We characterized sequences for nine pygmy chimpanzee *KIRs* (*Pp-KIR*), representing at least seven different genes. Each *Pp-KIR* belongs to one of the three lineages of *KIR* defined from study of human and common chimpanzee *KIR* and all three lineages are represented in the pygmy chimpanzee *KIR* (15, 32, 42). All three species have orthologs for *KIRCI*, *KIR2DL4*, and *KIR2DL5*, and their pairwise comparison indicates that pygmy chimpanzee *KIR* are more similar to common chimpanzee *KIR* than human *KIR*, consistent with the estimated separations from these species of ~2.3 and ~5 million years, respectively (35). Also supporting this hierarchy is the presence of pygmy chimpanzee orthologs (*Pp-KIR3DL4* and *Pp-KIR3DL5*) for two common chimpanzee *KIRs* (*Pt-KIR3DL4* and *Pt-KIR3DL5*), for which there are no human orthologs.

Table I. *Frequency of Pt-KIR3DL1/2 Variants and Pt/KIR3DL3 in a Panel of 48 Common Chimpanzees*

Pt-KIR variants	No. positives	Frequency
		%
Pt-KIR3DL1/2 generic	48	100
Pt-KIR3DL1/2-v1	38	79.2
Pt-KIR3DL1/2-v2 and *-v3*	23	47.9
Pt-KIR3DL3	8	16.7
Pt-KIR3DL1/2-v1 homozygous	22	45.8
Pt-KIR3DL1/2-v2 and *-v3* homozygous	5	10.4
Pt-KIR3DL1/2-v1 and *-v2* and *-v3* heterozygous	13	27.1
Pt-KIR3DL1/2-v1 and *Pt-KIR3DL3* heterozygous	4	8.3
Pt-KIR3DL1/2-v2 and *-v3* and *Pt-KIR3DL3* heterozygous	4	8.3

The two pygmy chimpanzee *KIR* genes without obvious orthologs in either common chimpanzees or humans are those of the *KIR3D* lineage that in common chimpanzees and humans is characterized by genes encoding receptors specific for MHC-A and MHC-B (22–25, 32). In common chimpanzee, this lineage is represented by a single gene (*Pt-KIR3DL1/2*) which encodes a receptor that binds both MHC-A and -B allotypes, whereas in humans there are two genes, *KIR3DL1* that encodes an MHC-B receptor and *KIR3DL2* that encodes an MHC-A receptor. All the evidence points to this lineage of *KIR* genes having undergone much recombination, both to change the number of genes as well as to produce allelic variation. Thus, it is difficult without direct comparison of haplotype sequences to discern whether particular pygmy chimpanzee, common chimpanzee, and human genes in this KIR3D lineage have orthologous or paralogous relationships. Within this lineage, the pattern of polymorphism within a species and of species-specific divergence is consistent with coevolution of these KIRs with MHC class I polymorphism.

The striking feature that distinguishes the pygmy chimpanzee *KIR* system from its common chimpanzee and human counterparts is the presence of small *KIR* haplotypes and their preponderance in the cohort of animals we studied. These small *Pt-KIR* haplotypes give relatively simple patterns on Southern blotting, three bands, and appear to contain just three *KIR* genes: *Pp-KIRCI*, *Pp-KIR2DL4*, and *Pp-KIR3DLb*. Of importance is that each of these genes is either orthologous or paralogous to one of the three genes that is a conserved component of otherwise divergent human KIR haplotypes, and which Wilson et al. have called "framework genes" (8). Thus, *Pp-KIR2DL4* and *Pp-KIRCI* are orthologous to human *KIR2DL4* and *KIRCI* (also designated *KIR3DL3*) and *Pp-KIR3DLb* is of the same lineage and most closely related to *KIR3DL2*. In the ~100-kb human *KIR* gene family, the *KIRCI* and *KIR3DL2* genes define the two ends, and the *KIR2DL4* gene is placed in the middle. In each of the two intervals defined by this framework the two human haplotypes sequenced by Wilson et al. (8) differ in having between one and five genes.

3 of the 12 pygmy chimpanzees studied (Bosondjo, Jill, and Zalia) are homozygotes for short *Pp-KIR* haplotypes. Bosondjo is the father of five of the other animals and by the criteria of fecundity in captivity, homozygosity for short *KIR* haplotypes does not seem an impairment. Similarly, in terms of health and longevity the three animals homozygous for the short haplotype do not appear compromised in comparison to the others. In conclusion, the short *KIR* haplotypes appear to provide the minimal essential functions of the KIR system that are needed to generate functional NK cells, live, survive, and reproduce. Whether the preponderance of the short *Pp-KIR* haplotypes seen in the animals studied here is representative of the natural situation is uncertain and difficult to assess because of the very small numbers of animals in captivity and the endangered status of the species.

Comparison of the *KIR* gene families in the two chimpanzee species and humans indicates that *KIRCI*, *KIR2DL4*, and a *KIR3D* gene have been conserved as framework genes of *KIR* haplotypes since divergence of the human and chimpanzee lines ~5 million years ago (35). The three genes of the short haplotypes represent all three KIR lineages, and include ones encoding receptors for nonclassical and classical MHC class I (KIR2DL4 is a receptor for MHC-G (26), and KIR3D includes receptors for MHC-A and -B [23–25]). For KIRCI, neither its function nor its pattern of expression have been defined (42). KIRCI is in the lineage of KIR that includes those human and common chimpanzee KIRs that have specificity for MHC-C determinants (32). This lineage of KIR appears to have been the most rapidly evolving during the last 5 million years, a possibility being that *KIRCI* was the first gene of this type and that other members of the lineage are derivatives of it.

We have been unable to assess functionally the MHC class I specificity of pygmy chimpanzee KIR because of the small quantities of pygmy chimpanzee blood available. However, some inferences as to the possible receptor specificities can be made from structural comparison with human and common chimpanzee KIR. Based on their phylogenetic conservation, Pp-KIR2DL4 is a candidate MHC-G receptor and Pp-KIR3DL4 a candidate receptor for the C2 MHC-C specificity. By analogy with their paralogs in the other species, Pp-KIR3DLa, Pp-KIR3DLb, Pp-KIR3DLc, and Pp-KIR3DSa are candidates for MHC-A and -B receptors. In the D1 domain, Pp-KIR3DLa is distinguished from the other Pp-KIR3DL by several residues (E21, D48, T49, E54, and H55) which it shares with human KIR2DL2. In the crystallographic structure of the complex of KIR2DL2 with HLA-Cw3, these residues contribute to the interaction surface (46), raising the possibility that Pp-KIR3DLa may have affinity for MHC-C allotypes with the C1 motif. No Papa-C alleles encoding the C1 motif have been found in the pygmy chimpanzees studied here (36), but the small number of animals does not mean that such allotypes are not present in the population at large.

The similarities in the KIR in the two chimpanzee species serve to emphasize how different they both are from human KIR. First, a major component of the human KIR family is a set of KIR2D with D1 plus D2 configuration, which are related to chimpanzee KIR3D of a lineage different from that containing MHC-A– and -B–specific KIRs, and which have exons 3 that are not used (8, 12, 47, 48). The number of these KIR2Ds is much reduced in common chimpanzee and we have no evidence for such KIRs in pygmy chimpanzee, although they were deliberately sought (32). Because of the number of pygmy chimpanzees studied (12 individuals), we cannot rule out that such genes do not exist in this species, but genes encoding KIR2D of D1 plus D2 configuration appear to be represented at low frequency in common chimpanzee and none of them are invariant components of human *KIR* haplotypes, although all haplotypes have at least one of them (8, 17, 18, 32). Second, the numbers of activating KIRs, as assessed from the size and sequence of the cytoplasmic tail, is considerably greater in humans compared with either chimpanzee species (15, 32). For pygmy chimpanzee only one activating receptor has been defined, Pp-KIR3Sa.

The presence of *KIR* genes in primates and their absence in rodents led to the hypothesis that the *KIR* gene family is of recent origin and perhaps specific to the primates (7, 49). This view is supported by the presence within the *KIR* region of Alu sequences that are mostly of a type that originated only 31–55 million years ago (8). As for other multigene families (50, 51), the modern *KIR* gene family is envisioned to have originated with duplication of a single gene followed by successive expansions in gene number. Thus, in these formative times there was a trend in which the size of the *KIR* family increased. Accordingly, it is possible that all the haplotypes now present in chimpanzees and humans are derived from an older form of haplotype containing just the three framework genes and that the short haplotypes present in the pygmy chimpanzee retain this ancestral configuration. The genomic structure of the modern *KIR* gene family is unusual in that the genes are closely juxtaposed and separated by short homologous sequences (8). As well as reciprocal recombination, this arrangement is particularly favorable for unequal crossing over, a process that can delete, expand, and hybridize members of the gene family. The evidence that such mechanisms are active is the extent and type of diversity seen in both human and chimpanzee *KIR* haplotypes (17, 18, 32, 52, 53). Thus, it is alternatively possible that the short haplotypes present in the pygmy chimpanzee are derived from more complicated haplotypes in which inessential genes were deleted by unequal recombination.

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