The D0 Domain of KIR3D Acts as a Major Histocompatibility Complex Class I Binding Enhancer

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Abstract

In contrast to the KIR2D:HLA-C interaction, little is known of KIR3DL1's interaction with HLA-B or the role of D0, the domain not present in KIR2D. Differences in the strength and specificity for major histocompatibility complex class I of KIR3DL1 and its common chimpanzee homologue Pt-KIR3DL1/2 were exploited to address these questions. Domain-swap, deletion, and site-directed mutants of KIR3DL1 were analyzed for HLA-B binding using a novel, positively signaling cell–cell binding assay. Natural 'deletion' of residues 50 and 51 from its D0 domain causes Pt-KIR3DL1/2 to bind $Bw4^+$ HLA-B allotypes more avidly than does KIR3DL1. Deletion of these residues from KIR3DL1, or their substitution for alanine, enhanced binding of $Bw4^+$ HLA-B. None of 15 different point mutations in D0 abrogated KIR3DL1 binding to $Bw4^+$ HLA-B. In contrast point mutations in the D1 and D2 domains of KIR3DL1, made from knowledge of KIR2D:HLA-C interactions, disrupted binding to $Bw4^+$ HLA-B. The results are consistent with a model in which D1 and D2 make the principal contacts between KIR3DL1 and HLA-B while D0 acts through a different mechanism to enhance the interaction. This modulatory role for D0 is compatible with natural loss of expression of the D0 domain, a repeated event in the evolution of functional *KIR* genes.

Key words: natural killer cells • killer cell immunoglobulin-like receptors • MHC class I • human • chimpanzee

Introduction

NK cells are key players in the innate immune response to pathogens (1). Their functions are controlled by multiple inhibitory and activating cell surface receptors (2, 3). A particular diverse family of NK cell receptors is the killer cell Ig-like receptors (KIR).* KIR have either two (KIR2D) or three (KIR3D) extracellular immunoglobulin domains and either short (S) or long (L) intracytoplasmic tails which transduce positive and negative signals, respectively (4–7). Within an individual, NK cells are distinguished by their stable expression of different combinations of KIR, which creates a diverse NK cell repertoire (8). The known ligands for KIR are all MHC class I molecules: HLA-C is recognized by KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1, and KIR2DS2 (9–11); HLA-B is recognized by KIR3DL1 (12, 13), and HLA-A is recognized by KIR3DL2 (14, 15), and HLA-G is recognized by KIR2DL4 (16). As both *KIR* and *MHC class I* are diverse and evolve rapidly, their functional binding relationships must be constantly challenged, through independent segregation of the two gene families in populations and by the production of new variants through recombination and mutation.

X-ray crystallographic analysis of complexes has given high-resolution images of KIR2DL2 bound to HLA-Cw3 and of KIR2DL1 bound to HLA-Cw4 (17, 18). In both complexes loops from the D1 and D2 domains of KIR2D bind with approximately orthogonal orientation across the COOH-terminal part of the α_1 helix and the NH_2-terminal part of the α_2 helix. The ligand–receptor interaction is dominated by charge complementarity with HLA-C speci-

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**Abbreviations used in this paper:* AP, alkaline phosphatase; KIR, killer cell immunoglobulin-like receptor; KIR2D, killer cell immunoglobulin-like receptor with two Ig-like domains; KIR2DS, killer cell immunoglobulin-like receptor with two Ig-like domains and short cytoplasmic tail; KIR2DL, killer cell immunoglobulin-like receptor with two Ig-like domains and long cytoplasmic tail; KIR3D, killer cell immunoglobulin-like receptor with three Ig-like domains; KIR3DL, killer cell immunoglobulin-like receptor with three Ig-like domains and long cytoplasmic tail; SEAP, secretion of alkaline phosphatase.

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ficity being determined by the residue at position 44, as was first shown in binding experiments (19).

In comparison to the interactions of KIR2D with HLA-C, little is known of the interaction between KIR3D and either HLA-B or HLA-A. On the basis of sequence comparison and modeling it was proposed that the D1 and D2 domains of KIR3D interact with MHC class I in a homologous manner to the KIR2D:HLA-C interaction (20). However this model neither explains the presence of the D0 domain nor does it account for the results of Rojo et al. demonstrating that all three of the Ig domains of KIR3DL1 are required for binding to HLA-B (21).

The *KIR2D* genes encoding HLA-C receptors form part of a larger group of *KIR* called lineage III *KIR* (22). Genomic analysis revealed that all *KIR2D* genes of lineage III contain a pseudoexon encoding a D0 domain that is not incorporated into mature RNA (23, 24). Thus, all the genes encoding these KIR2D have evolved from genes encoding KIR3D. Inactivation of the D0 domain appears to have happened on several occasions as the inactivating mechanism differs among *KIR2D* genes. The extent to which the D0 domains of lineage III KIR are inactivated varies between species. For example, in common chimpanzees it is uncommon, in comparison to humans, and in that species one MHC-C receptor is a KIR3D and the other a KIR2D (22). Thus, during the evolution of lineage III KIR there seem to have been circumstances when having a D0 domain was of benefit and others when it was better got rid of.

Human KIR specific for HLA-A and B form part of another KIR lineage, lineage II, which is comprised solely of KIR3D. Whereas in humans this lineage is represented by two genes, *KIR3DL1* and *KIR3DL2*, in the common chimpanzee there is a single gene, for which the common allele, *Pt-KIR3DL1/2*, combines structural elements and functional properties of both human genes with some unique features. Thus, Pt-KIR3DL1/2 combines an MHC-B specificity, which is overlapping but distinct from KIR3DL1, with a weak specificity for some MHC-A allotypes akin to that of KIR3DL2. In cytotoxicity assays Pt-KIR3DL1/2 appeared a more avid MHC class I receptor than either KIR3DL1 or KIR3DL2 (22). Because of these differences further comparison of HLA-B recognition by KIR3DL1 and Pt-KIR3DL1/2, provided a system that facilitated the investigation described here of the D0 domain's function. We find that D0 enhances functional interaction with $Bw4^+$ HLA-B ligand, with the chimpanzee D0 domain being a stronger enhancer than its human counterpart.

Materials and Methods

Recombination PCR for KIR-CD3ζ Chimeric Molecules. KIR-CD3ζ chimeric molecules were generated by recombination PCR (25). In these molecules the extracellular and transmembrane domains of the KIR are attached to the intracellular domain of CD3ζ. *Pt-KIR3DL1/2* was amplified from an error-free clone (M1.1–3-10) using sense primer 5′-^{1}ATG-TTGCTCATGGTCGTCAGCATGGCGTGTGTTGGGTTC-TTCTTGCTGCA-3′ and antisense primer 5′-TGCGCTC-CTGCTGAA^{1126}TTTGTTGGAGCACCAGCGATGAAG-3′. As the clone from which the *Pt-KIR3DL1/2* gene was amplified did not contain the full leader sequence, the leader sequence of KIR3DL1*002 (NKB1 [4]) was included in the sense primer (underlined) to ensure cell surface expression of the mature protein. The antisense primer contained 15 bp of *CD3ζ* at the 5′ end (underlined) to allow annealing to the *CD3ζ* sequence during the subsequent recombination PCR step. PCR conditions were: denaturation at 95°C for 2 min, followed by 15 cycles of 45 s at 60°C, 60 s at 72°C, 30 s at 95°C, then 7 min at 72°C. *CD3ζ* was amplified from a *CD3ζ*-containing plasmid (25) using the sense primer 5′-TGGTGCTCCAACAAA^{237}TTCAGCAGGAGCG-CAGAGCC-3′ which contains 15 bp of Pt-KIR3DL1/2 at the 5′ end (underlined) and antisense primer 5′-^{822}CGAGGA-ACCGCCAGGAGACA-3′ under the same PCR conditions. The *KIR* and *CD3ζ* were recombined in a PCR reaction using the *Pt-KIR3DL1/2* and *CD3ζ* amplification products as templates and the following primers; sense 5′-GGGCGCGGC-CGC^{-6}AGCAGTATGTTGCTCATGGTCGTCAGC-3′ (N1-fnot) antisense 5′-GGGCTCTAGA^{599}TGGCCTTTGAGTGG-TGAAATCC-3′, (zeta3xba) which incorporate the restriction sites NotI and Xba1 (underlined), respectively. PCR conditions were: denaturation at 95°C for 2 min, followed by 15 cycles of 45 s at 60°C, 90 s at 72°C, 30 s at 95°C, then 7 min at 72°C. The amplification product was cloned into *pcDEF* and sequenced to ensure fidelity.

An error-free clone was transfected into the Jurkat cell line by electroporation using a BTX electroporator with two pulses of 240 V at 100 μF and resistance 360 ohms. Transfectants were selected with G418 (Sigma-Aldrich) at a concentration of 2 mg/ml. After selection, cells expressing Pt-KIR3DL1/2-CD3ζ chimeric molecules were stained with the DX9 antibody, sorted, and cultured.

The *KIR3DL1-CD3ζ* construct was made in the same way as *Pt-KIR3DL1/2-CD3ζ* using the *KIR3DL1*002*-containing plasmid *LL349* (4) as template, except that the primers for the first amplification were 5′-$^{-16}$CGGCACCGGCAGCACCATGT-3′ (which sits in the 5′ untranslated region of *KIR3DL1*002*), and 5′-TGCGCTCCTGCTGAA^{1126}TTTGTTGGAGCACCAGAG-ATGAAG-3′, which includes 15 bp of the *CD3ζ* gene (underlined) at the 5′ end and amplifies the same region of the transmembrane and intracytoplasmic region as the corresponding primer for *Pt-KIR3DL1/2*. PCR conditions were the same as for *Pt-KIR3DL1/2*. The resulting stable transfectants were sorted by flow cytometry for high KIR expression using either the DX9 (a gift from Dr. L. Lanier, San Francisco, CA) or Z27 (Beckman Coulter) anti-KIR3DL1 monoclonal antibodies. Cultured transfectants were periodically checked for both KIR and CD3 expression.

Assay of Secreted Alkaline Phosphatase. 1×10^7 Jurkat-KIR transfectants were transfected with the alkaline phosphatase (AP) reporter construct *NFAT-SX* (10 μg) and an *SV40 TAg* (0.5 μg) expression construct driven by an *RSV* promoter (26). *NFAT-SX* contains sequences encoding an NFAT binding site and a minimal *IL-2* promoter cloned upstream of a cDNA encoding secreted AP (27). The *SV40 TAg* construct was used to increase the copy number of the reporter construct.

24 h after transfection of the reporter construct, cells were plated out at 10^6 cells per ml at a 2.5:1 ratio with stimulator cells, in a final volume of 200 μl. Incubation was for 18 h. Endogenous AP was heat inactivated at 67°C for 2 h and then 100 μl aliquots

of the cell supernatants harvested. 100 μl of 2 M diethanolamine containing 1 M methylumbelliferyl phosphate and 10 mM L-homoarginine were added to the supernatants and the mixture incubated overnight at 37°C. Fluorescence was measured in a Titertek Fluorescan II (Labsystems) at 355 nm excitation and 460 emission. AP activity was expressed as a percentage of stimulation by plate-bound anti-CD3 alone; with the baseline reading to calibrate the fluorometer at zero being from wells with "medium alone." All experiments were done in duplicate and performed at least three times. Anti-CD3 stimulation was usually between 600 and 1,200 fluorescence units. Results are expressed as the mean plus one SEM from all experiments performed with each transfectant. In blocking experiments the anti–class I antibody W6/32, or an isotype-matched control antibody, were added to give a final concentration of 10 μg/ml.

Domain Swap KIR3DLζ Constructs. The domain exchange constructs were made using the *pcDEF/KIR3DL1ζ* or *pcDEF/Pt-KIR3DL1/2ζ* constructs as PCR templates to amplify sequences encoding individual domains, which were then recombined by recombination PCR. Sequences encoding D0 domains were amplified using the primers sense N1fnot and antisense 5′-^{349}TGATCACCACGGGGTTGCTGG-3′. Sequences encoding D1D2ζ were amplified using sense primer 5′-^{215}CGGGGTTCACACCCACACTCC-3′ and antisense primer zeta3xba. The sequence encoding the D0 domain of KIR3DL1*002 was then recombined with that encoding the D1D2ζ domains of Pt-KIR3DL1/2 by PCR using primers N1fnot and zeta3xba to generate a chimeric gene, *HCCζ*. The resulting product was then cloned into *pcDEF* using NotI and XbaI. The *CHHζ* chimera was generated in the complementary manner.

To generate chimeric molecules in which the D2 domain was substituted, sequences encoding the D2 domain from *KIR3DL1ζ* and *Pt-KIR3DL1/2ζ* were amplified with the *CD3ζ* gene using sense primer 5′-^{505}CCTACAGATGCTACGGTTCTG-3′ and antisense primer zeta3xba. Sequences encoding the corresponding D0D1 domains were amplified using the sense primer N1fnot and the antisense primer 5′-^{565}GGGATCACTGGGAGCTGACAA-3′. The appropriate amplification products were then recombined by PCR and cloned into *pcDEF* to generate the *HHCζ* and *CCHζ* chimeras.

To generate chimeras in which the D1 domain was exchanged, *HHCζ* and *CCHζ* templates and primers 5′-^{505}CCTACAGATGCTACGGTTCTG-3′ and zeta3xba were used to amplify sequences encoding the D1D2ζ domains. The resulting products were recombined with sequences encoding the appropriate D0 and cloned into *pcDEF*.

PCR conditions were identical for all procedures: denaturation at 95°C for 2 min, followed by 20 cycles of 45 s at 60°C, 2 min at 68°C, 30 s at 95°C, then 7 min at 68°C. All chimeric constructs were sequenced on a 373A automated sequencer (Applied Biosystems) to ensure fidelity.

Mutagenesis. To make *KIR3DL1* mutants, a fragment was isolated from the *pcDEF/KIR3DL1ζ* construct using the restriction enzymes NotI and NdeI and subcloned into *pGEM*. Mutagenesis was performed in this vector using the Quick Change™ mutagenesis kit (Stratagene) and oligonucleotide primers containing the relevant mutations, per manufacturer's protocol. The fidelity of the procedures was checked by sequencing. The product from a plasmid containing the relevant mutation was cloned back into *pcDEF* using the NotI and NdeI restriction enzymes. The resulting constructs were again checked by sequence analysis.

To make HLA-B*3801 mutants, the complete coding sequence of *HLA-B*3801* was cloned into *pcDNA3.1* (Invitrogen) as described by Gumperz et al. (13). After digestion with BamHI and ApaI the resulting fragment was subcloned into *pBluescript*. Mutagenesis was performed as described above for KIR. Plasmids containing the relevant mutations were cloned back into *pcDNA-B*3801* using BamHI and ApaI. The resulting constructs were checked by sequence analysis and clones corresponding to the designed mutants transfected into 721.221 as described previously (28).

NK Cell Clones and Cell Killing Assays. Procedures were as described previously (22). NK cell clones chosen for analysis lysed untransfected 721.221 cells at levels >30% specific lysis.

B Cell Lines and 721.221 Transfectants. EBV transformed lymphoblastoid B cell lines were: YAR (HLA-A*2601, -B*3801, -C*1203), Blair (Patr-A*0401, -A*1101, -B*0101, -B*1301, -C*0301, -C*0401), Chatter (Patr-A*0101, -A*0404, -B*0501, -B*1602, -C*0501, -C*0601), Ross (Patr-A*0401, -A*1401, -B*1601, -B*1701, -C*0301, -C*0401), and Todd (Patr-A*0402, -A*0601, -B*1701, -B*2001, -C*0601, -C*1201). 721.221 transfectants expressing individual *HLA class I* alleles were generated as described previously (28), except for that expressing *HLA-B*3801*, which had been cloned into *pcDNA3.1* in order to perform mutagenesis.

Results

A Novel Cellular Assay that Detects KIR3D Binding to MHC Class I. Analysis of functional MHC class I interactions with KIR has largely relied upon detecting lack of lysis in cellular cytotoxicity assays. Because of the inherent limitations in measuring this negative effect, we developed a cellular assay which detects KIR3D binding to HLA-B with positive read-out. Constructs were made that encoded chimeric receptors containing the extracellular domains of KIR3DL1 or Pt-KIR3DL1/2 fused to the intracytoplasmic domain of CD3ζ. In these constructs the ligand recognition part of each KIR is retained but the negatively signaling cytoplasmic region is replaced by the corresponding, positively signaling, region of CD3ζ. Stable transfectants of Jurkat cells expressing chimeric *KIR3Dζ* constructs were then transiently transfected before each assay with the *NFAT-SX* reporter construct in which the *AP* gene is driven by an NFAT-binding promoter (26). In the assay Jurkat double transfectants expressing *KIR3Dζ* and *NFAT-SX* are incubated with B lymphoblastoid cells expressing MHC class I. Binding of KIR3Dζ on the Jurkat cells to MHC class I on the stimulator cells leads to secretion of AP which is quantified in the extracellular medium (27). For short this assay is called the SEAP assay, for secretion of AP.

Our analysis aimed first to compare results from the SEAP assay with those obtained from measurements of inhibition in conventional cellular cytotoxicity assays. Class I–deficient 721.221 B cells stimulated little response from Pt-KIR3DL1/2ζ or KIR3DL1ζ in the SEAP assay, whereas B cell lines expressing a normal complement of MHC-A, B, and C molecules stimulated a range of response (Fig. 1). Inclusion of the anti–MHC class I monoclonal antibody W6/32 in the assay abrogated the response, demonstrating its dependence upon MHC class I. Dependence upon expression of a *KIR3Dζ* construct was shown in assays performed with Jurkat cells transfected with the *pc-*

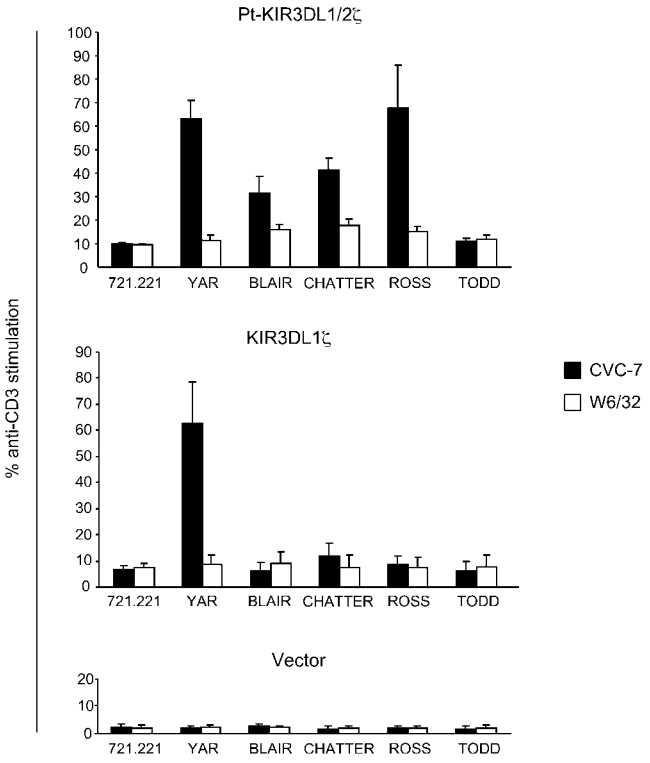

Figure 1. Binding of MHC class I ligands to Pt-KIR3DL1/2ζ and KIR3DL1ζ stimulates secretion of AP by Jurkat cells. Jurkat cells stably transfected with Pt-KIR3DL1/2ζ, KIR3DL1ζ or the pcDEF expression vector alone were transiently transfected with the NFAT-SX plasmid and then incubated with human (YAR) or chimpanzee (BLAIR, CHATTER, ROSS, and TODD) B cell lines, or the class I–deficient B cell line 721.221. Secreted AP was quantified and normalized to the release stimulated by plate-bound anti-CD3 antibody. Assays were performed either in the presence of the anti-MHC class I antibody W6/32 (white bars) or the control, isotype-matched antibody CVC-7 (black bars). Results shown are the means and standard errors of three independent experiments.

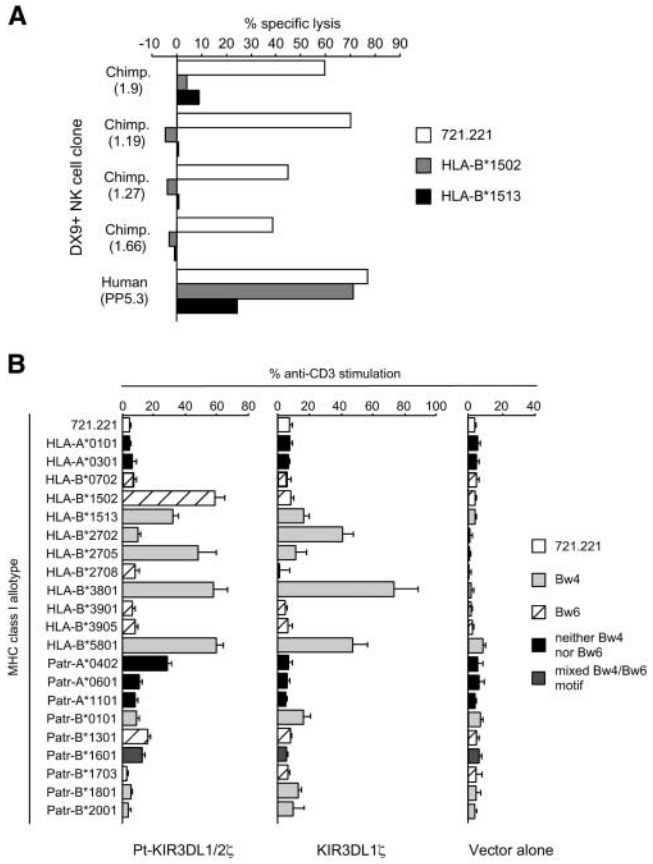

Figure 2. Pt-KIR3DL1/2 and KIR3DL1 exhibit similar HLA-B specificity in cytotoxicity and SEAP assays. (A) Chimpanzee NK cell clones that express Pt-KIR3DL1/2 (1.9, 1.19, 1.27, and 1.66) kill class I–deficient cells 721.221, but not 721.221 transfectants expressing either Bw6$^+$ HLA-B*1502 or Bw4$^+$HLA-B*1513. In contrast, the human NK cell clone PP5.3 expressing KIR3DL1 efficiently kills 721.221 cells and the transfectant expressing HLA-B*1502 but not the transfectant expressing HLA-B*1513. Cytolytic assays were performed at an effector to target ratio of 6:1. (B) Jurkat cells transfected with *Pt-KIR3DL1/2ζ*, *KIR3DL1ζ*, or vector alone were challenged with 721.221 cells and a panel of 721.221 transfectant expressing single human or chimpanzee MHC class I allotypes in SEAP assay. MHC class I allotypes with a Bw4 motif are depicted with bars shaded light gray, those with a Bw6 motif are depicted with hatched bars, and those with neither motif are depicted with black bars. The allotype Patr-B*1601, which has a mixed Bw4/Bw6 motif, is depicted with bars shaded dark gray. Results shown are the means and standard errors of three independent experiments.

DEF vector alone (Fig. 1). Whereas chimpanzee Pt-KIR3DL1/2ζ responded to both human and chimpanzee B cells lines human KIR3DL1ζ responded only to the human cell line. This differential was previously observed in cytotoxicity assays in which HLA class I were good inhibitory ligands for NK cells expressing either KIR3DL1 or Pt-KIR3DL1/2, whereas chimpanzee Patr class I were inhibitors of NK cells expressing Pt-KIR3DL1/2 but not of cells expressing KIR3DL1 (22).

In cytotoxicity assays KIR3DL1 and Pt-KIR3DL1/2 have different patterns of inhibition by HLA-B allotypes. A diagnostic difference is that KIR3DL1 only engages HLA-B allotypes having a Bw4 motif at residues 77–83 (13) whereas Pt-KIR3DL1/2 also engages the HLA-B*1502 allotype that has the alternative Bw6 motif (Fig. 2 A). In addition, Pt-KIR3DL1/2 has weak reactivity with certain MHC-A allotypes and for both receptors there is a range of response to different MHC-B allotypes having a Bw4 motif (22).

To assess and compare the MHC class I reactivity of KIR3DL1ζ and Pt-KIR3DL1/2ζ expressed on Jurkat cells, they were challenged with a panel of 721.221 transfectants expressing single human or chimpanzee MHC class I allotypes. Both KIR3DL1ζ and Pt-KIR3DL1/2ζ secreted AP in a manner that was dependent upon MHC class I polymorphism but with different specificity (Fig. 2 B). KIR3DL1ζ was stimulated by a subset of Bw4$^+$ HLA-B allotypes and within this group there was a gradation of response: B*3801 > B*5801 ~ B*2702 > B*1513 ~ B*2705. Pt-KIR3DL1/2ζ was stimulated by a slightly different subset of Bw4$^+$ HLA-B allotypes: B*3801 ~ B*5801 ~ B*2705 > B*1513 > B*2702, and also by the Bw6$^+$ HLA-B*1502 and by Patr-A*0402. Notable were the weak responses of Pt-KIR3DL1/2ζ and KIR3DL1ζ to transfec-

	Domain composition			SEAP release (% anti-CD3)				
				Responder cell line				
Construct	D0	D1	D2	721.221	B*1502	B*1513	B*3801	B*5801
Pt-KIR3DL1/2ζ	■	■	■	11±2	69±5	40±5	93±6	53±7
HCCζ	▨	■	■	16±5	15±5	29±9	144±8	57±5
CHHζ	■	▨	■	20±2	18±1	103±5	172±21	107±12
HCHζ	▨	■	■	25±1	36±4	31±3	76±6	38±5
KIR3DL1ζ	▨	▨	▨	12±2	11±1	14±1	100±6	18±2

Figure 3. The D0 domain of Pt-KIR3DL1/2 stabilizes the interaction with MHC-B. Domain swap constructs of *KIR3DL1ζ* and *Pt-KIR3DL1ζ* were tested against a panel of 721.221 transfectants expressing various HLA-B allotypes in SEAP assays. The Ig domain composition of the three constructs tested are shown. Human Ig domains are indicated with stippled boxes and chimpanzee Ig domains with filled boxes. The reactivities of the constructs in the SEAP assays are shown in comparison to stimulation with plate bound anti-CD3. The means and standard errors of three independent experiments are shown.

tants expressing chimpanzee MHC class I molecules. From these results we see that the MHC class I specificities of KIR3DL1 and Pt-KIR3DL1/2 as assessed by SEAP assay and inhibition of cytotoxicity are similar but not identical. The differences may be due to lower sensitivity of the SEAP assay, such that the weaker reactions detected by inhibition of cytotoxicity drop out, or by the absence on Jurkat T cell transfectants of additional NK cell receptors and coreceptors that lead to the additional reactions seen in cytotoxicity assays. Common to both assays is that chimpanzee Patr class I molecules are generally weaker ligands for human and chimpanzee KIR3DL than human HLA class I molecules.

Natural Variation in the D0 Domain Modulates Binding to Bw4⁺ HLA-B Allotypes. To assess the contribution of individual Ig-like domains to differences in HLA-B recognition by KIR3DL1 and Pt-KIR3DL1/2, we made six constructs from *KIR3DL1ζ* and *Pt-KIR3DL1/2ζ* in which the sequences encoding individual human and chimpanzee domains were recombined in all possible combinations. Of these, three failed to give a detectable cell-surface protein upon transfection into Jurkat cells. The three expressed recombinant mutants analyzed for HLA-B binding consisted of HCCζ and CHHζ in which the D0 domains were swapped, and HCHζ in which the D1 domain of KIR3DL1 was replaced with that of Pt-KIR3DL1/2 (Fig. 3). Mutant CHHζ, in which chimpanzee D0 replaces human D0, exhibited stronger reactions with all three Bw4⁺ HLA-B allotypes tested, but no reactivity with the Bw6⁺ HLA-B*1502. The reciprocal mutant, in which human D0 is associated with chimpanzee D1 and D2, gave a pattern of reactivity B*3801 >> B*5801 > B*1513, comparable to that of KIR3DL1ζ and lacking reaction with B*1502. Thus, for Pt-KIR3DL1/2, reactivity with Bw6⁺ B*1502, but not Bw4⁺ HLA-B, is dependent upon the D0 domain and upon features that distinguish the chimpanzee D0 domain from its human counterpart. The reactivity of mutant HCHζ was B*3801 > B*5801 ≈ B*1513 ≈ B*1502, a pattern distinct from that of KIR3DL1 and which includes the Bw6⁺ allotype B*1502. This suggests that substitutions in the chimpanzee D1 domain also contribute to the recognition of HLA-B*1502.

Features unique to the D0 domain of Pt-KIR3DL1/2 are deletion of what are residues 50 and 51 in KIR3DL1 (Fig. 4) and substitution of proline for serine at position 14. To determine the contribution these differences make to the enhanced binding due to the Pt-KIR3DL1/2 D0 do-

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Leader
          -21                 -1
PtKIR3DL1/2  ---------- --------c-
KIR3DL1*002  -l-m------ -----v---g-
KIR3DL2*001  -s-t------ -------g-w-
Consensus    M-L-VVSMAC VGFFLLQRA-P

D0 domain
             1                          31                                  61                                  97
PtKIR3DL1/2  ----h----- ---s------ ---------- -g-------- ---------. .--------- ---------- ---------- ---------- -------
KIR3DL1*002  -v-------- ---------- ---------- -hr------- ---------- ---l------ ---------- ---------- ---------- -------
KIR3DL2*001  l--------- --r--t---- ----a-q--- -r-------- ----s----- ----------i -g---p---- t-r-----r-- -l--------- -l-----
Consensus    HMGGQDKPFL SAWPSAVVPR GGHVTLRCHY R-GFNNFMLY KEDRIHVPIF HGRIFQESFN MSPVTTAHAG NYTCRGSHPH SPTGWSAPSN PVVIMVT

D1 domain
             98                         128                                 158                                 197
PtKIR3DL1/2  ---------- ------r--- ---------- ---------- ---q------ ---------- ---------- ---------- ---------- -------m--
KIR3DL1*002  ---------- ---------- r---------i --------k- ---k------ ---------- ---------- ---------- ---------- ----------
KIR3DL2*001  ---------- -----l---- ---------- ---------- ---e ------ ---------- ------l-pv ---------- -p-s------ --------i-
Consensus    GNHRKPSLLA HPGPLVKSGE TVILQCWSDV MFEHFFLHRE GIS-DPSRLV GQIHDGVSKA NFSIGPMMLA LAGTYRCYGS VTHTPYQLSA PSDPLDIVVT

D2 domain
             198                        228                                 258                                 295
PtKIR3DL1/2  ---------- ---------- ---------r -------f-- -----c---- ---------- ---------r ---------- ---------- --------
KIR3DL1*002  -p-------- ----k----- ---------- ---------- rg-------- ---------- ---------- ---------- ---------- --------
KIR3DL2*001  ---------- ---------- n-------w- ---i------ --------r- -p-------- ---------- ---------- al-cv--ns- --------
Consensus    GLYEKPSLSA QPGPTVQAGE SVTLSCSSRS SYDMYHLSRE GEAHERRLPA VRKVNRTFQA DFPLGPATHG GTYRCFGSFR HSPYEWSDPS DPLLVSVT

Stem/transmembrane domains
             296                        326         339
PtKIR3DL1/   ---------- ---------r ---------- -------ii- ----
KIR3DL1*002  ---------- --------np ----i----- ---------- ----
KIR3DL2*001  ---------- ---------c ---------- ---f------ ----
Consensus    GNPSSSWPSP TEPSSKSGI- RHLHVLIGTS VVIILFILLL FFLL

Intracytoplasmic domains
             340                         370                                 400                                 435
PtKIR3DL1/2  ---------- ----g----- ---------- ---------- ---------- g--------- ---------- ---------- ---------- ----*
KIR3DL1*002  -l-------- --------n- -a-se----- --e------ -----t---- t--------- p---il---- ---k------ ---*
KIR3DL2*001  y--------- ---------- ---------- ---------- ---------- s--------- ---------- ---------- ---------- ----*
Consensus    HRWCSNKKNAA VMDQEPAGDR TVNRQDSDEQ DPQEVTYAQL DHCVFIQRKI -RPSQRPKTP LTDTSVYTEL PNAEPRSKVV SCPRAPQSGL EGVF
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Figure 4. Comparison of the amino acid sequences of KIR3DL1*002, Pt-KIR3DL1/2, and KIR3DL2*001. The sequences were aligned using the Wisconsin package version 10.1 (Genetics Computer Group). Positions identical to the consensus sequence are indicated by dashes (-). Residues deleted in comparison to the consensus sequence are indicated by dots (.). Residues indicated with the symbol "■" were mutated to generate the panel of KIR3DL1ζ mutants.

main, a mutant KIR3DL1ζ was made that lacks residues 50 and 51 and has serine substituted for proline at position 14. This Δ50,51,P14Sζ mutant gave a pattern of HLA-B response like that of CHHζ, in that there was enhanced reactivity with the $Bw4^+$ HLA-B allotypes but no reactivity with HLA-B*1502 (Fig. 5 A). Thus, the enhanced response appears specific for allotypes reactive with the wild-type KIR3DL1 molecule. A mutant having just deletion of residues 50 and 51 (Δ50,51ζ) was then made and shown to have the same binding phenotype as Δ50,51,P14Sζ (Fig. 5 B). These results demonstrate that absence of residues 50 and 51 is responsible for the enhanced binding of $Bw4^+$ HLA-B by Pt-KIR3DL1/2 compared with KIR3DL1.

To investigate further the binding of the D0 domain to MHC class I we examined the properties of KIR3DL1ζ mutants in which individual residues of the D0 domain, including positions 50 and 51, were substituted for alanine. Residues were chosen for mutagenesis using the criterion of being shared by KIR3DL1 and Pt-KIR3DL1/2 but absent from any D0 domain of a lineage I or III KIR. All 15 of the D0 mutants tested gave positive reactions with HLA-B*3801. Although some of the mutant KIR3DL1 molecules gave lower reactivities than the wild-type, these differences were small and scattered in comparison to the enhanced binding observed for mutants having alanine at positions 49, 50, 51, or 52 (Fig. 6). The peak of this enhancement was for alanine substitutions at positions 50 and 51. Thus reduction in side-chain size as well as deletion at residues 50 and 51 has an enhancing effect. For those mutants giving enhanced response to HLA-B*3801 and B*1513 there was also increased reactivity with B*1502 and untransfected 721.221 cells (Fig. 6). These reactivities were unaffected by addition of the HLA class I–specific antibody W6/32 showing they are not dependent on KIR interaction with MHC class I (data not depicted). It is possible that these mutations increase aggregation of KIR3DL1ζ in the Jurkat cell membrane, which leads to an increased basal level of AP secretion.

The interactions of three KIR3DL1ζ mutants having changes at residues 50 and/or 51 in D0 with four point mutants of HLA-B*3801 were assessed (Fig. 7). Three of the B*3801 mutants: T73A, Y74A, and E76A, were designed to study the influence on KIR binding of HLA-B residues in the α_1 helix but outside the Bw4 motif; of these

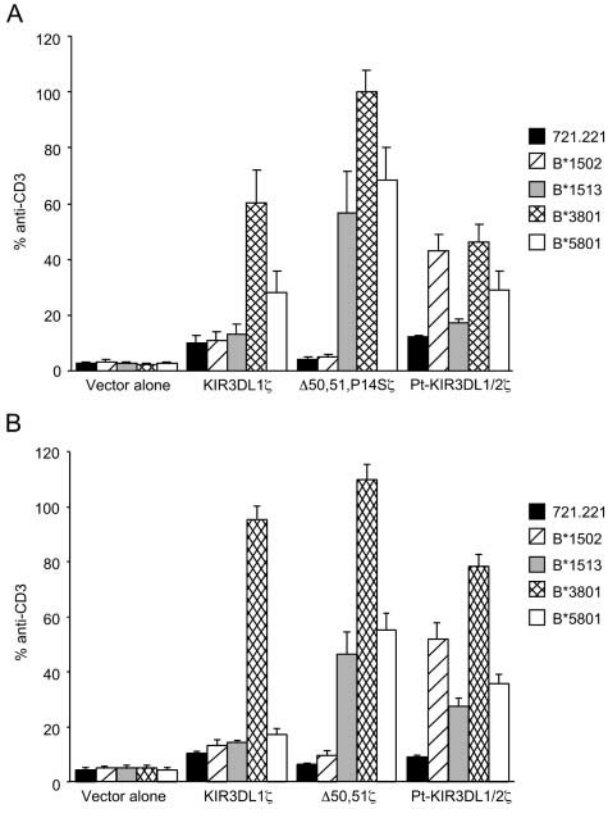

Figure 5. Naturally occurring variations in D0 stabilize the KIR-MHC class I interaction. (A) Comparison between the HLA-B specificities of KIR3DL1ζ, Pt-KIR3DL1/2ζ, and KIR3DL1-Δ50,51,P14Sζ in the SEAP assay. Jurkat transfectants stably expressing these molecules were incubated with 721.221 transfectants expressing HLA-B*3801, B*1513, B*1502, and B*5801, or untransfected 721.221 cells. In B a further comparison is made with KIR3DL1ζ, Pt-KIR3DL1/2ζ, and KIR3DL1-Δ50,51ζ in the SEAP assay against the same panel of cell lines. The data shown are the means and standard errors of three independent experiments in comparison to plate-bound anti-CD3.

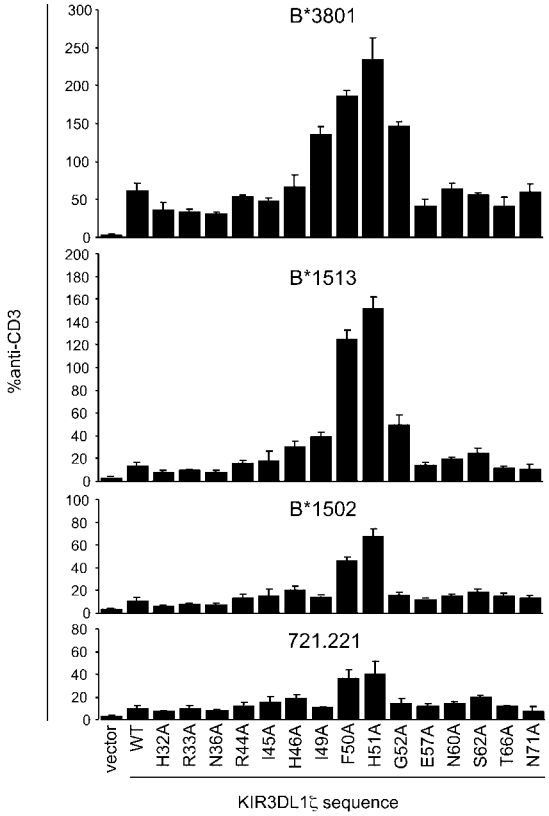

Figure 6. Mutation at residues 49–52 in the D0 domain enhance binding of KIR3DL1 to $Bw4^+$ HLA-B. A panel of Jurkat transfectant expressing KIR3DL1ζ mutants having point substitutions in the D0 domain were challenged with 721.221 transfectants expressing HLA-B*3801, B*1513, and B*1502, as well as untransfected 721.221 cells, in SEAP assays. The means and standard errors of three independent experiments in comparison to plate-bound anti-CD3 are shown.

E76 is highly conserved among $Bw4^+$ and $Bw6^+$ MHC-B allotypes. The fourth mutation, substitution of alanine for glutamate at position 46, was predicted to have no affect on KIR3D binding and this mutant (E46A) was designed as a negative control. For mutants E46A, T73A, and Y74A, the interactions with KIR3D were indistinguishable from wild-type B*3801. The fourth B*3801 mutant, E76A, was weakly recognized by KIR3DL1ζ, a diminution that was less severe for KIR3DL1ζ mutants having alanine at residue 50 or 51. Intermediate responses were seen for Pt-KIR3DL1/2ζ and the KIR3DL1ζ mutant having residues 50 and 51 deleted and proline 14 replaced by serine. Thus, from this test system we see that changes in the D0 domain of KIR3DL1 can compensate for reductions in KIR binding caused by substitutions in HLA-B, an illustration of how KIR3D and MHC-B might coevolve to preserve ligand–receptor interaction.

Mutations in the D1 and D2 Domains Reduce KIR3DL1 Interaction with HLA-B. Because none of the point mutations in the KIR3DL1 D0 domain diminished binding of HLA-B it was important to investigate whether mutations having such an effect could be introduced into KIR3DL1ζ. To do this we characterized nine KIR3DL1 mutants with point substitutions in either the D1 domain (four mutants) or the D2 domain (five mutants). Mutants were chosen on the basis of different criteria with the overall aim of giving some that would diminish binding to KIR3DL1 and others that would not. Four mutations made in the D2 domain (Y200A, E201A, D230H, and H278A) correspond to mutations previously characterized for KIR2DL2 (Y105A, E106A, D135H, and D183A, respectively) and shown to disrupt its binding to HLA-Cw3 (17). Position 139 in the D1 domain of KIR3DL1 is the equivalent of KIR2DL1 position 44, at which substitution determines specificity for either the C1 (lysine) or C2 (methionine) group of allotypes (19). In mutation I139K the isoleucine present in KIR3D is replaced by lysine. Position 140 is equivalent to position 45 at which Winter et al. (29) found substitution of tyrosine for phenylalanine converted KIR2DS2 from null-binding phenotype to C1 binding phenotype; mutant S140F replicates this change in KIR3DL1. At position 141 KIR3DL1, KIR3DL2 and Pt-KIR3DL1/2 all differ; mutant K141Q introduces the glutamine characteristic of Pt-KIR3DL1/2 into KIR3DL1. Mutations M128A in D1 and Q256A in D2 were designed as negative controls, chosen because their equivalents in KIR2DL2 (positions 33 and 161, respectively) do not contribute to the interaction seen with HLA-Cw3 in the crystallographic structure of the complex (17). The Y200A mutant bound the Z27 antibody but not the DX9 antibody, the first observation to distinguish the reactivities of the two monoclonal antibodies and showing that tyrosine 200 of the D2 domain is an important determinant of DX9 binding.

As anticipated the nine mutations in the D1 and D2 domains exhibited a range of response in the cell-binding assay (Fig. 8). Binding reactions were preserved in the two negative controls mutations (M128A and Q256A) as well as in the mutations at positions 140 and 141 (S140F and K141Q). Mutation at positions 200 (Y200A) and 230 (D230H) caused abrogation of HLA class I binding, as seen

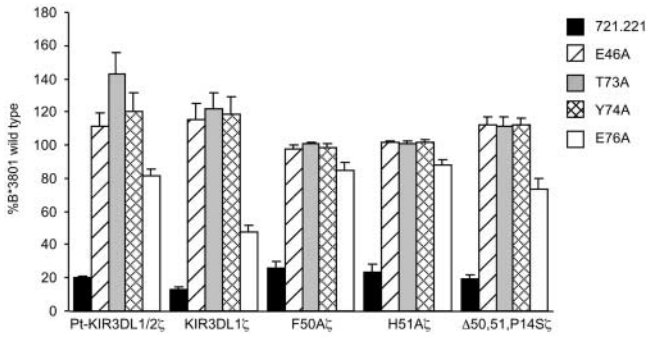

Figure 7. Complementary mutation in HLA-B*3801 and the D0 domain of KIR3DL1 can stabilize ligand-receptor interaction. Jurkat transfectants expressing KIR3DL1ζ, Pt-KIR3DL1/2ζ, or KIR3DL1ζ with substitutions in D0 were challenged with 721.221 transfectants expressing wild-type and mutated HLA-B*3801 molecules. The mean and standard errors of three independent experiments are expressed as a percentage of the SEAP release in comparison to the wild-type HLA-B*3801 transfectant.

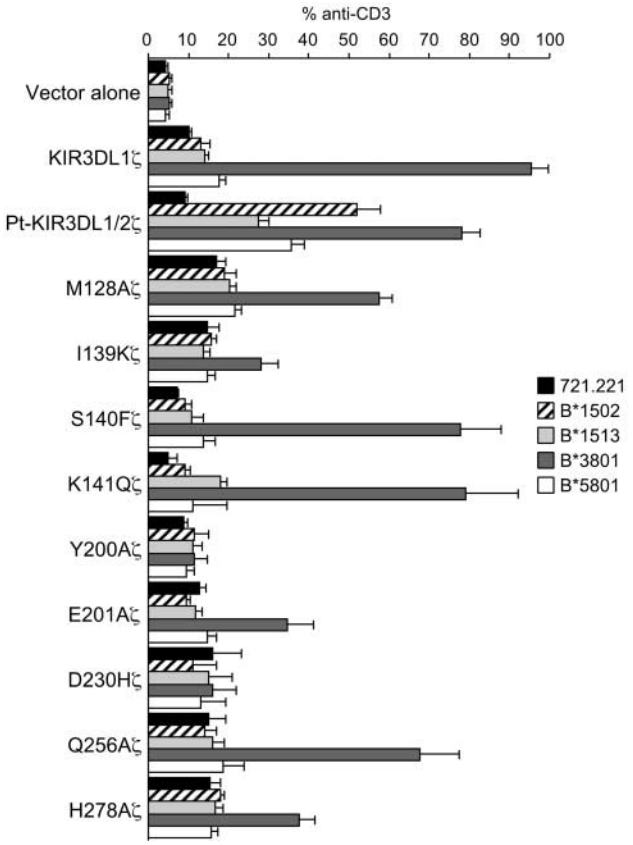

Figure 8. Point mutations in the D1 and D2 domain abrogate binding of KIR3DL1 to HLA-B. A panel of Jurkat transfectants each expressing a KIR3DL1ζ mutant with a point substitution in the D1 and D2 domains were challenged with 721.221 transfectants expressing HLA-B*3801, B*1513, B*1502, and B*5801, as well as untransfected 721.221 cells in SEAP assays. The means and standard errors from three independent experiments are shown.

previously for the equivalent mutations in KIR2DL2. Reduced but significant levels of binding were observed for mutants I139K, E201A, and H278A. For E201A this intermediate result matches that seen in surface plasmon resonance experiments for the equivalent mutation in KIR2DL2; for the other two mutations the KIR2DL2 equivalent preserved no demonstrable binding to HLA-Cw3 (17). Of note, alanine is the naturally occurring residue at position 278 in KIR3DL2.

Discussion

Human KIR3DL1 and chimpanzee Pt-KIR3DL1/2 are homologous MHC class I receptors of NK cells with different inhibitory specificity. Ligands for KIR3DL1 are $Bw4^+$ MHC-B allotypes; ligands for Pt-KIR3DL1/2 include $Bw4^+$ MHC-B allotypes, a $Bw6^+$ MHC-B allotype, and MHC-A allotypes. Previous analysis suggested that chimpanzee class I molecules are weaker inhibitors of NK cell cytotoxicity than their human counterparts, but that chimpanzee Pt-KIR3DL1/2 is a stronger receptor for shared $Bw4^+$ HLA-B ligands than human KIR3DL1 (22). To study these differences we developed the SEAP assay. Like the cytotoxicity assay it involves interaction between two cell types but was designed to eliminate the well known complications that can confound the interpretation of data obtained from inhibition assays of NK cell cytotoxicity. First, the SEAP assay limits interaction to one type of classical MHC class I molecule and one type of KIR, whereas NK cells express multiple receptors; second, the SEAP assay measures a positive effect – secretion of AP, whereas in the cytotoxicity assay the critical parameter is lack of killing, i.e.: no effect.

Signals generated in the SEAP assay were shown to be dependent upon interaction between MHC class I and KIR. Major elements of the KIR3DL1 and Pt-KIR3DL1/2 MHC class I specificity as dissected from analysis of inhibition of NK cell cytotoxicity were reproduced in the SEAP assay. Thus, KIR3DL1 was only stimulated by $Bw4^+$ HLA-B allotypes, Pt-KIR3DL1/2 also reacted with the $Bw6^+$B*1502 and within the subsets of positively reactive allotypes there was variability in the strength of the response (as is also characteristic of cytotoxicity assays). Moreover, in the SEAP assay Pt-KIR3DL1/2 behaved as a stronger receptor for $Bw4^+$HLA-B allotypes than human KIR3DL1, whereas chimpanzee MHC class I molecules were much weaker inhibitors of both KIR than their human counterparts.

By analysis of domain-swap, deletion, and point mutants of KIR3DL1 and Pt-KIR3DL1/2 we have demonstrated that structural difference in their D0 domains is a major contributor to their differential avidity for MHC class I. Specifically, a natural two amino acid deletion in Pt-KIR3DL1/2 corresponding to residues 50 and 51 of KIR3DL1 is responsible for its enhanced response to $Bw4^+$ HLA-B allotypes. Deletion of these residues in KIR3DL1 was not essential to produce enhanced response, substitution of alanine at positions 50 or 51 had similar effect.

In this study 24 point mutations in the extracellular domains of KIR3DL1 were characterized. Mostly alanine mutations, they comprised 15 in D0, 4 in D1, and 5 in D2. None of the mutations in D0 significantly reduced the response to $Bw4^+$ HLA-B. Conversely several mutations in the D1 and D2 domains diminished or abrogated the response. The pattern of residues that did or did not reduce the response in the SEAP assay correlated well with the crystallographic structures of KIR:HLA-C complexes, suggesting that the effects are largely at the level of ligand binding. Consequently, our mutational analysis of KIR3D is consistent with a picture in which the D1 and D2 domains of KIR3DL1 interact with $Bw4^+$ HLA-B with orientation similar, but not necessarily identical, to that seen in the KIR2D:HLA-C complexes (17, 18). Within this context the data described here indicates that the enhancing function of the KIR3DL1 D0 domain involves interaction distinct from the contact made between the D1 and D2 domains and $Bw4^+$HLA-B. Three models for this interaction will be considered. In the first model the D0 domain makes an additional contact with cognate $Bw4^+$ HLA-B ligand, thereby increasing the overall strength of the individual ligand–receptor interaction. In the second model the D0 domain contacts the $Bw4^+$ HLA-B ligand bound by the D1 and D2 domains of a different KIR3DL1 molecule. This mode of interaction could facilitate the aggregation of ligand–receptor complexes in the cell membranes at the point of cell-cell contact. In the third model the D0 domain makes contact with other KIR molecules, also facilitating aggregation of receptors and/or receptor–ligand complexes in the NK cell membrane. None of these models is either eliminated or strongly favored by the data as it stands. Whatever the mechanism the enhancing contacts made by the D0 domain appear necessary for the function of KIR3DL1, as also suggested by Rojo et al.'s analysis of domain-deletion mutants in which all three Ig-like domains were needed for binding to $Bw4^+$ HLA-B (21).

Residues 49–52, that appear most important for D0 domain mediated enhancement, are predicted to form part of the external C′ strand of the β-pleated sheet comprising strands A′, G, F, C, and C′. In considering model 1, the homologous strands in the D1 and D2 domains of KIR2D do not contact HLA-C in the crystallographic structures of the complexes. One possible effect of the enhancing mutations and deletions could be to distort the CC′ loop, which is important for binding of the 2 Ig domain KIR to MHC class I. In considering the second model, a second binding site on MHC class I has been proposed for the 2 Ig domain KIR (30). However, in the cocrystal of KIR2DL2 with HLA-Cw3 this interaction involves the B and E β-strands not the C′ strand. If KIR-KIR aggregation is required for efficient signal transduction, then the increased MHC class I–independent, "background" release of AP by the F50A and H51A transfectants provides data consistent with model 3.

Although deletion or alanine substitution within residues 49–51 of the D0 domain enhanced binding of KIR3DL1 to $Bw4^+$ HLA-B allotypes, none of these changes pro-

duced a positive response to Bw6$^+$ HLA-B*1502. Indeed, of all the KIR3DL1 mutants, the only one showing a response to B*1502 was the domain swap mutant HCHζ in which the D1 domain was replaced by its chimpanzee counterpart, and its response was weak (Fig. 3). Thus, although replacement of the chimpanzee D0 domain with its human counterpart abrogates interaction with B*1502, the species-specific differences in the D0 domain do not account for all the differences in the specificity of KIR3DL1 and Pt-KIR3DL1/2. Substitutions in the D1 domain are also likely to make a contribution and a role for D2 is not ruled out.

The SEAP assay measures the product of both ligand binding and signaling by KIR. In using chimeric KIR3Dζ molecules it was possible that the KIR interaction with MHC class I would be altered in ways not relevant to the biology. Our results indicate that is unlikely. Response in the SEAP assay was demonstrated to be dependent upon interaction between KIR and MHC class I. Comparison showed that KIR exhibited similar but not identical responses in the cytotoxicity and SEAP assays. The difference being that not all of the MHC class I allotypes which can inhibit NK cell cytotoxicity could stimulate in the SEAP assay and that those allotypes tended to be the weaker, more variable inhibitors of cytotoxicity. Importantly, comparison with previous cytotoxicity experiments (12, 13, 21, 22) revealed no false positives in the SEAP assay. Lower sensitivity in the SEAP assay could be due to reduced intensity of the signals emanating from CD3ζ compared with the cytoplasmic tail of KIR3D; reduced efficiency of the chimeric molecules to form signaling complexes within the cell membrane; or the absence of additional NK cell receptors or coreceptors for MHC class I interaction that not are expressed by the Jurkat T cells used in the SEAP assay. As in cytotoxicity assays, there is interexperiment variation in the SEAP assay data. Accordingly, experiments were designed so that important comparisons were always made within the same experiment, and each experiment was performed on at least three occasions. Importantly, the main conclusions of the investigation were reached by separate analysis of three distinct types of mutant KIR: domain swap, deletion, and point mutants.

An advantage the SEAP assay has over binding assays that use soluble, recombinant KIR (29) is its basis on cell–cell interaction. In addition to being more physiological all the molecules contributing to the assay should be correctly folded and modified by mammalian cellular machinery, making it unlikely that nonphysiological binding sites will engage. Thus, we were unlikely to observe false positive reactions for mutant KIR3DL1 molecules. The configuration of the SEAP assay reduces the probability of confounding false positive reactions due to other receptor–ligand interactions, as can occur when measuring inhibition of NK cell cytotoxicity. That certain point mutations in the D1 and D2 domains abrogated KIR response to MHC class I demonstrated that the assay system is sensitive to changes in KIR-MHC binding caused by single amino acid substitutions. Of note in this regard, the point mutations at positions 49 and 50 in the D0 domain increased SEAP release in response to the MHC class I–negative cell line, 721.221. This MHC class I–independent signal could have been caused by increase in receptor-receptor aggregation, which may have physiological significance. Indeed, a model for KIR2D aggregation to augment signal transduction has been proposed by Snyder et al. (20) and a similar phenomenon may occur for KIR3D.

In humans and apes (hominoids) the expressed KIR comprise three lineages each represented in all the species (31). Only lineage I, comprising KIR2DL4 and KIR2DL5, is shared with the rhesus monkey, which has an additional lineage not present in hominoids (32). Lineages II and III, which include MHC-A and -B, and MHC-C specific KIR, respectively, therefore appear to have originated with the hominoids. Whereas all lineage II genes encode 3Ig KIR, lineage III genes can encode 2Ig and 3Ig KIR and their relative numbers vary between species. Importantly, all the 2Ig *KIR* genes in lineage III contain a 'pseudoexon' encoding an unexpressed D0 domain, showing that all 2Ig *KIR* in this lineage derive from ancestral genes encoding 3Ig KIR. The mechanism underlying the lack of expression of the pseudoexons differs amongst the genes encoding 2Ig KIR, indicating that loss of expression of D0 is a property that has been either selected or tolerated on multiple, independent occasions (23).

The tendency to lose the D0 domain is particularly evident in human lineage III *KIR* genes, all of which encode 2Ig KIR. In comparison the common chimpanzee lineage III comprises both 2Ig and 3Ig KIR, with the latter being in the majority (22). More specifically the KIR specific for group C2 MHC-C allotypes is a 3Ig KIR. In contrast, there is no evidence of any tendency for any lineage II KIR, including those specific for MHC-A and -B, to lose expression of the D0 domain. The data accumulated from studying inhibition of NK cell cytotoxicity indicate that HLA-C allotypes are more potent and more frequently used inhibitors of NK cells than MHC-A or -B (8–10). The distinguishing properties of the *MHC-C* locus suggest it has evolved specializations that facilitate interaction of its products with KIR, and in reciprocal fashion that may also be true for the cognate KIR2D. Such specializations might include strengthening direct D1 and D2 domain interaction with MHC-C, with concomitant loss of the D0 domain and its enhancing function. Such progression could exemplify specialization toward particular function with accompanying loss of flexibility and adaptability.

This tendency can also be seen in the lineage II *KIR* where it is apparent that the human and chimpanzee *KIR* have taken quite different evolutionary trajectories. In chimpanzee this lineage is represented by a single gene with a high frequency allele, *Pt-KIR3DL1/2*. This allele encodes a receptor with broad specificity for MHC-A and -B and has a strongly enhancing D0 domain. By contrast this lineage is represented by two human genes, *KIR3DL1* and *KIR3DL2*, both of which are highly polymorphic (33). In structure *Pt-KIR3DL1/2* is a pastiche of sequence elements present in either *KIR3DL1* and *KIR3DL2*. In function the

two human genes are specialized in elements of Pt-KIR3DL1/2's broader specificity: KIR3DL1 has specificity for $Bw4^+$ HLA-B allotypes, whereas KIR3DL2 is reported to be an HLA-A receptor (14, 15). As demonstrated here, the D0 domain of KIR3DL1 is a poorer enhancer than that of Pt-KIR3DL1/2. The difficulty of demonstrating MHC class I–mediated inhibition through KIR3DL2 and the consequent debate concerning its function as an inhibitory HLA-A receptor (8), could be explained by the D0 domain of KIR3DL2 being an even weaker enhancer.

Of relevance to the human and chimpanzee KIR3D differences are observations, from cytotoxicity and SEAP assay, that human MHC class I molecules are stronger inhibitors than chimpanzee MHC class I. This suggests coevolution of receptors and ligands in the two species has followed different paths to maintain the avidity of their interaction within a similar range. The difference in the MHC class I molecules of the two species remains enigmatic, as there are no obvious species-specific characteristics to their structures (34). Thus, the phenomenon may not be attributable to a single cause that similarly affects all MHC class I allotypes. Alternatively the effect may not be due to the MHC class I genes themselves, but to other species-specific factors that influence cell-surface expression of MHC class I.

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