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The centromeric part of the human natural killer (NK) receptor complex: lectin-like receptor genes expressed in NK, dendritic and endothelial cells

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Summary: The human natural killer (NK) receptor complex encompasses a region of about 2 Mb on the short arm of chromosome 12. It contains at least 18 lectin-like receptor genes, of which some are expressed in NK and NK/T cells and function as NK receptors. Close to the CD94 and NKG2 NK receptor genes in the centromeric part, a novel family of genes, expressed in myeloid, dendritic and/or endothelial cells, recently became evident. These genes encode a receptor for oxidized low density lipoprotein in endothelial cells and three other receptors potentially serving regulatory functions in dendritic cells. Although the overall structure of the human NK receptor complex is similar to the syntenic rodent regions, the centromeric part lacks the cluster of *Ly49* genes. This supports the notion that recognition of MHC class Ia molecules has evolved separately in rodents and humans in the lectin-like *Ly49* and the killer immunoglobulin-like receptors, respectively. In the telomeric part, other lectin-like genes expressed in different hematopoietic lineages are found. The receptors of the NK receptor complex apparently serve important functions in several leukocytes and in endothelial cells, and the exact role of these receptors, their ligands, and their distinct and co-ordinate regulation in different cell lineages warrants further investigation.

Introduction

The immune response relies on receptors that can sense pathogenic invasion. Aside from the immunoglobulin domains, lectin domains are modular structures frequently used in binding domains of cellular receptors of the immune system (1). One class of lectin-like receptor genes encodes type II transmembrane proteins with a C-type lectin-like domain (CTLD) in their extracellular part. A significant number of these receptor genes cluster within a region on the short arm of human chromosome 12p12.3-p13.2 (2–4). This region has been known for some time to contain the NKG2, CD94, NKR-P1 and CD69 genes (5–8). In mice, the corresponding

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Nomenclature: According to the Human Gene Nomenclature Database (www.gene.ucl.ac.uk/nomenclature) some of the genes described have obtained the following symbols: KLRA1 (*LY49L*), KLRB1 (*NKR-P1A*), KLRC1 (*NKG2A*), KLRC2 (*NKG2C*), KLRC3 (*NKG2E*), KLRC4 (*NKG2F*), KLRD1 (*CD94*), KLRG1 (*MAFA-L*), OLR-1 (*LOX-1*).

syntenic region, located on chromosome 6 (9), was initially found to contain the NKR-P1 and *Ly49* genes, which gave rise to the term natural killer (NK) gene complex (10). A similar syntenic region is found in rats on chromosome 4 (11). In the rodent genomes, loci mediating resistance to cytomegalovirus (*Cmv1*), mouse pox virus (*Rpm1*) or mediating lysis of CHO cells (*Chok*) and allogeneic lymphocytes (*Nka*) map to the NK gene complex (11–14) supporting the general importance of the region for natural immunity and NK-cell cytotoxicity.

NK cells are well recognized as an important component of the natural immune system involved in the defense of pathogen invasion and the rejection of tumor cells and allogeneic cells (15, 16). They are particularly important in the earliest phase of infection by viruses or intracellular bacteria and parasites, where they respond with immediate cytotoxicity and cytokine production. These cytokines appear to contribute significantly to the development of the subsequent adaptive or specific immune response (17, 18). Furthermore, NK cells are thought to be involved in the regulation of hematopoiesis (19) and the development of placentation (20, 21). The work of the recent years has shown that activation of human NK cells is controlled by an intricate balance of inhibitory and activating receptors of the immunoglobulin (Ig)-like and the lectin-like receptor families (22–27) encoded within the leukocyte receptor complex (28) and the NK receptor complex (3), respectively. Whereas the killer Ig-like receptors (KIRs) recognize a narrow range of MHC class Ia alleles (29), the lectin-like NKG2/CD94 receptors bind to the MHC class Ib molecule HLA-E (30–32). As initially predicted by the “missing self” hypothesis (33), both systems are obviously designed to complement the cytotoxic T-cell receptor system and to sense the reduction of MHC molecules on the surface of cells, as viruses frequently downregulate MHC class I expression to avoid the cytotoxic T-cell response (34, 35). In contrast to human NK cells, available evidence suggests that the function of MHC class Ia binding resides in rodents solely in the family of the lectin-like *Ly49* receptors, and no KIR receptors with comparable function have been detected (36, 37). Evidence for functional *Ly49* receptors is missing in humans (4, 38).

Several isoforms of NKG2 receptor chains can form covalently linked heterodimers with the CD94 chain (39). NKG2A contains immunoreceptor tyrosine-based inhibition motifs (ITIMs) in its cytoplasmic tail and forms the inhibitory NKG2A/CD94 receptor, which recruits SH2-containing tyrosine phosphatases. In contrast, NKG2C, E and H lack the ITIM motif and associate via a charged amino acid in their trans-

membrane domain with the DAP12 molecule, which contains an immunoreceptor tyrosine-based activation motif (ITAM). These NKG2/CD94 receptor forms can activate NK cells following ligand binding via activation of tyrosine kinases.

Another kind of activating NK-cell receptor is the lectin-like NKG2D receptor. Originally classified as a member of the NKG2 family, NKG2D displays only distant homology to the other NKG2 isoforms (40, 41). Homodimers of human NKG2D bind to the MHC class I-related molecules MICA and MICB, which seem to be upregulated on virally infected cells and many tumor cells (42, 43). In addition, it might recognize cell-surface glycosylphosphatidylinositol-linked molecules, called UL16-binding proteins (44), which are homologs of MICA/B and bind the human cytomegalovirus (CMV) glycoprotein UL16. Activation of NK cells is mediated via association with the ITAM-bearing DAP10 molecule.

Mainly on the basis of data obtained in the rodent systems, NKR-P1 is thought to be another activating NK-cell receptor (45). The human NKR-P1 gene is localized in the telomeric half of the human NK receptor complex, and its ligand and function for human NK cells are so far not well understood (7). Further lectin-like receptor genes localized to the NK receptor complex are activation-induced C-type lectin (*AICL*) (46) and *CD69* (47). *AICL* and *CD69* are expressed more widely in the hematopoietic lineage. Whereas it was previously thought that *CD69* had mainly carbohydrate-binding properties (48), recent evidence suggests that *CD69* and its closest relatives lack the typical features involved in Ca^{2+} and carbohydrate binding, which suggests a proteinaceous ligand (49). More recently, additional NK complex genes of unknown function, such as *KLRF1* (50) and *LLT1* (51), have been reported.

We have recently linked another lectin-like gene, *LOX-1* (52), to the NKG2/CD94 NK receptor genes (4). *LOX-1* is a receptor expressed on myeloid and endothelial cells and binds oxidized low density lipoprotein (oxLDL) ligands (53). As such it has scavenger function and was proposed to be involved in the atherosclerotic process (54). Close to *LOX-1* we have detected three further genes, *CLEC-1*, *CLEC-2* (55) and a novel human lectin-like gene (Y. Sobanov, A. Bernreiter, S. Derdak, D. Mechtcheriakova, M. Döchler, F. Kalthoff, E. Hofer, manuscript submitted) most closely related to murine *Dectin-1* (56). Whereas *CLEC-2* appears to be more widely expressed, *CLEC-1* and especially *DECTIN-1* are preferentially expressed in dendritic cells. They contribute to the number of lectin-like genes with potential important function in dendritic cells, such as the previously detected *DCIR* (57), which is located on the distant telomeric side of the NK complex.

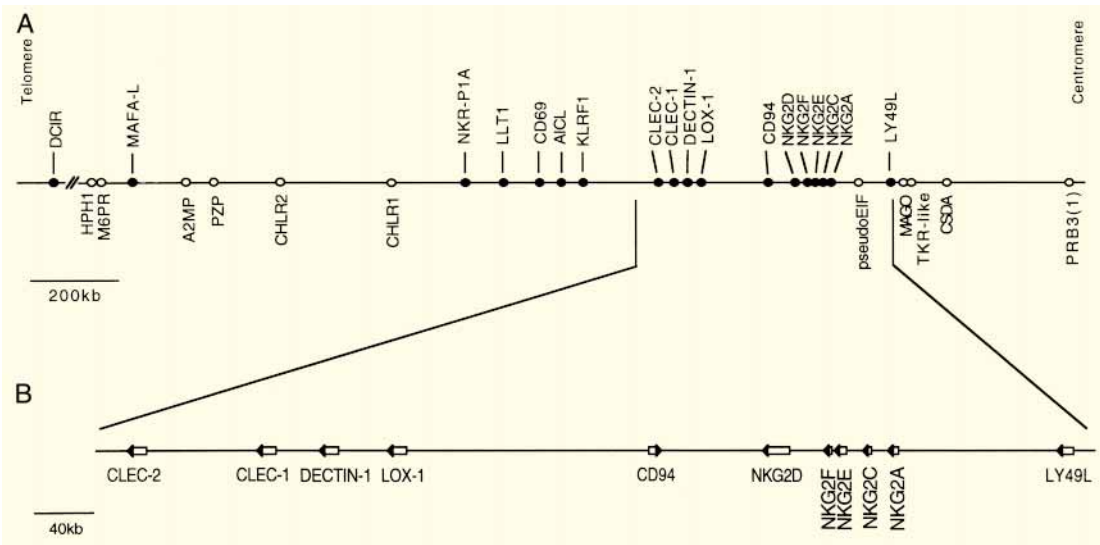


Fig. 1. Organization of the human NK receptor complex on chromosome 12. A. Physical map of the 2.5 Mb region extending from the DCIR gene on the telomeric side to the PRB gene cluster on the centromeric side. B. Linkage and orientation of the lectin-like genes in the centromeric part of the NK receptor gene complex. In the telomeric part, DCIR and MAFA-L are separated by a region containing several unrelated genes from a cluster of lectin-like genes containing NKR-P1A, LLT-1, CD69, AICL and KLRF1. In the centromeric part, a recently detected cluster of four genes consisting of CLEC-2, CLEC-1, DECTIN-1 and LOX-1 (4) (Y. Sobanov, A. Bernreiter, S. Derdak, D. Mechtcheriakova, M. Döchler, F. Kalthoff, E. Hofer, manuscript submitted) is followed by the previously analyzed cluster of the CD94

and NKG2 NK receptor genes (3, 58). No additional lectin-like gene is found centromeric of the single human LY49L pseudogene, but the region between LY49L and PRB3 again contains several unrelated genes. The map of the telomeric part was constructed based on the data of Renedo et al. (2) and partial sequence information available from the human genome project (<http://sequence.aecom.yu.edu/chr12/>). Linkage, orientation and distance between the genes in the centromeric part were delineated from our mapping and partial sequencing efforts (3, 4, 61) (Y. Sobanov, A. Bernreiter, S. Derdak, D. Mechtcheriakova, M. Döchler, F. Kalthoff, E. Hofer, manuscript submitted), the data of Plougastel et al. (94) and partial sequence information available from the human genome project.

In this review, we summarize data on the structure of the centromeric part of the human NK receptor complex, including the CD94 and NKG2 genes. We discuss the obvious absence of functional Ly49 genes and the presence of a novel family of genes expressed in myeloid, dendritic and endothelial cells, which seem to be related to the NK receptor genes by similarity of their CTLDs and by their close genomic localization, thus suggesting co-evolution. Furthermore, we show the extension of the complex and briefly discuss the additional lectin-like genes in the telomeric part and the unrelated genes in the border regions.

The CD94 and NKG2 NK receptor genes

The human NKG2 gene family comprises five members. Seven transcripts have been reported, referred to as NKG2A, B, C, D, E, F and H (40, 58), with A/B and E/H being splice variants of the same genes (see Fig. 2). Although occasionally generated via splicing from a transcript reaching from NKG2F to NKG2D, NKG2D is only remotely related to the other NKG2 family members. Structurally and functionally it constitutes a

more separate form of lectin-like NK receptor. We have previously characterized gene structure and genomic organization of the human NKG2 members (58). We found NKG2A, C, E and F to be closely linked (as listed) and of the same transcriptional orientation, with distances of 5 to 10 kb between the individual genes (Fig. 1). The NKG2D gene is located on the 3' side of the NKG2F gene, about 20 kb away and is of identical transcriptional orientation. The CD94 gene is about 50 kb telomeric of NKG2D and of opposite transcriptional orientation. The close linkage of all six genes within a genomic region of about 150 kb and the identical transcriptional orientation of the NKG2 genes is reminiscent of other genomic regions under co-ordinate control during differentiation, such as the globin gene cluster (59, 60). It appears possible that a common locus control region allows NK and T-lymphocyte-specific transcription and is activated during differentiation of these cells. Alterations in expression of the genes between subclones of NK and NK/T cells could then be controlled by the individual promoter regions of the genes.

To identify potential regulatory gene regions, we analyzed the DNA sequences extending 3 kb upstream of the first cod-

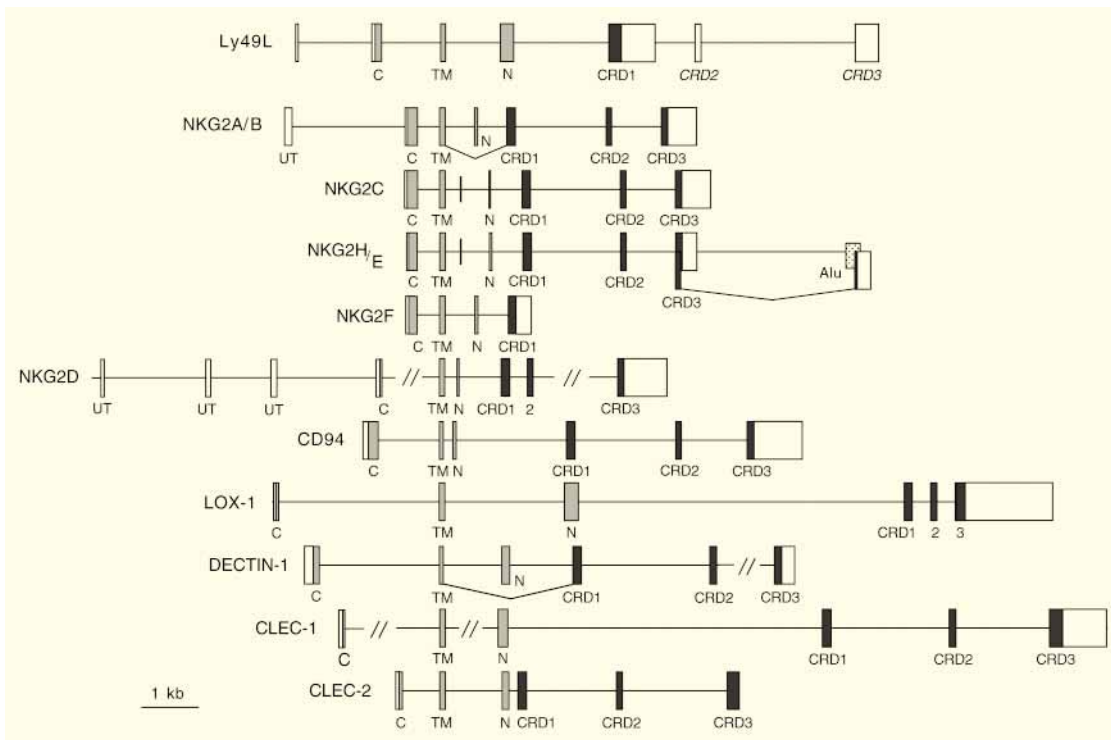


Fig. 2. Exon–intron structures and splicing variations of lectin-like genes of the NK gene complex. The exon–intron structures for the lectin-like genes of the centromeric part are displayed. While all genes are divided in exons containing a cytoplasmic, transmembrane, neck and three CTLD domains, there are some variations in transcription start sites and splicing. Some genes are transcribed preferentially from an upstream promoter preceding a 5′-untranslated exon, whereas some appear to be transcribed from promoters preceding the cytoplasmic

exon. Splicing variations include the partial omission of the stalk exon and the activation of cryptic splice sites for some transcripts leading to either the addition of unrelated amino acid sequences to the C-terminal end or to terminal truncation of the proteins. Exon and intron sizes are drawn to approximate scale when corresponding information was available from published data (52, 58, 94) or the human genome project. Observed splicing variations are indicated by connecting V-shaped variations in the genes.

ing exons of NKG2A, C, E and F (61). NKG2A contains a 5′ upstream untranslated exon not detected in transcripts of the NKG2C, E and F genes (58, 62). Comparable untranslated exons are observed in the murine *Ly49* genes (63, 64). The potential promoter regions of the NKG2 genes revealed substantial homology among all family members. The corresponding regions of NKG2C and E were 98% identical to each other and 75% identical to the NKG2F region. NKG2A differed to a greater extent, showing somewhat closer homology to NKG2F (60%) than to NKG2C and E (57%). All four upstream gene regions show a characteristic pattern of repetitive element insertion. NKG2A shows a single Alu insertion about 200 bp upstream of the start codon in the proposed region of transcriptional initiation of NKG2C, E and F. NKG2F has a unique Alu insertion at the site corresponding to the NKG2A promoter, and all three activating receptor genes lack a 57 bp element of the region of NKG2A transcript initiation. These structural data support the proposal of a preferential use of separate promoter regions for the inhibitory NKG2A receptor

gene and the activating receptor genes as outlined below. The occurrence of a common Alu Jb insertion at about −3,000 bp for NKG2C, E and F and of a mammalian-wide interspersed repeat (MIR) element at about −1,000 bp in all four genes is in line with the evolution of all four genes by consecutive gene duplications. The duplication of a precursor gene containing the MIR element most likely gave rise to NKG2A and a precursor gene for NKG2C, E, and F and was followed by an Alu insertion at −3,000. A further duplication step likely led to the generation of NKG2F and a gene that very recently duplicated to give NKG2C and E.

The exact identification of the transcriptional start sites and characterization of the relevant promoters of the various genes has turned out to be tedious because of the very low transcription rates of the genes. By primer extension analysis and 5′ RACE mapping, most NKG2A transcripts were found to initiate within a region of 100 bp at a distance of about 2.2 kb from the ATG translation initiation codon, and the 5′-untranslated exon is subsequently spliced at −2,042/−31

(61, 62). Since transcript levels for the triggering *NKG2C*, *E* and *F* genes were over 10-fold lower in the cell lines available for the analysis, any determination of transcript start sites had to rely on a combination of 5' RACE and computational predictions. All evidence obtained by these methods point to a transcription start site for *NKG2C*, *E* and *F* at $-186/-188$ with respect to the ATG in a region corresponding to *NKG2A* intron 1. No indication of an upstream untranslated exon could be obtained, suggesting the preferential use of distinct promoter regions for the inhibitory *NKG2A* and the triggering *NKG2C*, *E* and *F* genes (61). Similarly, in the case of human *CD94*, no 5'-untranslated exon has been identified, and in the absence of a functional TATA box multiple start sites from a promoter preceding the first translated exon have been described (65). *NKG2D* transcripts are unique in that they can contain three upstream untranslated exons, which are sporadically fused to *NKG2F* exon 4. This suggests the existence of primary transcripts originating at *NKG2F* and reaching beyond 20 kb to the *NKG2D* exons (58). No data are currently available on a potential promoter immediately preceding the *NKG2D* exons.

Development of an NK-like phenotype, i.e. *de novo* expression of *CD94/NKG2* receptors, has been reported to be induced in thymic precursor cells or T-cell clones by cytokines such as interleukin (IL)-2, IL-15 and transforming growth factor (TGF)- β (66, 67). On the other hand, in the established NK-cell lines *NK92* and *NKL*, transcript levels for *CD94* and *NKG2* proteins seem to be upregulated and cannot be further modulated by a large series of cytokines tested (61). Thus, the understanding of transcriptional regulation of the *NKG2* genes will need experiments with NK precursor clones and cellular differentiation systems. In this context it is of interest to mention available evidence for clonal expression of the *NKG2* isoforms. Our recent data using quantitative real time RT-PCR show that, whereas *CD94* and *NKG2D* seem to be similarly expressed in all NK clones tested, certain individual clones can express large levels of either inhibitory *NKG2A* or triggering *NKG2C*, *E*, *F* transcripts. This points towards differential high expression of triggering or inhibitory forms of *NKG2* in certain clones and suggests functional specification of subclones (C. Brostjan, T. Bellon, M. López-Bottet, E. Hofer, in preparation). It is likely that this differential expression is controlled by the promoter regions of the different genes (61, 65); however, the functional significance of the numerous predicted transcription factor binding sites in the promoters will have to be determined in future transcriptional studies.

Our previous work has further analyzed the exon-intron

structures of the *NKG2* genes. The exon-intron structure principally follows the domain pattern observed for all C-type lectin-like receptors with a type II transmembrane orientation. An exon containing the translation initiation codon and encoding the N-terminal cytoplasmic domain is followed by a transmembrane exon and an exon coding for the so-called stalk or neck domain of the receptor, which presumably provides a flexible connection to the ligand-binding CTLD. The CTLD in *NKG2A*, *C*, *D* and *CD94* is encoded by the three exons typically observed for most C-type lectin-like receptors. However, frequent splicing variations are observed for the transcripts of the NK receptor genes (Fig. 2). This feature is shared by some other lectin-like genes of the NK receptor complex and is also seen in rodent equivalents and the *Ly49* genes (68). One example is the occurrence of the *NKG2B* transcript which is identical to *NKG2A* with exception of the stalk exon that is missing. In this case it was shown that the *NKG2B* protein is still functional in terms of HLA-E binding, since the CTLD part is intact (30). A similar stalkless transcript was also detected for the murine homolog (69). Within intron 2 of the *NKG2C* and *E* genes, a short duplicated piece of DNA contains a second version of the stalk exon. This version, if alternatively spliced, would lead to a stalk peptide with one amino acid change, but this transcript has not been observed so far. Furthermore, the *NKG2E* gene can give rise to two transcripts differing in the 3' parts. In the more frequent transcript an additional splice event connects a cryptic 5'-splice site within the third CTLD exon to a splice acceptor site in an Alu-type repeat several kb downstream. This Alu repeat occurs only in the *NKG2E* gene and this splice variation gives rise to an *NKG2E* protein that has the last 17 amino acids derived from the Alu sequences. The less frequent transcript, termed *NKG2H* (70), does not use this splice site, and the C-terminal end of the *NKG2H* protein is therefore encoded completely by the third CTLD exon. In the *NKG2E* as well as in the *NKG2H* protein, the last cysteine in the CTLD, absolutely conserved among all other NK receptor complex members, is missing (Fig. 3). Despite these variations, evidence for the functioning of both isoforms have been obtained (70). In contrast, the *NKG2F* protein is predicted to be truncated on its 3'-end. About 100 bp after the beginning of the first CTLD exon, an insertion of two nucleotides leads to a shift of reading frame and a stop codon 20 nucleotides before the splice donor site used in the *NKG2C* and *E* genes. *NKG2F* is thus only 158 amino acids long and lacks the entire second and third CTLD domains. Whereas it is likely that the variant *NKG2B* and *NKG2E* transcripts give functional proteins (30, 71), and the variation at the C-ter-

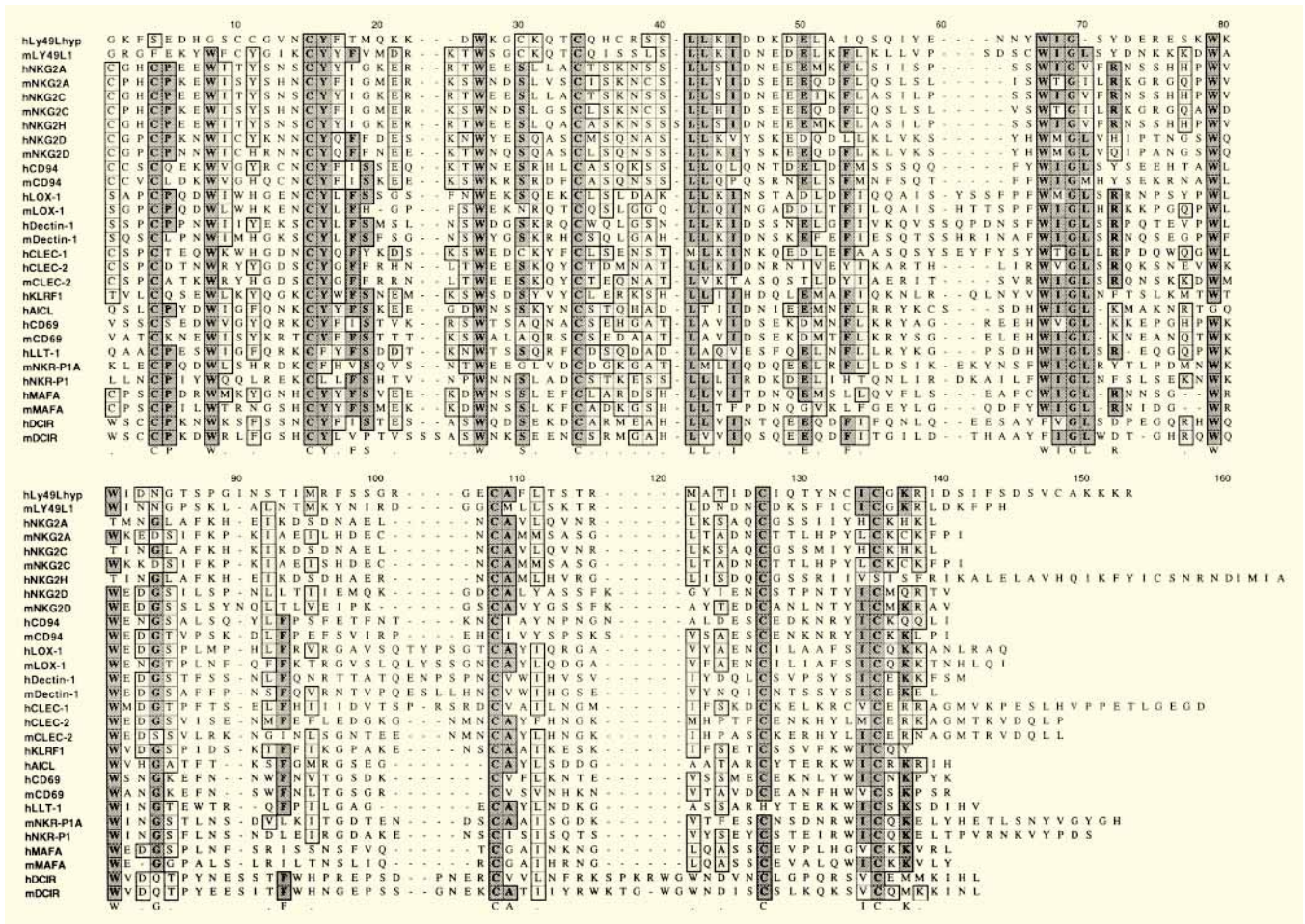


Fig. 3. Alignment of the CTLDs of the lectin-like receptors. The CTLDs of all lectin-like receptors encoded in the human NK receptor complex and, if available, of their murine homologs were aligned using the ClustalW algorithm of the McVector version 6.5 software (Oxford Molecular, Inc.) with the parameters open gap penalty and extend gap penalty set to 7.0 and 1.0, respectively. Peptides were chosen to start three amino acids before the first cysteine conserved in the CTLDs of all receptors besides the Ly49. The hLY49hyp sequence shows the

hypothetical sequence translated from the three CTLD exons present in the human gene and ignores the observed defective splicing. Highly conserved residues are displayed in dark shaded boxes and as consensus amino acids below the alignment. Positions with similar amino acids in most receptors are shown in light shaded boxes and are indicated by dots below. Human and murine receptors are indicated by h and m respectively.

minimal end of NKG2E could result in modified binding properties, it appears unlikely that *NKG2F* encodes a ligand-binding receptor, although a function in the activation of NK cells cannot be excluded.

The combined data on the genomic structures of the *NKG2A*, *C*, *E* and *F* genes support a history of subsequent gene duplications, some of which have occurred more recently in evolution. The data are in accordance with the idea that recent modifications of the triggering *NKG2* isoforms led to three closely related genes as well as transcript variants. It is tempting to speculate that this might be connected to their potential function as triggering receptors and changing ligands such as viral peptides in a complex with HLA-E. It is further

conceivable that allelic differences may exist in the *NKG2* and *CD94* genes, even though little evidence has been reported so far. The prevalence of allelic differences remains to be established.

Based on their chromosomal arrangement and a sequence comparison of the different CTLDs of the *CD94*, the *NKG2D* and the other more closely related *NKG2* genes, it is evident that, although *CD94* and *NKG2D* are relatively far diverged from the others, all these genes belong to a distinct subgroup of lectin-like receptor genes with a common evolutionary history (Fig. 4). It is interesting to note further that there is an exceptional similarity of the CTLD between the *NKG2A* and *NKG2C* genes in different species, such as human, macaque,

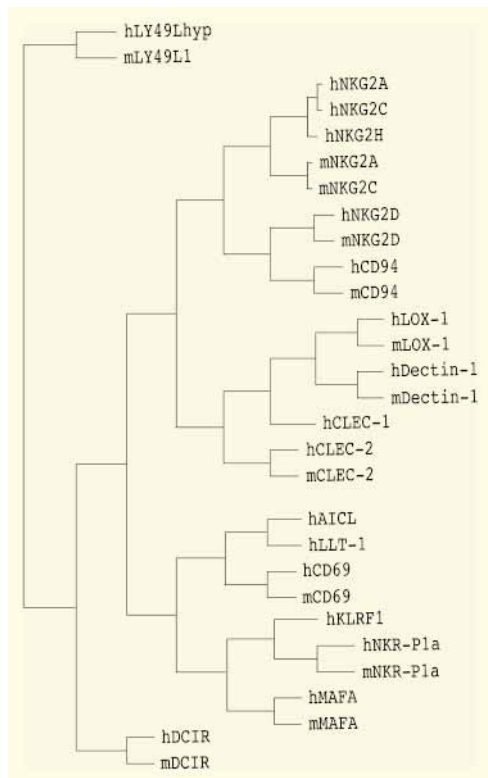


Fig. 4. Phylogenetic tree of lectin-like receptors based on CTLD sequences. The sequence alignment displayed in Fig. 3, but omitting the highly variable C-terminal amino acids past the last conserved cysteine, was used to construct a phylogenetic tree using the UPGMA algorithm of the GCG software version 8.1 (Genetics Computer Group, Inc.). The various CTLDs of the receptors display a relationship largely according to their clusterwise arrangement within the NK receptor complex. Human (h) and murine (m) homologs of the various receptors are displayed.

rat and mouse (Fig. 5). This suggests that NKG2A and C genes may maintain similarities by concerted evolution in the different species, possibly involving mechanisms of gene conversion as previously suggested (72). It also supports the finding of very similar ligands for both receptors such as HLA-E/peptide complexes (30).

The region centromeric of NKG2 contains a single *LY49* gene

Given the presence of more than 14 *Ly49* genes between the NKG2 genes and the *Prp* locus in mice (9, 68), it was of interest to search the syntenic human region for the potential presence of genes related to the rodent *Ly49* genes. Since the same region in mice further contains the viral resistance loci *Cmv1* and *Rmp1* between the known *Ly49* genes and *Prp* (12, 13), additional lectin-like or possibly unrelated genes important for NK-cell function are proposed to be located in this

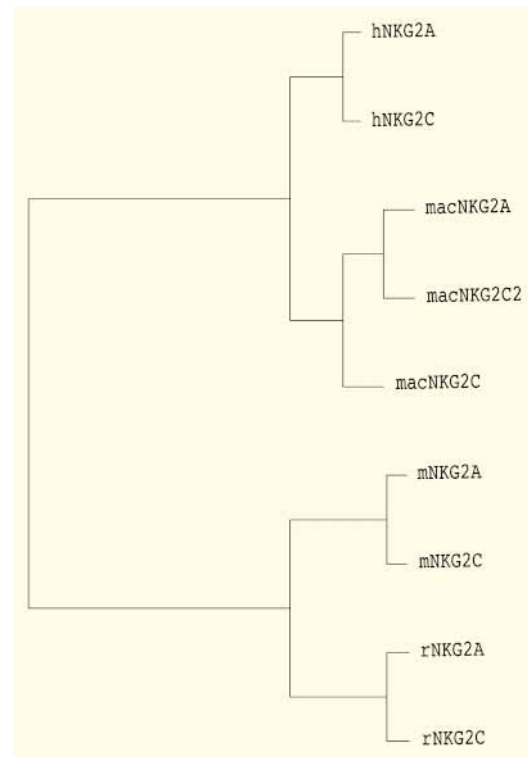


Fig. 5. Phylogenetic tree of NKG2A and NKG2C proteins from different species based on CTLD sequences. Sequence alignment and tree construction was performed as described in legends to Fig. 4 using the available NKG2A and NKG2C sequences available for the corresponding human (h), macaque (mac), mouse (m) and rat (r) proteins. In all four species NKG2A and NKG2C receptor chains retained high similarity to each other, suggesting mechanisms of gene conversion acting on the genes.

part of the rodent NK gene complex and could also be present in humans. Alternatively, since the known *Ly49* receptors recognize MHC class Ia molecules and the virus resistance in mice could be mediated by *Ly49* isoforms recognizing MHC class Ia or related proteins, comparable functions might be carried out in humans by the KIR encoded on chromosome 19 rather than by lectin-like receptors. In either case, analysis of the region centromeric of NKG2 and comparison to the syntenic rodent regions is expected to give some important answers to the extension and evolution of the NK receptor complex.

We have therefore analyzed the area using a PAC contig encompassing the genomic human region between NKG2A and *PRB3* (4). This region was estimated by us to be about 0.7 Mb, which is substantially smaller than the comparable region in mice, suggested to be about 3 Mb (12). While this work was in progress, the finding of a *Ly49*-like transcript in a human expressed sequence tag (EST) database by Westgaard

et al. (73) showed that at least one *Ly49* gene was present in humans. The corresponding human gene, termed *hLY49L* because of its closest homology to the murine *Ly49L1* gene, was localized by us about 100 to 200 kb centromeric of *NKG2A* (Fig. 1) in the PAC contig (4). The analysis of the gene and of several cDNA sequences isolated from NK and fetal cDNA libraries showed that this gene in fact gives only defective mRNA and protein products. The only detectable transcripts were aberrantly spliced using a cryptic 5'-splice site in the intron between CTLD exons 1 and 2 (Fig. 2). These transcripts encode proteins with shorter unrelated C-terminal peptide sequences replacing the second and third CTLD. For this reason it is to be assumed that the *LY49L* protein translated from this transcript will not be functional. A closer inspection of the respective exon/intron boundaries revealed that in all murine *Ly49* genes a potentially crucial G nucleotide is absolutely conserved at the very 3'-end of the first CRD exon, whereas a C is found in *hLY49L*. Since involvement of the 5' and 3' nucleotides of exons has been postulated, it can be speculated that this silent mutation interferes with proper 5'-splice site recognition (4). However, in view of the fact that i) the *hLY49L* gene is of substantial similarity to the rodent *Ly49L1* and ii) the sequences of the second and third CTLD domain present in the gene do not show any further abnormalities (Fig. 3), it is likely that the gene served an important function until recently in evolution. Alternatively, it cannot be excluded that the truncated protein still has some function via its functional cytoplasmic signaling domain or that so far undetected levels of correctly spliced products can be produced in certain cell types.

In addition to searching several NK and fetal cDNA libraries, we have further tested the PAC contig between *NKG2A* and *PRB3* for the presence of potential human *LY49* genes by homology screening using probes from conserved regions of the rodent *Ly49* and the human *Ly49L* genes. Furthermore, a minimal contig of the region was used for exon trapping in collaboration with E. Dissen and S. Fossum (Oslo). However, no additional lectin-type gene could be detected with any of the probes or by exon trapping. Similarly, no further lectin-like sequence is detected in the BAC sequences available from the human genome project. In view of the significantly smaller size of the human when compared to the rodent region between *NKG2* and the cluster of genes for the proline-rich proteins, it has therefore to be assumed that this human region never developed to the same complexity observed in rodents. It is likely that the *hLY49L* gene constitutes the equivalent of a primordial *Ly49* gene existing before the human to rodent separation. Subsequently, the *Ly49* gene cluster de-

veloped in rodents to the complexity seen today, whereas the same function developed in humans through the evolution of the *KIR* gene locus on chromosome 19, which again appears to have no comparable equivalent in rodents. Alternatively, it is also possible that a pre-existing *Ly49* gene cluster was deleted in humans when the function had been sufficiently replaced by the *KIR* genes. Thus the development of the *Ly49* and *KIR* genomic regions is the rare example of convergent evolution leading to the execution of a similar function by structurally different molecules in rodents and humans. This points towards a relatively rapid and recent evolution of the *Ly49* and *KIR* NK receptor genes.

Whereas so far no further lectin-like gene has been detected in the region between *hLY49L* and *PRB3*, several structurally unrelated genes were identified by our mapping analysis and from data recently released from the human genome project. However, before the availability of the complete sequence of the region and a further characterization of the murine genes mediating viral resistance, it is hard to judge whether any of these genes could potentially contribute to viral resistance.

A gene encoding a receptor for oxidized low density lipoprotein (oxLDL) is localized in the NK receptor complex

Analyzing the region telomeric of *CD94*, we localized the lectin-like oxLDL receptor gene (*LOX-1*) just within 100 kb of the sequence-tagged site marker *D12S77* (4), i.e. about 200 kb telomeric of *CD94*. *LOX-1* was originally identified as an endothelial receptor for oxLDL (53). It is a protein homologous to the C-type lectin-like receptors with a type II transmembrane orientation and has no similarity to any of the known oxLDL-binding scavenger receptors. The corresponding *LOX-1* gene spans a chromosomal region of about 15 kb and displays the usual exon/intron structure of the C-type lectin-like genes (Fig. 2): the cytoplasmic domain, neck domain and transmembrane domain are encoded by single exons, while the CTLD is encoded by three exons (52).

LOX-1 has been found to be expressed by vascular endothelial cells and macrophages. It is dynamically regulated *in vivo* by inflammatory stimuli such as tumor necrosis factor- α , lipopolysaccharide and oxLDL, but also by TGF- β , angiotensin II and by fluid shear stress in hypertension. A number of potential transcription factor binding sites were found in the 5'-flanking region of the *LOX-1* gene, including two *GATA-2*-binding sites and one *c-ets-1* site, which might be involved in regulating expression of *LOX-1* (74). In addition,

several phorbol 12-myristate 13-acetate (PMA)-responsive elements and a shear stress-responsive element were identified as control elements required for the observed upregulation by phorbol esters and hypertension, respectively.

Importantly, LOX-1 has been found to be upregulated in atherosclerotic lesions, where it is believed to contribute to foam cell formation and endothelial dysfunction. It was described to bind oxLDL and anionic phospholipids as well as aged red blood cells and apoptotic cells (75). One of the earliest events in aged or apoptotic cells is the exposure of phosphatidylserine on the outside of the plasma membrane, which is recognized and bound by LOX-1. It has therefore been proposed that LOX-1 is involved in the removal of those cells from the bloodstream. In contrast to the signaling events initiated upon ligand binding of other lectin-like receptors such as the CD94/NKG2 receptors, molecules and cellular fragments bound to LOX-1 are internalized by endocytosis.

A family of lectin-like genes expressed in myeloid, dendritic and endothelial cells

Whereas our search centromeric of the NKG2 genes did not detect additional lectin-like genes, the telomeric side revealed several lectin-like genes represented in various EST databases. Next to LOX-1, three additional genes are localized in this area. These appear to form a distantly related family together with the LOX-1 gene. This is suggested by their occurrence within a region of 200 kb and by the similarity of their CTLD domains (Fig. 3). The first gene telomeric of LOX-1 is the human version of the murine *Dectin-1* gene. The murine cDNA has recently been isolated by a differential screen and described by Arizumi et al. (56). hDECTIN-1, like its murine counterpart, is transcribed abundantly in dendritic cells only (Y. Sobanov, A. Bernreiter, S. Derdak, D. Mechtcheriakova, M. Döchler, F. Kalthoff, E. Hofer, manuscript submitted). The other two genes are named CLEC-1 and CLEC-2. Their corresponding cDNAs have recently been identified in EST databases and described by Colonna et al. (55). CLEC-1 mRNA was shown to be preferentially expressed in dendritic cells and was also found in placenta, lung and thymus. CLEC-2 mRNA was also detected in dendritic cells, but in addition showed wider expression in the hematopoietic lineage. CLEC-2 mRNA levels were especially high in liver.

Our analysis revealed that all three genes, hDECTIN-1, CLEC-1 and CLEC-2, occur within 150 kb on the telomeric side of LOX-1 and are of identical transcriptional orientation (Fig. 1). The exon-intron structures of the genes follow the

principal organization of the C-type lectin-like genes (Fig. 2). In the case of hDECTIN-1, the major transcript lacks the stalk exon, although it is present in the genomic sequence. This is likely due to a nucleotide change at the 5'-end of intron 3 from GT to CT. Preliminary evidence suggests, however, that the stalk exon is spliced in a minority of transcripts of dendritic cells (Y. Sobanov, A. Bernreiter, S. Derdak, D. Mechtcheriakova, M. Döchler, F. Kalthoff, E. Hofer, manuscript submitted). A similar G to C change has also been observed at the 5'-end of intron 4 in the CD94 gene; however in this case the splice is usually performed properly (65). In the absence of any available data about transcriptional initiation, we currently assume in analogy to the LOX-1 gene (52, 74) that transcription may start in front of the exon containing the cytoplasmic domain. However, the presence of an upstream untranslated exon cannot be ruled out at the moment.

Our recent data have further shown that hDECTIN-1 mRNA can be detected at a low level in primary monocytes but is expressed at high levels only in primary dendritic cells. CLEC-1, on the other hand, when compared to hDECTIN-1, is expressed at low levels in dendritic cells, but appears to be transcribed in endothelial cells as well. This is similar to the LOX-1 gene, which has been reported to be expressed in endothelial and myeloid cells.

It is of further interest that a closer inspection of the cytoplasmic tail of hDECTIN-1 shows the presence of an ITAM-like sequence (Y. Sobanov, A. Bernreiter, S. Derdak, D. Mechtcheriakova, M. Döchler, F. Kalthoff, E. Hofer, manuscript submitted). Taking into consideration the finding that mouse *Dectin-1* was reported to bind to surface molecules on T cells, a role for DECTIN-1 in the control of T-cell activation can be envisioned. CLEC-1 also contains tyrosine residues in the cytoplasmic tail, which might be involved in signaling; however, no clear known activating or inhibitory motif can be recognized. It remains to be seen which functions might be exerted by CLEC-1 in dendritic cells and endothelial cells. It is noteworthy that neither CLEC-1 nor the stalkless hDECTIN-1 is transported to the cell surface upon overexpression of the recombinant proteins, suggesting that they require the association with an heterologous protein chain for functional surface expression similar to the NKG2 proteins (55) (Y. Sobanov, A. Bernreiter, S. Derdak, D. Mechtcheriakova, M. Döchler, F. Kalthoff, E. Hofer, manuscript submitted).

A comparison of the CTLD domains reveals that all four genes, LOX-1, hDECTIN-1, CLEC-1 and CLEC-2, are more similar to each other than to the NKG2 and CD94 NK receptor genes, suggesting that they form a separate subfamily of lec-

tin-like receptors (Fig. 4). Taken together, the similarities in sequence, the similarities in cell type expression, the close chromosomal linkage and their identical transcriptional orientation argue strongly that all four genes form a distinct subfamily of lectin-like genes in the NK receptor complex with specialized functions in myeloid cells, dendritic cells and/or endothelial cells. Furthermore, these findings increase the number of related or identical genes expressed by myeloid and endothelial cells, suggesting several shared functions, and supports the relatedness of endothelial cells to the hematopoietic lineage (76). It will be interesting to evaluate whether these novel receptors will have peptide ligands such as the NK receptors or whether they would share the property of oxLDL and apoptotic cell binding with the LOX-1 receptor.

Additional lectin-like genes in the telomeric part of the NK receptor complex

Several additional lectin-like genes have been mapped to the short arm of chromosome 12 further telomeric of CLEC-2. Part of these lectin-like genes have been identified recently by searching human EST databases with consensus sequences common to known C-type lectin-like receptors. The genes in the telomeric part include killer cell lectin-like receptor family gene 1 (KLRF1) (50), AICL (46), CD69 (47), lectin-like transcript-1 (LLT-1) (51), NKR-P1 (7), mast cell function-associated antigen-L (MAFA-L) (77) and dendritic cell immunoreceptor (DCIR) (57) (see Fig. 1). The CD69 (47) receptor is one of the first described members of the NK receptor complex family. It is expressed on the surface of most hematopoietic lineages where it represents one of the earliest activation markers. Although its ligands are not known so far, cross-linking of CD69 by specific antibodies has been shown to activate cell-specific functions of lymphocytes, granulocytes, monocytes and platelets (78).

In short term-stimulated lymphocytes, activation-induced C-type lectin (AICL) mRNA is highly expressed. Also identified by an EST search (46), AICL shows highest similarity with CD69 (56%). Its gene maps proximal to the CD69 gene, and its RNA is rapidly upregulated by PMA with a transcriptional kinetic similar to CD69 mRNA. Although no AU-rich destabilizing elements have been identified in the 3'-untranslated region, mRNA turnover is regulated by fast degradation of the highly unstable mRNA. AICL expression was found in addition to lymphocytes in monocytes and granulocytes. Taken together, AICL seems to constitute another broadly expressed activation antigen similar to CD69.

Comparably, LLT-1 was identified in an EST database search

(51). The LLT-1 protein shows highest similarity in the CTLD to AICL (67%) and CD69 (57%), and its gene is localized between NKR-P1 and CD69. In Northern blots, multiple bands were obtained, which might indicate splice variants of the LLT-1 mRNA. LLT-1 mRNA is expressed at high levels in NK cells and to a lesser extent in T and B cells.

Another CTLD receptor identified recently by screening a human EST database is KLRF1 (50). The KLRF1 gene is located between AICL and CLEC-2 and shows the same genomic structure as the other lectin-like genes in the NK receptor complex. Based on sequence comparisons it seems likely that the CTLD of KLRF1 probably does not bind carbohydrates or Ca²⁺. Its cytoplasmic domain contains two ITIM-like sequences, and no charged residue is found in the transmembrane domain. Therefore, an inhibitory role is likely for the KLRF1 receptor. Two alternatively spliced forms of KLRF1 mRNA have been identified, the shorter of which lacks the stalk region. KLRF1 mRNA was found to be expressed in activated NK cells, monocytes and myeloid cell lines.

NKR-P1 was the first lectin-like receptor detected on rat NK cells (79). The gene for a human equivalent, which was first described by Lanier et al. (7), is found telomeric of LLT-1. The human NKR-P1 gene is expressed not only in NK cells but also in part of CD4⁺ T lymphocytes as well as in resting monocytes and dendritic cells. It was shown to mediate upregulation of IL-1 and IL-12 in monocytes and dendritic cells and was suggested to be involved in transendothelial migration of resting CD4⁺ T lymphocytes.

MAFA was identified in the rat by its ability to inhibit mast cell degranulation upon binding of IgE to FcεRI (80). Contrary to the expression of Ly49, MAFA is downregulated in MHC class I-deficient mice (81). MAFA-L is the human homolog of rat MAFA (54% identity). Its gene maps to the most telomeric part of the NK receptor gene complex and is separated by an over 400 kb long region containing some genes unrelated to the lectin-like genes described above. The MAFA-L receptor contains an ITIM sequence motif in its cytoplasmic domain (77). It is expressed in lung mast cells and basophils, as well as in NK cells from peripheral blood, but not in decidual NK cells or in T cells. Similar to some other lectin genes, different mRNA variants were identified which might represent alternatively spliced forms (82).

DCIR was also recently identified by a genome search using a sequence motif conserved among C-type lectins (57). Its sequence shows highest homology to macrophage lectin, hepatic lectins and CD23. In the extracellular lectin domain, DCIR contains the amino acid residues that are critical for Ca²⁺ binding. The cytoplasmic tail contains an ITIM motif.

DCIR was found to be highly expressed in blood monocytes and granulocytes, and a low expression was seen in tonsillar B cells. No expression was detected in T or NK cells purified from blood, but monocyte-derived dendritic cells strongly expressed DCIR after 7 days of culture with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Several unrelated genes are detected in the border regions of the complex

Based on the currently available sequence and mapping data, the central region of the NK receptor complex seems to contain exclusively type-II lectin-like genes. In this region, extending for 1 Mb between NKR-P1 and *Iy49L*, only one processed pseudogene (pseudo-EIF) is found close to *Iy49L* in addition to the lectin-like receptors. Flanking this central region several non-lectin genes have so far been mapped. The function and expression of most of these seem to be not directly connected to the lectin-like receptors.

Starting at the most telomeric part of the described region, the first non-lectin gene detected is a human homolog of the polyhomeotic 1 (HPH1) gene. In *Drosophila melanogaster* the corresponding protein is part of a large multimeric, chromatin-associated protein complex, which plays a crucial role in the maintenance of the transcriptional repression state of Hox genes. This repressor is part of a cellular memory system that is responsible for stable transmission of gene activity to progeny cells (83).

A following gene encodes the Ca²⁺-dependent mannose 6-phosphate receptor, which is a dimeric transmembrane protein participating in the intracellular transport of lysosomal enzymes. It recognizes terminal mannose-6-phosphate on N-linked oligosaccharides in newly synthesized proteins in a pre-Golgi department; the proteins are then redirected from the secretory to the lysosomal targeting pathway (84).

The next lectin-like gene is *MAFA-L*, which is followed by the α 2-macroglobulin (α 2M) and the pregnancy zone protein (PZP) genes. α 2M and PZP proteins share 70% sequence identity and are functional homologs. They are potent proteinase inhibitors exhibiting a unique trapping mechanism (85, 86). Proteinase cleavage of a peptide bond in the bait region of α 2M or PZP leads to a conformational change, whereby the protease is entrapped and covalently linked to α 2M/PZP. A receptor recognition site thus becomes exposed, allowing rapid clearance of the α 2M-protease complex from the circulation. α 2M is always present in high concentrations in circulation, whereas PZP is the prevalent pregnancy-associated plasma protein.

Two genes, *CHLR1* and *CHLR2*, located between PZP and the lectin-like receptor NKR-P1, code for helicases. Helicases transiently destabilize the hydrogen bonds between complementary nucleic acid strands, which is critical for multiple cellular processes, e.g. DNA replication or transcription (87). Many different helicases have been identified, which all share seven conserved domains. *CHLR1* and *CHLR2* appear to be the human counterparts of the yeast *CHL1* gene.

The ensuing array of lectin-like receptors between NKR-P1 and *NKG2A*, which extends for about 1 Mb, seems to be uninterrupted by other genes. A complete cDNA copy for the eukaryotic translation initiation factor 2 γ subunit (88) was then found between *NKG2A* and *IY49L*, which is the most centromeric member of the lectin-like genes detected. Furthermore, adjacent to *IY49L* at the end of the human NK receptor complex, several additional unrelated genes are found. The first is a gene that shows homology to *mago nashi*, a protein required for normal germ plasm development in the *Drosophila* embryo (89). Interestingly, a so far uncharacterized putative tyrosine kinase receptor gene (TRK-like) maps close to this region, followed by the gene for cold shock domain protein A (CSDA). CSDA is a DNA-binding protein which seems to act as a repressor e.g. in the strictly regulated transcription of GM-CSF (90).

Finally, the NK receptor complex is flanked by the at least 700 kbp spanning the proline-rich protein (PRP) gene cluster, which is also found on mouse chromosome 6 at the corresponding position (9). PRPs are the major protein constituents of saliva; their amino acid sequence shows a predominance of proline, glycine and glutamic acid/glutamine (91). At least 11 different PRPs have been described, which seem to be encoded by six genes. Differential RNA splicing and post-translational proteolytic cleavage is thought to generate multiple protein products from various PRP genes.

Concluding remarks

The current review presents an analysis of the genes in the human NK receptor complex with a special focus on the centromeric half of the complex based on our previous mapping and sequencing data (3, 4, 58) and on partial sequence information from the human genome project (<http://sequence.aecom.yu.edu/chr12/>). The centromeric part is of particular interest since it contains the CD94 and NKG2 NK receptor genes, the region syntenic to the rodent complex containing the *Iy49* gene family and viral resistance loci (9, 12). It also contains a family of additional genes expressed in myeloid, dendritic and endothelial cells, which have only recently be-

come known (52, 55) (Y. Sobanov, A. Bernreiter, S. Derdak, D. Mechtcheriakova, M. Döchler, F. Kalthoff, E. Hofer, manuscript submitted).

Overall, it appears that the lectin-like genes of the NK gene complex evolved by gene duplication from a primordial gene encoding a C-type lectin domain; this most likely contained a lectin fold binding Ca^{2+} and corresponding carbohydrate ligands, such as the hepatic asialoglycoprotein receptor (1). During several rounds of gene duplications and modifications the carbohydrate recognition domain finally evolved to fold independently of Ca^{2+} and was used as a modular structural element in the various receptors of the NK receptor complex to bind a wide variety of different ligands, including peptides and oxLDL. Several genes and gene families with distinct binding properties and cellular expression pattern developed. Generally, the similarity between the receptors is most pronounced in the three CTLDs, whereas a similarity between the cytoplasmic domains is barely detectable for the more distant members, suggesting that many of the receptors acquired widely differing cytoplasmic signaling functions. Some parts of the NK receptor complex appear to have changed more recently in evolution. This is exemplified by several genes that produce protein products with altered or truncated C-terminal ends and which presumably duplicated recently, such as the triggering NKG2 isoforms.

The CD94 and NKG2 genes constitute a crucial component in the analyzed part. Their protein products form an intricate system of receptors, which can only be exposed on the surface upon heterodimerization with the CD94 chain; in certain cases, an additional third chain such as the signaling adapter DAP12 is necessary (27, 92). The close linkage of these genes and the same transcriptional orientation of the NKG2 genes suggests co-ordinate regulation, possibly via a locus control region specific for NK and NK/T cells. The insertion of repetitive elements may have contributed to the change in transcriptional initiation and regulation between the activating NKG2 isoforms and the inhibitory NKG2A member (61). Besides the questions of why the activating and inhibitory forms appear to be co-expressed on some NK subclones and why certain clones selectively express high levels of either the inhibitory or activating forms, the control mechanisms that evolved to allow this kind of differential expression specific for subclones of NK and NK/T cells are important future areas of research. The triggering NKG2 genes seem to be under evolutionary pressure, since they originated from two recent duplications and display several possibilities for splicing variations. The potential generation of truncated protein products (NKG2F) or receptors that have an unrelated sequence added

to their C-terminal portions (NKG2E) could indicate that these genes are in the process of changing to acquire modified binding functions, as could be the case for activating receptors with a role in the recognition of viral or other parasite infection.

The region between the NKG2 genes and the PRB3 locus was found to be less complex in humans when compared to the rodent system. While in mice this region appears to span a distance of 3 Mb (12), the human part is estimated to be about 700 kb. In rodents it contains over 14 members of the Ly49 gene family encoding MHC class Ia receptors as well as the viral resistance markers *Cmv* and *Rmp*. In contrast, a careful analysis of the human part did not reveal any LY49 genes apart from LY49L, for which only defective transcripts have been detected and which thus most likely constitutes a pseudogene. We have been unable to detect further genes by a combination of hybridization protocols using reduced stringency and exon trapping procedures for a PAC contig established from the region (4). Furthermore, a close inspection of the partial sequences available from the human genome project, which by now covers at least 75% of the region, did not reveal any further relatives of Ly49. The obvious absence of further functional Ly49 genes suggests that this locus never developed to the complexity seen in rodents, or that a region containing these receptors was deleted in humans when they lost functional importance. It supports the hypothesis that the function of the rodent Ly49 genes resides in humans in the KIR genes of chromosome 19, as previously suggested (93). The notion that the function of specific MHC class Ia receptors has evolved in distinct gene complexes in rodents and humans is further independently corroborated by the findings that the KIR gene complex does not exist in mice in a form comparable to the human (37). Whereas no further Ly49 relatives were detected in humans, several genes encoding in part proteins with likely housekeeping or so far uncharacterized function can be found between NKG2-A and PRB3. It will need further data from the murine system to see whether homologs of any of these genes are also present on mouse chromosome 6 and might be connected to the *Cmv* and *Rmp* resistance loci or whether these loci represent certain forms of Ly49 genes and are not present in humans.

Opposite to the centromeric side, in the region flanking the CD94 and NKG2 genes on the telomeric side, a new family of receptors, expressed preferentially on myeloid, dendritic and/or endothelial cells, became known more recently. These genes can be grouped in one subfamily based on their similarities and chromosomal arrangement. This is reminiscent of the leukocyte receptor complex on chromosome 19, where

the KIR locus is similarly flanked by the cluster of related ILT genes; these are more widely expressed in leukocytes, and some seem to play a role in dendritic cells (28). Whereas ligands and function of CLEC-1, CLEC-2 and DECTIN-1 remain to be established, LOX-1 is preferentially expressed on macrophages and endothelial cells and has been reported to bind ligands such as oxLDL and phosphoserine. This constitutes an additional class of ligands for lectin-like receptors. DECTIN-1 is preferentially expressed in dendritic cells and to a lesser extent in monocytes, whereas CLEC-1 mRNA is found in dendritic cells, monocytes and endothelial cells. CLEC-2 is more widely expressed in the hematopoietic lineage (55) (Y. Sobanov, A. Bernreiter, S. Derdak, D. Mechtcheriakova, M. Döchler, F. Kalthoff, E. Hofer, manuscript submitted). Judging from the perspective of immunoregulation and based on the suggested co-evolution of the genes, it is conceivable that these lectin-like genes serve important functions in dendritic cells, comparable to the importance of the lectin-like NK receptors for NK-cell function. It remains to be seen what kind of ligands and functions can be attributed to these receptors.

At the moment we can only speculate on the function and relation of the non-lectin genes occurring within or in the border regions of the NK receptor complex. Whereas some apparently fulfill housekeeping functions and are unrelated in expression and function, others such as the TKR-like gene could possibly play a role in the growth and differentiation of hematopoietic lineages. However, before any conclusions can be drawn, the cell type-specific expression and functional data on these genes have to be established.

While several of the lectin-like genes in the NK receptor complex are specifically expressed in NK and NK/T cells, and others, although expressed in several cell types, contribute to NK cell function, several genes appear to have preferential roles in other leukocytes or even in endothelial cells. The total cluster of lectin-like genes may therefore be seen as a complex providing important receptors to hematopoietic and endothelial cells, a major part of these being involved in functions related to the innate immune response or the interface of the innate and adaptive immune response.

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