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Novel immunoglobulin-like transcripts in teleost fish encode polymorphic receptors with cytoplasmic ITAM or ITIM and a new structural Ig domain similar to the natural cytotoxicity receptor NKp44

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Abstract Members of the immunoglobulin superfamily (IgSF) include a group of innate immune receptors located in the leukocyte receptor complex (LRC) and other small clusters such as the TREM/NKp44 cluster. These receptors are characterised by the presence of immunoglobulin domains, a stalk, a transmembrane domain, and a cytoplasmic region containing either an immunoreceptor tyrosine-based inhibitory motif (ITIM) or are linked to an adapter molecule with an activation motif (ITAM) for downstream signalling. We have isolated two carp cDNA sequences encoding receptors in which the extracellular Ig domain structurally resembles the novel V-type Ig domain of NKp44. This is supported by a homology model. The cytoplasmic regions contain either an ITAM (*Cyca-NILT1*) or ITIMs (*Cyca-NILT2*). The tissue expression of these receptors is nearly identical, with the highest expression in the immunological organs. Peripheral blood leucocytes showed no detectable expression, but upon in vitro culture expressed NILT1, the activating receptor, and not the inhibitory NILT2 receptor. Southern blot analysis indicated that the *NILT1* and *NILT2* sequences belong to a multigene family. Analysis of the *NILT* Ig domain-encoding sequences amplified from both genomic DNA and cDNA revealed extensive haplotypic and allelic polymorphism. Database mining of the zebrafish genome identified several homologs on Chromosome 1, which also contains a cluster of class I major histocompatibility genes. This constellation is reminiscent of the *TREM/NKp44* gene cluster and the HLA complex located on human Chromosome 6. The carp *NILT* genes form a unique cluster of innate immune receptors, which are highly polymorphic, and characterised by a new Ig structural subfamily and are distinct from the novel immune-type receptors (Nitrs) found in other fish species.

Keywords Fish leucocyte receptor · NKp44 · TREM · ITAM · ITIM

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Introduction

The innate immune system uses a wide range of non-rearranging receptors to recognise largely unknown ligands collectively denoted as pathogen-associated molecular patterns (Janeway and Medzhitov 2002). Several clusters of innate immune receptors have been identified in mammals (Barten et al. 2001). Well-studied clusters of receptors comprise the leucocyte receptor cluster (LRC), which among others include the killer cell Ig-like receptors (KIR), Ig-like transcripts (ILTs), leucocyte Ig-like receptors (LIRs) and the natural cytotoxicity receptor (NCR) NKp46 (Martin et al. 2002). This cluster is located on human Chromosome 19. The ligands for these receptors are largely unknown, with the exception of the KIRs and some LIRs, which interact with major histocompatibility complex class I molecules (Biassoni et al. 2001). The KIRs are particularly

interesting as they exhibit both haplotypic and allelic variation, an uncharacteristic feature of the innate immune receptors (Trowsdale and Parham 2004). The LRC proteins have a common structure, as they are composed of a variable number of Ig-domains of the C2 set, a transmembrane region and a cytoplasmic region. The cytoplasmic regions of activating receptors are short and provide their signal through adaptor molecules, which are associated with the receptor through interaction of positive and negative charged residues in the transmembrane regions. The adaptor molecules contain immunoreceptor tyrosine-based activating motifs (ITAMs). The inhibitory receptors have long cytoplasmic regions with one to four immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Barten et al. 2001; Trowsdale et al. 2001).

In addition to the LRC, two other small clusters have been identified in the human genome on Chromosomes 6 and 17, which contain a limited number of innate immune receptors. Chr 6 harbours the *TREM* gene cluster and includes the NCR *NKp44* (Allcock et al. 2003). These genes encode inhibitory and activating receptors comprised of a novel V-type Ig domain (Cantoni et al. 1999; Radaev et al. 2003). Several *CMRF35* genes are located on Chr 17, which encode receptors with opposing signalling abilities (Clark et al. 2000, 2001). The activating receptors in these two clusters use adaptor molecules for signal transduction leading to cell activation (Lanier and Bakker 2000).

When comparing these clusters in humans and mice the most striking observation is that a common framework of genes exists in both species, but different selective pressures have resulted in unique gene arrangements evolving independently (Barten et al. 2001; McQueen and Parham 2002; Martin et al. 2003). The findings reported in this study are an attempt to resolve whether the receptors encoded in the above mentioned receptor clusters are recent innovations or descendants of a phylogenetically ancient group of innate immune response genes. The origin of some of the innate immune system receptors like the toll-like receptors (TLR), also present in teleost fish, can be traced back to invertebrates (Janeway and Medzhitov 2002). Others like the novel immune-type receptors (Nitrs) have been found in several fish species, but their mammalian homologs have yet to be identified (Litman et al. 2001). The Nitrs are composed of a V domain or a V domain and a V/C2 (or intermediate) domain, which either possess a joining (J) or an GXG motif.

We have isolated two novel immunoglobulin-like transcripts (*NILTs*) from carp, which encode receptors comprising an extracellular domain structurally related to the novel V-type domain of NKp44 (Cantoni et al. 1999, 2003). The cytoplasmic regions of the NILTs contain either an ITAM or an ITIM. Detailed analyses revealed that these receptors belong to a multigene family, which exhibit both haplotypic and allelic variation. The extensive polymorphism of the NILTs is unique for innate immune receptors, with the notable exception of the KIRs. To further explore the syntenic relationship between the *NILT* genes and the *TREM*/*NKp44* gene cluster or the *CMRF35* cluster we performed database mining of the zebrafish genome. Several homologs were identified on zebrafish Chr 1, 7 Mb downstream of the cluster of major histocompatibility class I genes (*Dare-ZE*) previously identified by Kruiswijk et al. (2002). This suggests that the carp *NILTs* are related to the *TREM*/*NKp44* genes encoded on Chr 6, which are located 10 Mb downstream of the *HLA* complex. The NILTs form a novel cluster of innate receptors that are not related to the previously described Nitrs located on zebrafish Chromosome 7.

Materials and methods

Animals

Carp (*Cyprinis carpio* L.) of the R3R8 and Ukraine strains (Irnazarow 1995) were kept in the central fish facility "De Haar-Vissen" in recirculating UV-treated tap water at 23°C. Animals were euthanised by immersion in tricaine methane sulfonate buffered with sodium carbonate. Organs were removed and snap frozen in liquid nitrogen and stored at −80°C for further use.

Cells and cell lines

Carp peripheral blood leukocytes (PBL) were isolated by density separation using Lymphoprep (Nycomed Pharma, Norway). Head kidney cells were obtained by Percoll density gradient separation as described by Verburg-van Kemenade et al. (1995). Cells were cultured for 12 h under standard conditions in RPMI in the presence or absence of 0.5% pooled carp serum and subsequently stimulated with phytohemagglutinin (PHA) for 3 h. After 24 h cells were harvested and RNA isolated with the SV Total RNA isolation system (Promega, Leiden, The Netherlands) according to the manufacturer's specifications.

Carp cell lines used represent two cell types: epithelial cells (EPC) and macrophage-like cells (CLC) and were obtained from the ATCC cell bank. RNA was isolated as described above.

PCR amplification

Carp sequences were obtained by anchored PCR using a macrophage cDNA library (Saeij et al. 2000) as template. Specific primers (see Table 1) were designed based on the initial sequence and were combined with LambdaZAP primers (SK and T7). To amplify the sequences from cDNA, two specific primers were used. The reactions were performed in *Taq* buffer, using 1 U *Taq* polymerase (Eurogentec, Seraing, Belgium) supplemented with $MgCl_2$ (1.5 mM), dNTPs (200 μM) and primers under the following PCR conditions: initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s, and a final extension at 72°C for 10 min.

The organisation of the carp *NILT1* and *NILT2* genes was determined by PCR amplification of genomic DNA

Table 1 Primers sequences used to amplify *Cyca-NILT* sequences from a carp cDNA library, genomic DNA, and cDNA

Primer	Sequence
1. NILT1-244F	GTG GTT AAA TCA GGA TCT CCA GCT
2. NILT1-397R	CCA GCA ACA AAA TCT CTG TGT AGA C
3. NILT1-67F	TGT GGG GCT TTC TTC TTC TCT GGT C
4. NILT1-1043R	GAA CAA TAC AGG TAT GAT CTT TAC AGC
5. NILT1-70F	GGA CAC AGA GGA GAG CGG CTT GAC A
6. NILT1-686R	CAG CAG AAC CGT CAT CAC TTT GAG
7. NILT1F	TGC CCC TAT AAA TCT GGA TAT GAA TC
8. NILT1R	CCA GTA TTG TCC CTC ATC CTC TGT TC
9. NILT1IGF	CGG CTT GAC ATC AGA TGC CCC TA
10. NILT1IGR	TGT GTA GAC ATC AGT AGT AAA TAA
11. NILT1CYTF	AAT GCT TAC GGG AAA GGA AAC TCA
12. NILT1CYTR	GTG TTC AGG TGT TTG ATC AAT ATT
13. NILT2-007F	ATG TTC AGA ATG TGT GTC GCT
14. NILT2-456R	TTA CAG AAC CGA ACC CAG ACC ACC
15. NILT2-635R	AGG CGT CTG ATT GGC TGA AGA TGC TG
16. NILT2-1038R	TCT GTC CTG CTG TCT GTT GTT GCT TCA
17. NILT2F	AGA GCT GAC ATC AGA TGC ACA TA
18. NILT2R	ACT CCT CCT CAC TCC ACA CCA GTA
19. NILT2IGF	GGT GCT CCA GTT AAA GTC ACA GGA
20. NILT2IGR	CGT TTT AAC CAG CAA CAC AAT CTC
21. NILT2CYTF	CAG CAA AAT GTG CTG CAC AAC ACG
22. NILT2CYTR	ATG TTT GAT GAC TGA ATA TAT CGG

from a single carp individual using different sets of carp-specific primers (Table 1) under the conditions described above or using Expand long template PCR (Boehringer Mannheim, Ingelheim, Germany).

Polymorphism of the *NILTs* was studied by analysing PCR products obtained from genomic DNA and cDNA of single individuals using different combinations of *Cyca-NILT1*- and *Cyca-NILT2*-specific primers (see Table 1).

Cloning and sequencing

Fragments amplified by PCR and Expand long PCR were ligated into the pGEM-T Easy vector (Promega) and the plasmids were transformed into JM109 cells according to standard protocols. Plasmid DNA was isolated from single colonies using the QIAprep Spin miniprep kit (Qiagen, Leusden, The Netherlands). Sequences were determined for both DNA strands using the Big-Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, Calif., USA) and an ABI 377 Automated Sequencer. Sequence analysis was performed using the Sequencher software (Gene Codes, Ann Harbor, Mich., USA). Nucleotide and amino acid data obtained were analysed for identity to other sequences using BLAST. Comparisons between sequences were performed with CLUSTALX (Thompson et al. 1994) and percentage identity was determined by the FASTA program. Predictions of the signal peptide, transmembrane region and glycosylation patterns were performed at http://www.cbs.dtu.dk. Phylogenetic analyses were performed using MEGA 2.1 (Kumar et al. 2001).

Expression studies

To study gene transcription, total RNA was isolated from different organs or cells using the SV total RNA isolation system (Promega). The RNA samples were used as template in a RT-PCR. Gene-specific primers (see Table 1) were used in the SuperScriptOne-step RT-PCR system (GibcoBRL, Breda, The Netherlands). To control for equal use of the RNA templates, control PCR experiments were performed using primers based on actin (Table 1). Templates were subjected to 25 PCR cycles to avoid saturation of the actin control amplification. The resulting products were visualised on agarose gels.

Southern hybridizations

Carp genomic DNA was digested overnight with *Hae*III at 37°C, separated on a 1% agarose gel. DNA was transferred to a nylon filter (Roche Diagnostics, Manheim, Germany) and fixed using UV light. The filter was prehybridized in hybridization buffer (Roche Diagnostics) at 42°C and hybridized overnight with a digoxygenin (DIG)-labeled probe at 42°C. Probes were prepared by PCR using genomic DNA as the template with primers specific for the Ig domain or the cytoplasmic region (see Table 1). Probes were sequenced prior to their use and were each shown to represent the desired sequence. Filters were washed at different stringencies (2× SCC, 1× SSC, 0.1× SSC) at 55°C and hybridizations were visualised by DIG High Prime DNA detection solution and recorded on chemiluminescent detection film (Roche Diagnostics).

Protein modelling of Cyca-NILT1

Recently, the structure of the human NK cell receptor NKp44 (PDB entry: 1HFK) has been determined (Cantoni et al. 2003). This is the only template available in the SWISS-PROT database that allowed us to model the Cyca-NILT1 extracellular Ig domain. Model building of Cyca-NILT1 was performed with MODELLER (Sali and Blundell 1993) using the CVFF force field (Dauber-Osguthorpe et al. 1988). The model was verified after several rounds of energy minimisation and the stereochemical quality of the homology model was assessed by PROCHECK. The protein folding was verified by PROSAII (Sippl 1993), which evaluates the compatibility of each residue to its environment independently. In addition, molecular dynamics (MD) simulations were carried out using the GROMACS program (Lindahl et al. 2001) to assess the molecular stability of all Cyca-NILT1 models in an aqueous environment.

Each protein structure obtained with the Modeller program was first energy minimised using a steepest descent algorithm. The resulting protein structure was solvated by generating a cubic box of spc water molecules, such that the minimum distance between the protein and the edge of the periodic box would be 0.9 nm, resulting in a cubic box of 5.8 nm. All water molecules with the oxygen atom

closer to any protein atom than the sum of their respective Van der Waals radii were removed, leaving 5,884 water molecules around the protein. The resulting conformation was energy minimised with harmonic constraints on the atomic coordinates of the protein. Subsequently a round of 10 ps of MD was performed, also with harmonic constraints on the atomic coordinates of the protein to relax the water orientation near the protein. The final conformation was used as starting conformation for simulations of the protein in water. Long simulations of 1 ns of the protein in water were performed to determine the long-term properties of the protein using a time step of 2 fs.

Results

Carp Ig-like transcripts resemble mammalian leucocyte receptors

We serendipitously isolated a partial cDNA sequence as a result of an anchored PCR using carp *IL-1*-specific primers. In a BLAST analysis this sequence showed significant similarity to the 5′ end of the CMRF-35 leucocyte receptor (Clark et al. 2000, 2001), a triggering receptor expressed on myeloid cells (TREM) (Bouchon et al. 2000), and the natural cytotoxicity receptor NKp44 sequence (Cantoni et al. 1999). This prompted us to further characterise this partial sequence. Two primers (NILT1-244F and NILT1-397R) were designed based on the available sequence data and an anchored PCR was performed on the carp macrophage cDNA library. This resulted in two different fragments when the specific forward primer was combined with the vector primer. Sequence analysis of the two fragments revealed the presence of 3′ ends of two distinct sequences, and included a poly(A)$^+$ tail. Again, specific primers (Table 1: primers 3–6 for *NILT1* and primers 13–16 for *NILT2*) were designed to obtain overlapping sequences. This analysis resulted in the isolation of two sequences designated carp novel immunoglobulin-like transcripts *Cyca-NILT1* and *Cyca-NILT2*. The *Cyca-NILT1* cDNA sequence (1,143 bp) encodes a protein consisting of an 18 amino acid leader peptide, an immunoglobulin-like domain (103 aa), a connecting peptide (40 aa), transmembrane region (23 aa) and a cytoplasmic region (123 aa) (Fig. 1a). The immunoglobulin domain has the characteristic two cysteines spaced 65 amino acids apart, and two additional cysteines at positions 30 and 34. The connecting peptide contains a preponderance of serine and threonine residues (13/40 aa). The cytoplasmic region has no similarity to any known sequence in the EMBL database. How-

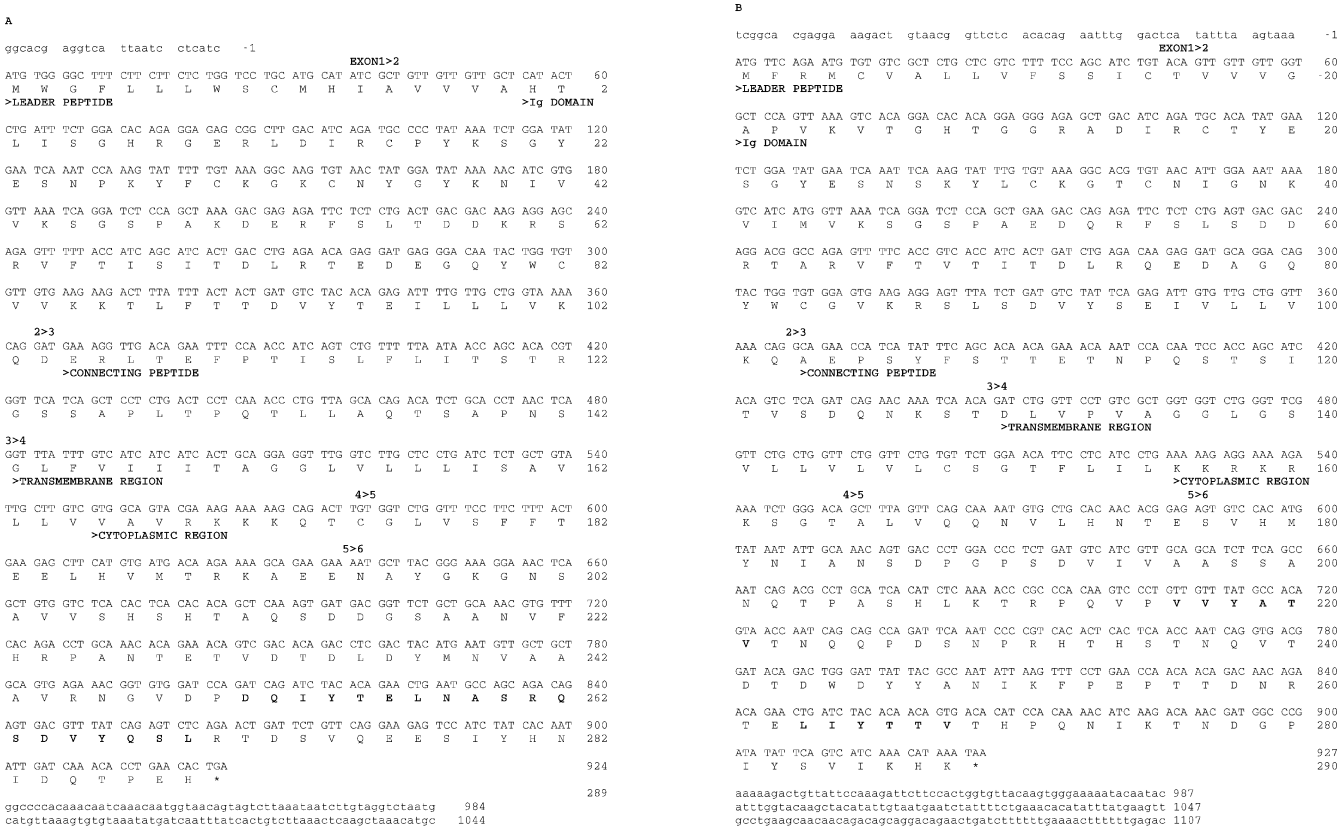


Fig. 1 cDNA and deduced protein sequences of Cyca-NILT1 (**a**) and Cyca-NILT-2 (**b**). The different protein domains (leader peptide, immunoglobulin domain, connecting peptide, transmembrane and cytoplasmic region) are indicated *below* the deduced protein sequence. The putative ITAM (Cyca-NILT1) and ITIM (Cyca-NILT2) located in the cytoplasmic region are in *bold*. Exon boundaries are shown *above* the nucleotide sequences and the polyadenylation sites in the 3′ untranslated regions are *underlined*. Genomic and cDNA sequences have been deposited in the EMBL database (accession numbers AJ811994–AJ811997)

ever, in this region a motif is present at positions 251–269 (DQIYTELNASRQSDVYQSL) which conforms to the consensus motif of an immunoreceptor tyrosine-based activating motif (ITAM; D/EXXYXXL(X)$_{6–8}$YXXL/I). The second carp cDNA sequence (*Cyca-NILT2*) encodes a protein which is similar in structure to Cyca-NILT1 (Fig. 1b). The most striking difference is the absence of the ITAM in the cytoplasmic region of Cyca-NILT2. Instead of this activating motif, two immunoreceptor tyrosine-based inhibiting motifs (ITIMs) are present at positions 216–221 (VVYATV) and 263–268 (LIYTTV). These motifs follow the consensus ITIM (V/LXYXXV/L). The identity (68%) between the Cyca-NILT1 and Cyca-NILT2 proteins is confined to the Ig-like domain (Fig. 2). The connecting peptide, transmembrane region and cytoplasmic regions showed no significant identity. BLAST searches indicated the highest similarity to the CMRF35 (identity 35% and similarity 60%), TREM (identity 35% and similarity 56%), and NKp44 (identity 34% and similarity 50%) Ig domain sequences. Alignment of Cyca-NILT1 and Cyca-NILT2 with NKp44 revealed the conservation of four out five residues defining the V-type Ig subfamily (Cys22, Cys91, Arg62, Asp85). The conserved tryptophan (Trp36) is not found in the NILT sequences. In NKp44 the two additional cysteines (Cys37 and Cys45) are separated by seven residues, whereas in Cyca-NILT1 and Cyca-NILT2 they are separated by only three residues.

Genomic organisation of the *Cyca-NILT* genes

The fact that the two carp sequences were similar in their Ig-like domains, but completely dissimilar in the remainder of the coding sequences, prompted us to investigate the organisation of the genes to examine the possibility of alternative splicing or gene rearrangement. PCR fragments were generated using *Cyca-NILT1*-specific primers (Table 1: primers 3–6). This resulted in single overlapping fragments, which were cloned and sequenced. The *Cyca-NILT1* gene consists of six exons and five introns (Fig. 1a). The Ig-like domain, connecting peptide and transmembrane region are encoded in exons 2, 3 and 4, respectively. Exon 4 also contains part of the cytoplasmic region, while the remainder of the cytoplasmic region and the 3′ untranslated region are encoded in exons 5 and 6. Introns 1–4 are phase 1, whereas intron 5 is phase 0. The length of introns 1–5 are 117, 279, 112, 381 and 91 bp, respectively.

The elucidation of the genomic organisation of *Cyca-NILT2* gene was performed by extra-long PCR. This resulted in the amplification of a fragment of approximately 4.5 kb, which was shown by PCR with *Cyca-NILT2*-specific primers (Table 1: primers 13–16) to contain the gene of interest. Sequence analysis revealed a similar exon/intron organisation and phasing of the introns as the *Cyca-NILT1* gene (Fig. 1b). The only notable difference is the large size of intron 3 (2,381 bp) of *Cyca-NILT2*, whereas introns 1 (169 bp), 2 (276 bp), intron 4 (88 bp) and intron 5 (110 bp) are comparable in length to the introns found in *Cyca-NILT1*. To exclude the possibility that the intron 3 sequence could potentially harbour a complete *NILT* gene, a GENSCAN http://www.genes.mit.edu) was performed. This analysis indicated that this 2,381 bp sequence contains no exons.

Expression of the *Cyca-NILT* sequences

Expression profiling of the *Cyca-NILTs* was performed using different tissues. cDNA was prepared from both immune and non-immune tissues and specific primers for either *Cyca-NILT1*or *Cyca-NILT2* were used in PCR amplification (Table 1: primers 9, 10, 19, 20). *Cyca-NILT1* has a similar expression profile as *Cyca-NILT2,* with the notable exception of the absence of *NILT2* expression in the thymus. The highest expression was found in immune tissues, such as (head) kidney, spleen and thymus (Fig. 3). Low expression was observed in the intestine, whereas the liver, muscle and PBL showed no demonstrable expression of the *NILT* genes with the number of PCR cycles

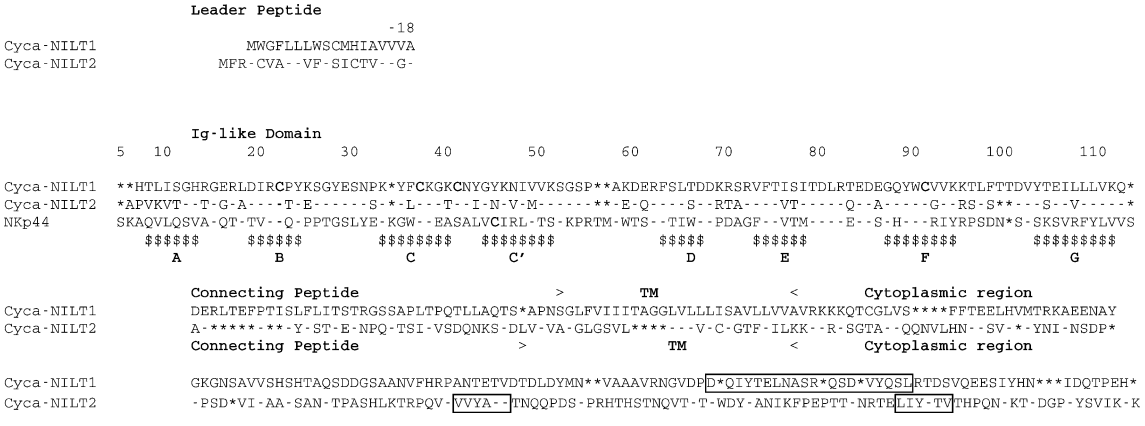


Fig. 2 Multiple alignment of the deduced protein sequences of Cyca-NILT1, Cyca-NILT2, and NKp44 (accession no. AJ225109). Only the immunoglobulin domain of NKp44 is aligned with the Cyca-NILT protein sequences. Numbering is based on the NKp44 protein sequence. The eight beta-strands of the NKp44 immunoglobulin domain are denoted by *dollar symbol*s. The putative ITAM of Cyca-NILT1 is *boxed* and the putative ITIMs of Cyca-NILT2 are boxed in *grey*. *Dashes* indicate identity and *asterisks* denoted gaps introduced for optimal alignment

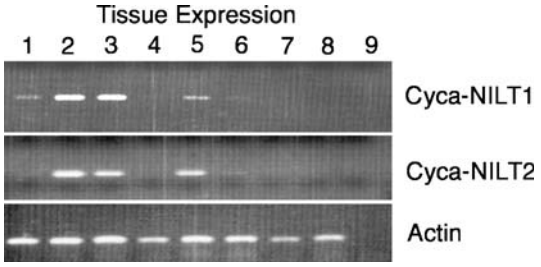


Fig. 3 mRNA expression as detected by RT-PCR of the *Cyca-NILT1* and *Cyca-NILT2* genes in different organs; *1* thymus, *2* head kidney, *3* spleen, *4* liver, *5* trunk kidney, *6* intestine, *7* muscle, *8* PBL, and *9* non-template control. Actin gene expression was used as positive control

used. These expression studies were extended to an examination of the expression patterns in different cell types. Investigations into the nature of the cell types responsible for the expression seen in head kidney was hampered by the fact that as soon as cell suspensions were prepared by macerating the tissue or when fractionated head kidney cells were cultured, expression of *Cyca-NILT1* and *Cyca-NILT2* was detected under all experimental conditions, including in the controls. Apparently, the extensive manipulations needed to obtain cell suspensions from tissue seem to induce expression of both *NILT* genes. Initial expression profiling using cDNA of freshly isolated PBL indicated that these cells do not express the *NILT* sequences. To further investigate the expression of the *NILTs* in PBL isolated from blood and cultured under different conditions, their expression was determined by RT-PCR with *Cyca-NILT1*- and *Cyca-NILT2*-specific primers (Fig. 4). Freshly isolated PBL express neither *Cyca-NILT1* nor *Cyca-NILT2*. However, culturing PBL in the either the presence or absence of pooled carp serum induced the expression of *Cyca-NILT1* only. PBL cultures stimulated with PHA showed relatively lower levels of expression of *Cyca-NILT1* compared to the unstimulated cultures, especially in the absence of pooled carp serum. No expression of *Cyca-NILT2* was observed under any of the experimental conditions, in contrast to cells isolated from the head kidney. These results suggested that expression of *Cyca-NILT1* in PBL is induced shortly after the cells are placed into in vitro culture, whereas under the the same experimental conditions *Cyca-NILT2* expression could be induced in head kidney cells only. In addition to the experiments using primary cultures, expression was also studied in immortalised carp cell lines (CLC and EPC), and neither of these cell lines expressed the *Cyca-NILTs* (data not shown).

Carp *NILT1* and *NILT2* genes are representatives of a multigene family

Extensive analysis of the *Cyca-NILT1* and *Cyca-NILT2* cDNA sequences amplified using specific primers located in the leader peptide and the end of the coding region revealed a single sequence in both cases. To confirm that these genes are present as a single copy gene, a Southern blot experiment was performed using four different probes encoding either the *NILT1* or *NILT2* Ig domains and the *NILT1* or *NILT2* cytoplasmic regions. The probes were obtained by PCR using *NILT1*- and *NILT2*- specific primers (Table 1: primers 9–12 and 19–22) We anticipated that under low stringency conditions the *NILT1* Ig domain probe might cross hybridize with the *NILT2* Ig domain-encoding regions or with other Ig domain-encoding exons. However, this would not be the case when using *NILT1* and *NILT2* cytoplasmic region probes, as they are highly divergent.

High stringency hybridizations with the *NILT1* and *NILT2* Ig domain probes revealed the presence of two to four hybridizing bands (Fig. 5). When the stringency was lowered multiple bands were observed, indicating either cross hybridization with exons encoding Ig-like domains or the presence of multiple copies of each *NILT* gene. To further investigate this, the filters were stripped and rehybridized with *NILT1* and *NILT2* cytoplasmic region probes. Under high stringency conditions, two to four hybridizing fragments were observed with the *NILT* cytoplasmic region probes. Similar to the results with the Ig domain probe, both *NILT1* and *NILT2* cytoplasmic probes hybridized at lower stringencies generated multiple bands, suggesting the presence of multiple *NILT1* and *NILT2* genes.

Analyses of the *NILT* multigene family in carp

To amplify the different *NILT1* and *NILT2* genes from a single individual we adopted an alternative strategy since the previous experiments using *NILT1*- and *NILT2*-specific primers (Table 1: primers 7 and 8, 17 and 18) resulted

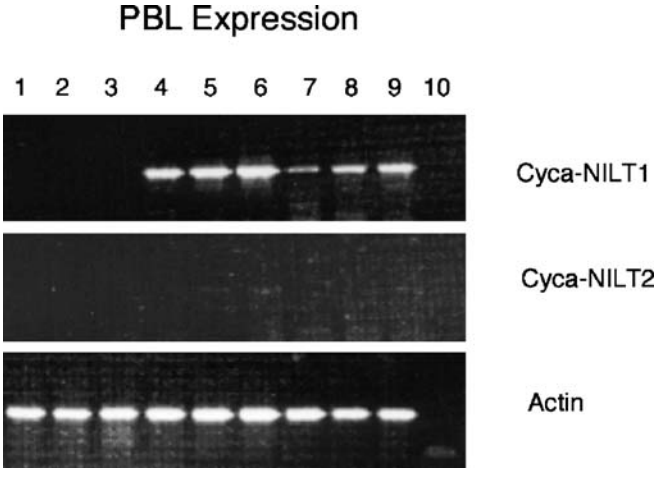


Fig. 4 mRNA expression as detected by RT-PCR of *Cyca-NILT1* and *Cyca-NILT2* genes in PBL under different conditions. Lanes *1–3* freshly isolated PBL in culture medium (*1*), culture medium supplemented with glutamine and antibiotics (*2*), and culture medium supplemented with glutamine, antibiotics, and 0.5% pooled carp serum (*3*). Lanes *4–6* PBL cultured overnight in the three different culture media as described for lanes *1–3*, lanes *7–9* PBL cultured overnight in the three culture media described above with the addition of the mitogen PHA. Lane *10* non-template control. Actin gene expression was used as positive control

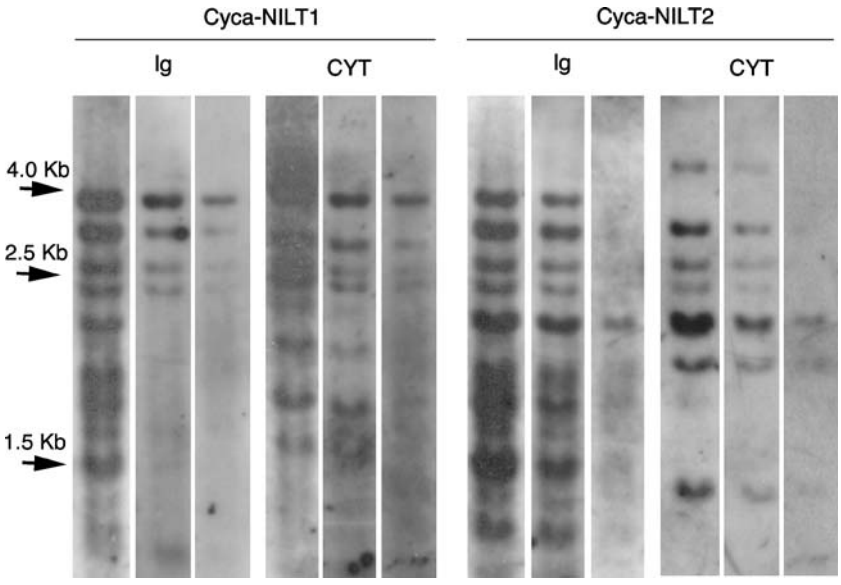


Fig. 5 Hybridization of carp genomic DNA digested with *Hae*III using an immunoglobulin (Ig) domain probe and a cytoplasmic (CYT) domain probe obtained from the *Cyca-NILT1* and *Cyca-NILT2* cDNA sequences. Final washes were performed under different stringencies (from left to right: 2× SSC, 1× SSC and 0.1× SSC)

in an amplicon containing a single cDNA sequence. In addition, sequence analyses of the probes used for the Southern hybridizations revealed that the primers designed to either amplify the exon encoding the Ig domain or the cytoplasmic region resulted in the specific amplification of either *Cyca-NILT1* or *Cyca-NILT2* sequences. However, the fact that we were able to amplify *NILT* sequences from two different teleost species (channel catfish and rainbow trout; R.J.M. Stet, unpublished observations) using different combinations of non-degenerate primers based on the sequences surrounding the conserved cysteines of the Ig domain of *Cyca-NILT1* (CPYKSGYE; YWCVVKSG) and *Cyca-NILT2* (RADIRCTY; YWCGVKRS) suggested that combining *NILT1*- and *NILT2*-specific forward and reverse primers might yield the maximum number of different *NILT* sequences. The genomic DNA of several carp individuals was analysed and one individual that gave an amplicon with all four primer combinations (NILT1F-NILT1R, NILT1F-NILT2R, NILT2F-NILT2R, NILT2F-NILT1R) was chosen for further analyses. cDNA was prepared from different organs of this individual to determine the expression of the *NILT* sequences. Amplicons were cloned and sequenced and 331 clones from two independent PCRs were analysed. This revealed the presence of 53 different *NILT* sequences, which in a phylogenetic tree were divided into two groups; 14 *NILT1*-like and 39 *NILT2*-like sequences (data not shown). The analyses were extended using genomic DNA and cDNA from six different carp. Essentially, this yielded similar results as described above with more *NILT2*-like sequences isolated compared to *NILT1*-like sequences per individual. In total, 30 *NILT1*-like (*Cyca-NILT1*001* to *Cyca-NILT1*030* and 95 *NILT2*-like (*Cyca-NILT2*001* to *Cyca-NILT2*095*) sequences were identified (see Electronic Supplementary Material). Out of all sequences identified in seven different individuals only four sequences (*Cyca-NILT1*001*, *Cyca-NILT1*002*, *Cyca-NILT2*001*, and *Cyca-NILT2*002*) were found in all individuals.

The NILT protein sequences described above are partial sequences obtained from genomic and cDNA and comprise 61 amino acids in between the conserved cysteines of the immunoglobulin domain. To describe the diversity of the sequences in relation to their possible function, the Shannon entropy (*H*; http://www.mifoundation.org/Tools) was calculated for both the NILT1 and NILT2 sequences. Positions with *H* values above 1.3 are considered to be variable, while those with *H* smaller than 1.3 are identified as conserved. The Shannon plot revealed that positions 30 and 33, and 71 and 74 of the Cyca-NILT2 deduced protein could be considered as variable (Fig. 6). The Shannon analysis of the available Cyca-NILT1 protein sequences showed variability in comparable positions, although the number of different Cyca-NILT1 sequences is much lower compared to the Cyca-NILT2. Most notably, positions 30 and 33 and 71 and 74 are variable in both NILT protein sequence. The relevance of variability at these positions can further be interrogated by determining the putative tertiary structure of the extracellular Ig domain.

Homology modelling of Cyca-NILT1

Recently, the structure of the natural cytotoxicity receptor NKp44 has been elucidated (Cantoni et al. 2003). This PDB entry (1HKF) was the only template from the SWISS-PROT database that allowed homology modelling. The programme MODELLER was used to generate several models of Cyca-NILT1 based on the NKp44 Ig domain structure. The NKp44 ribbon presentation was superimposed on the best-optimised model of Cyca-NILT1 (Fig. 7a). This showed an extensive congruence between the NKp44 structure and the Cyca-NILT1 model with only two deviations. First, an additional strand (D′) is seen in Cyca-NILT1, which is not present in the NKp44. Second and more importantly, the additional disulphide bride formed by residues Cys37 and Cys45 stabilising the C-C′ beta hairpin

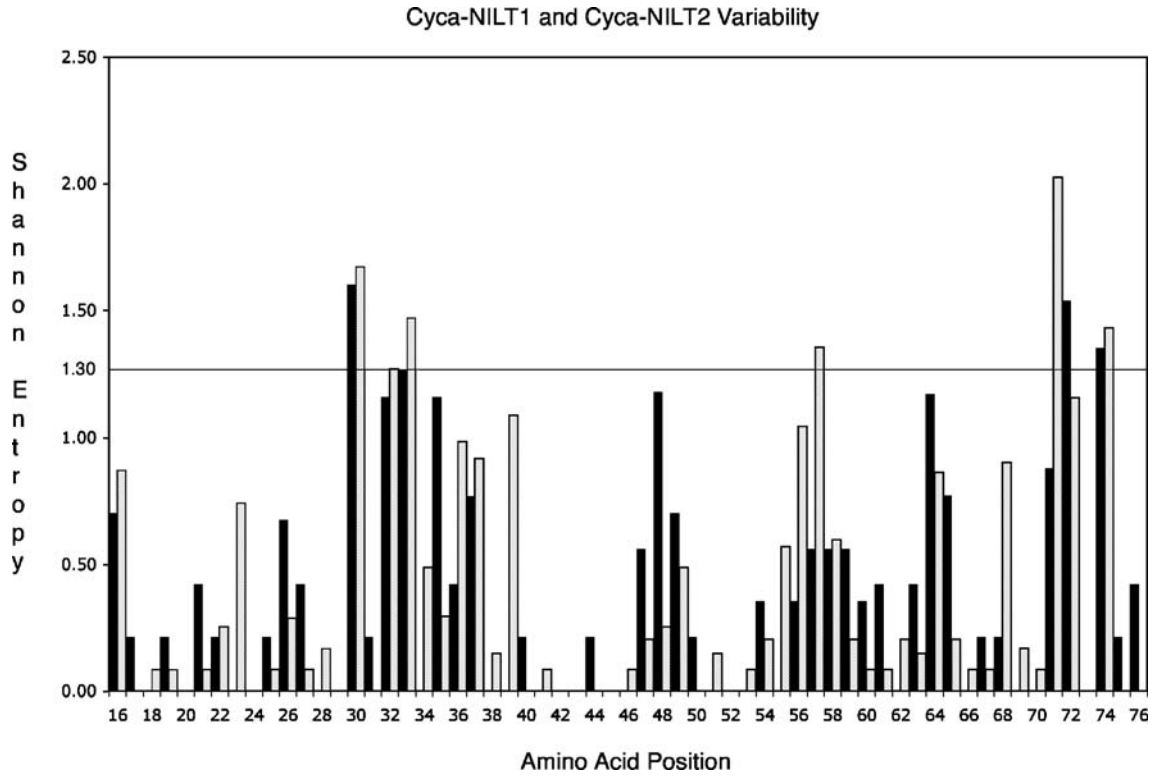


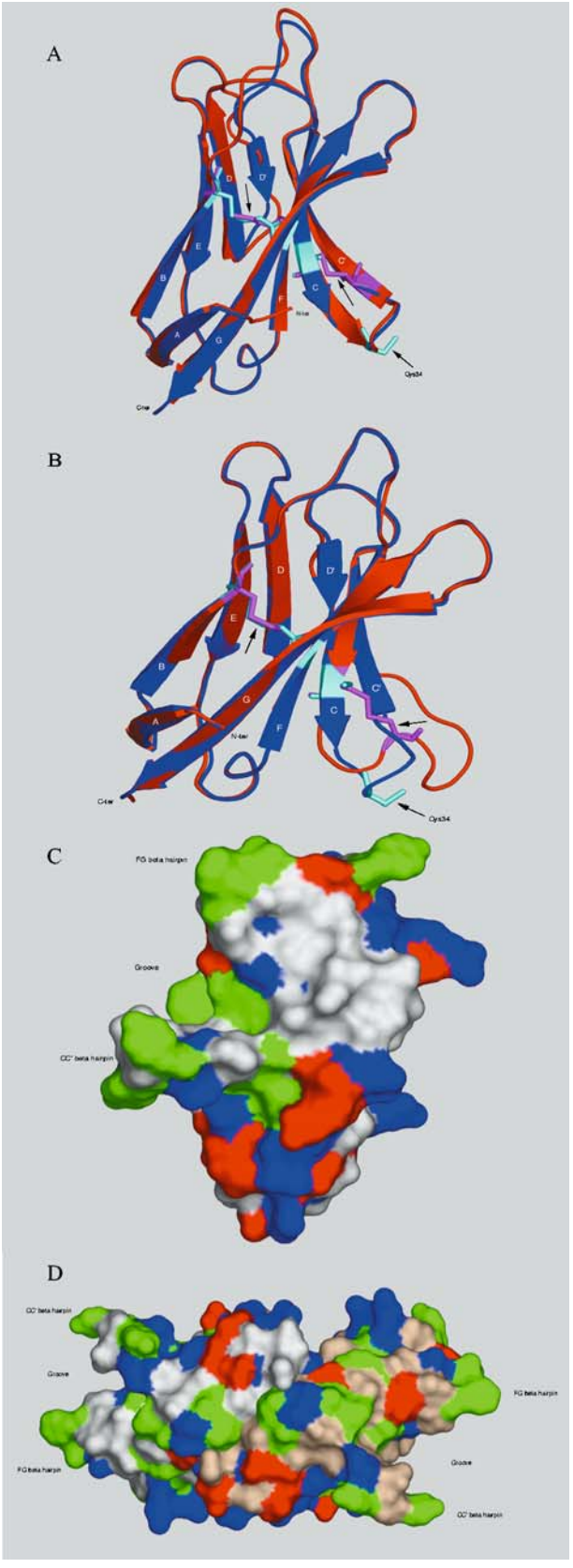
Fig. 6 Analysis of the variability of the partial Cyca-NILT1 and Cyca-NILT2 immunoglobulin domain protein sequences using Shannon entropy calculations. Positions with a Shannon entropy higher than 1.3 are considered significantly variable

of NKp44 is not present in the model. Although these two additional cysteines residues are present in all Cyca-NILT1 protein sequences, they are separated by only three residues instead of the seven residues in NKp44. It has been suggested that for the NKp44 protein this additional disulphide bridge is essential for the stability of the structure of the prominent groove formed by the protruding C-C′ and F-G beta hairpins from the ellipsoidal beta sheet consisting of the C-C′ F-G antiparallel beta strands. We therefore further investigated this proposal by constructing a model in which the additional disulphide bridge is formed (Fig. 7b). The ribbon presentation of the new model with the additional disulphide bridge was superpositioned on the initial model. The new model showed two major deviations from the first model; namely the absence of the D′ strand and the extension of the C-C′ beta hairpin. This, however, did not affect the characteristic groove, which retained its open conformation in both models. Subsequently, we tested the molecular dynamics indicative of the stability of both protein models using GROMACS. This demonstrated that both Cyca-NILT1 models, i.e., with and without the additional disulphide bridge and the open conformation of the groove are stable (data not shown).

Although the overall structure of the groove of Cyca-NILT1 is very similar to that of NKp44, the surface characteristics are quite distinct. The NKp44 protein has four basic residues (Arg and His) lining the groove surface, whereas Cyca-NILT1 has predominantly non-polar residues in the groove (Fig. 7c, d). The surface of the opposite side of the sheet forming the groove is flat and depleted of charged residues, which may result in dimerisation (Fig. 7d).

Database mining

Several genome databases http://www.ensemble.org) were searched for the presence of homologous sequences. No positive hits using both the nucleotide and protein sequences of the immunoglobulin domain of *Cyca-NILT1* and *Cyca-NILT2* were found in *Ciona intestinalis* (sea squirt) and *Caenorhabditis elegans* (nematode) genomes. However, mining of the zebrafish (*Danio rerio*) genome identified two regions on Chr 1 spanning 270 kb containing multiple exons encoding immunoglobulin domains with identity ranging from 42% to 79%. Nine exons were identified which were closely related to the *Cyca-NILT1* and *Cyca-NILT2* sequences, with the characteristic cysteines stabilising the immunoglobulin fold and two additional cysteines separated by three residues. This region on Chr 1 has not yet been fully annotated. However, this chromosome also contains the *Dare-ZE* loci, which encode major histocompatibility class I heavy chains (Kruiswijk et al. 2002), approximately 7 Mb upstream. An additional single significant hit was found on Chromosome 18, but no additional exons encoding Ig domains could be identified. In contrast to the zebrafish genome, searching the Fugu (puffer fish) genome data base v3.0 resulted in no significant positive hits.



◀ **Fig. 7** Putative protein model of the Cyca-NILT1 extracellular immunoglobulin domain using the human NKp44 (PDB entry 1HKF) structure as template. **a** Ribbon presentation of the putative model of Cyca-NILT1 superpositioned over the structure of NKp44 (*blue*). *Arrows* point to established disulphide bridges between the Cys residues in the B and F and C and C′ β strands, with the exception of Cys34 of the Cyca-NILT1 model. β Strands are labelled following the standard nomenclature. **b** Ribbon presentation of the first Cyca-NILT1 model (*blue*) superpositioned over a Cyca-NILT1 model in which the additional cysteines were aligned with the Cys37 and Cys45 of NKp44. This resulted in a new Cyca-NILT1 model (*red*) in which a disulphide bridge is formed. *Arrows* point to the two disulphide bridges between the cysteines located in the B and F and C and C′ β strands. **c**, **d** Surface model of Cyca-NILT1 as a monomer (**c**) and dimer (**d**). Colours represent different amino acids: Arg, Lys, His (*blue*), Asp, Glu (*red*), Phe, Trp, Tyr, Ile, Leu (*green*)

In contrast, BLAST searches of the human genome yielded several significant hits on Chr 17q25.1 with the highest score with *IREM1*. This receptor belongs to the CMRF35 family (Clark et al. 2000, 2001), which has four members. Significant hits were also obtained on Chr 6p21.1 in the region that contains the TREM family members of activating myeloid receptors [TREM1, TREM2 (Bouchon et al. 2000)], C6ORF76, and NKp44 (Allcock et al. 2003).

Discussion

Immunoglobulin superfamily genes encoding the tertiary structure known as the Ig domain, consisting of two beta-pleated sheets composed of antiparallel beta strands and connecting loops, are found throughout the animal kingdom (Barclay et al. 1997). The Ig domain can be found in a large variety of receptors and adhesion molecules. Recently, the number of genes encoding receptors consisting of a single or multiple Ig domains has expanded rapidly. Most notable are the genes found in the leucocyte receptor complex (LCR) encoding the killer inhibitory receptors (KIR), Ig-like transcripts (ILT), leucocyte-associated inhibitory receptors (LAIR) and the activating natural cytotoxicity receptors (NCR) NKp46 (Martin et al. 2002). The latter belong to a group of receptors (NKp30, NKp44) intimately involved in the activation of NK cells. These belong to an ever-expanding number of activating and inhibitory lymphoid or myeloid receptors described recently (Moretta and Moretta 2004).

The sequences that we have described in this study, *Cyca-NILT1* and *Cyca-NILT2* , showed significant similarity to the human receptors TREM, CMRF35, and one of the NCRs, namely NKp44. No hits were found with receptors belonging to the LRC. The similarity is confined to the extracellular domain, whereas the remainder of the carp *NILT* sequences showed no similarity to each other or with any database entry. However, there are some characteristics which the two *NILT* sequences share with TREM-2, CMRF35, and NKp44. The extracellular domains of Cyca-NILT1 and Cyca-NILT2 have the two characteristic cysteines spaced 65 amino acids apart. In addition, there are two cysteines at position 30 and 34. The human re-

ceptors also have these additional cysteines, but they are separated by seven residues, which is important as it allows the formation of a disulphide bridge stabilising the C and C′ beta strands of the NKp44 Ig domain (Cantoni et al. 2003). Comparison of the cytoplasmic regions of NKp44, Cyca-NILT1 and Cyca-NILT2 revealed some interesting differences. NKp44 has a short cytoplasmic region with no signalling ability because it lacks activation motifs such as an ITAM. Its signalling ability is provided by the ITAM-bearing adaptor molecules KARAP/DAP12 (Cantoni et al. 1999). In contrast, Cyca-NILT1 has a putative ITAM in its cytoplasmic region, whereas the cytoplasmic region of Cyca-NILT2 has two putative ITIMs. Both the ITAM and the ITIMs in the Cyca-NILT sequences conform to known inhibitory and activation motifs (Vely and Vivier 1997; Davis et al. 2002), although we have to establish their functionality. The other receptor with a high similarity to both Cyca-NILT1 and Cyca-NILT2 is CMRF35, of which two isoforms are known, CMRF35A and CMRF35H (Clark et al. 2000, 2001). These molecules may play a regulatory role in leucocyte function and have an inhibitory function indicated by the presence of ITIMs (CMRF35H), while CMRF-35A lacks these ITIMs. The latter may use an as yet unidentified adaptor molecule containing an ITAM, providing an activating signal. Human TREM-1 and TREM-2 are structurally related to NKp44 and are found in a cluster on Chr 6. TREM-1 is involved in activation of inflammatory responses, although the ligand is still unknown (Bouchon et al. 2000). TREM-1 does not have the additional two cysteines as seen in the NILTs and NKp44. TREM-2 is hypothesised to regulate chronic inflammatory responses and contains the two additional cysteines. Both TREM molecules and the NKp44 associate with DAP12 to induce activation. In this respect the NILT receptors are distinct and the only feature the carp and human receptors have in common is the V-type Ig domain, which as determined for NKp44 (Cantoni et al. 2003), represents a novel structural subfamily.

Comparison of the genomic organisation of the two *Cyca-NILT* genes with those of *NKp44*, *CMRF35A* and *CMRF35H* may reveal commonalities indicative of orthologous or paralogous relationships. The *Cyca-NILT1* and *Cyca-NILT2* genes have an almost similar structure with only one major difference, the length of intron 3. Interestingly, in *Cyca-NILT1* and *Cyca-NILT2* exon 6 encodes the protein region that contains the ITAM and ITIM, respectively. Exons bearing ITIM sequences are always in phase 0 (Alley et al. 1998). This suggests that the ITIM in *Cyca-NILT2* is likely to be functional. Genes belonging to the immunoglobulin superfamily have exons encoding the Ig domain that are in phase 1 (Radley et al. 1994). In this respect the *Cyca-NILT* genes conform to this paradigm. The genomic organisations of the *Cyca-NILTs* are very similar to *CMRF35H* with only one additional exon encoding part of the cytoplasmic region (Clark et al. 2000). However, the complete lengths of the *Cyca-NILT1* and *Cyca-NILT2* genes differ from *CMRF35H* and *NKp44* by 12 and 15 kb, respectively. In contrast, the *CMRF35A* gene (4.5 kb) is comparable in length, but has an exon-intron organisation distinct from all others discussed above (Clark et al. 2001). In conclusion, no clear relationship between the *Cyca-NILTs* and the *CMRF35A* and *NKp44* receptor genes is apparent based on their genomic organisation, with the possible exception of that of *NILT2* with *CMRF35H*.

The tissue expression profiles of the *Cyca-NILT1* and *Cyca-NILT2* mRNAs are remarkably similar. Expression was found in mainly in immunological organs known to contain different leucocyte subpopulations (Verburg-van Kemenade et al. 1994). NKp44 is expressed only on activated NK cells and a minor subset of gamma-delta T cells (Vitale et al. 1998; Cantoni et al. 1999). In contrast, the CMRF35 antibody reacts with a subset of T cells, monocytes, granulocytes, dendritic cells and NK cells (Clark et al. 2000). TREM-1 is expressed on neutrophils and monocytes, while TREM-2 can be found on dendritic cells and macrophages but not monocytes (Bouchon et al. 2000). The tissue expression profiles of the *Cyca-NILTs* are consistent with the profile of CMRF35 and the TREMs, but not with that of NKp44. The detection of the expression of the two NILT receptors on isolated cells is hampered by the fact that apparently the procedure required to obtain cell suspensions from, e.g., head kidney induces the expression of both receptors within a matter of hours. The exception is the expression profiling of PBL, which are negative for both receptors, but upon culturing express *Cyca-NILT1* and not *Cyca-NILT2*. Apparently, the in vitro conditions induce the expression of the activating receptor, but the nature of the inducing agent has not been identified yet. It may be cytokines, which are released when cells are brought under culture conditions. Studies on the expression of IL-1 and TNF have shown basal levels of these cytokines in control cultures of carp head kidney cells (Engelsma et al. 2003, Saeij et al. 2003). Alternatively, internal proteins from disrupted cells could act as danger signals that may activate the expression of the NILTs.

Initially we were only able to amplify single sequences representing either *Cyca-NILT1* or *Cyca-NILT2* using specific primers from cDNA. To confirm the single copy nature of these genes we performed Southern hybridizations. We expected the detection of multiple bands based on the degree of homology between the immunoglobulin domains of both NILT receptors and perhaps other immunoglobulin domains. This was indeed the case, and is evidenced by the similar pattern of multiple bands under intermediate stringency (Fig. 5). However, we anticipated that the probe representing the cytoplasmic region would be gene-specific and would result in a single or at the most two bands even under low stringency conditions, based on the fact that carp is a tetraploid fish (Larhammar and Risinger 1994). Both *Cyca-NILT1* and *Cyca-NILT2* cytoplasmic probes detected multiple bands at intermediate stringency. This suggests that *Cyca-NILT1* and *Cyca-NILT2* are representatives of a family of related genes. Our results indicated that each individual carp has a unique set of *NILT1* and *NILT2* sequences, with the exception of four sequences that were found in each individual. None of the partial sequences have frameshifts leading to in-frame stop

codons. However, we are aware that the sequences are partial and defects in other parts of the complete sequence may render them pseudogenes. The sequence analysis revealed that the repertoire of the *NILT1* and *NILT2* genes in each individual carp is the result of allelic and haplotypic variation. This is reminiscent of the *KIR* genes, where framework genes together with a variable number of polymorphic genes comprise the KIR repertoire of any given individual (Martin et al. 2003; Yawata et al. 2002).

The polymorphism of the NILT1 and NILT2 deduced partial protein sequences is extensive and most prominent in two regions (positions 30 and 33, and 71 and 74). These regions, according to the homology model, correspond to the C-C′ and E-F hairpins. The model we constructed was based on the NKp44 template (Cantoni et al. 2003) and showed a high degree of congruence with only two deviations. The major deviation is the absence of a second disulphide bridge between the C and C′ strand. Assessing the molecular stability of the two models, with and without the additional disulphide bridge, indicated that both models are stable. Thus, the actual presence of the additional disulphide bridge stabilising the C-C′ strands in the NILT model can neither be refuted nor validated.

Although the structural similarity between the Cyca-NILT1 and NKp44 is striking, the charge distribution, especially in the groove formed by the protruding C-C′ and F-G hairpin, is different. Although the actual ligand is still unknown, Cantoni et al. (2003) have suggested that due to the presence of charged residues lining the groove NKp44 most likely has an anionic binding specificity. The groove formed by the protruding hairpins of NILT1 is essentially apolar, suggesting a different binding specificity. In addition, NILT1 and NILT2 are highly polymorphic, in contrast to NKp44, which is monomorphic. The polymorphisms are located mainly between the C and C′ strands, which may attribute different binding specificities. As each individual carp has multiple *NILT1* and *NILT2* sequences this may result in a repertoire of binding specificities for an as yet unknown ligand. The presence of the ITAM and ITIMs in NILT1 and NILT2, respectively, may provide a balanced signal which is either predominantly activating or inhibitory. This situation is similar to the signalling ability of different KIRs, some of which have ITIMs in their cytoplasmic regions, whereas others with truncated cytoplasmic regions provide an activating signal using adaptor molecules such as DAP12 (Lanier and Bakker 2000). NKp30, NKp44, and NKp46 are activating receptors using adaptor molecules CD3 or DAP12 containing ITAMs and are intimately involved in the killing activity of human NK cells (Moretta and Moretta 2004). The NILTs therefore form a unique set of receptors, which have maintained their own opposing signal ability without the need for adaptor molecules bearing ITAMs. Invariably the receptors in the LRC have their own ITIMs, but activating receptors all use adaptor molecules (Fc receptor, CD3 or DAP12) containing ITAMs (Barten et al. 2001). The functionality of the signal motifs in NILT1 and NILT2 is corroborated by the difference in phase of the introns encompassing the exon encoding cytoplasmic region containing these motifs.

In fish, one receptor has been identified which has been implicated in natural cytoxicity, namely the non-specific cytotoxicity cell receptor protein-1 (NCCRP-1) (Jaso-Friedmann et al. 1997; Ishimoto et al. 2004). This receptor shares a significant level of identity with another group of proteins belonging to an F-box subfamily. These proteins share an F-box domain in the N terminus (not present in NCCRP-1) and an extremely conserved C-terminal region that has been termed the F-box-associated domain (FBA). The function of the FBA domain is unknown (Jaso-Friedmann et al. 2002). Thus, the structure of the NCCRP-1 is completely dissimilar from the NILT sequences.

The only group of receptors of which some members resemble the NILT sequences are the novel immune-type receptors (Nitrs), which have been described in pufferfish, zebrafish, channel catfish and rainbow trout (Strong et al. 1999; Yoder et al. 2001, 2002, 2004; Hawke et al. 2001). The Nitrs come in different prototypes with a majority composed of a V domain or a V domain and V/C2 (or intermediate) domain, which either possess a joining (J) or a GXG motif, a transmembrane region and a cytoplasmic region containing only ITIMs. In all Nitrs reported to date only the *Nitr5* family has a putative ITAM-related sequence in the cytoplasmic region. There are a number of *Nitr* genes within families (*Nitr3*, *Nitr6*, *Nitr7*, *Nitr10* and *Nitr11*) that encode a receptor with a single V domain (Yoder et al. 2004). Comparison of Nitr3r amino acid sequence with the NILT1 and NILT2 sequences revealed a low percentage identity (20%) or similarity (30%). In addition, the *Nitr* cluster, which has been estimated to be around 350 kb, is located on Chr 7. Database mining the zebrafish genome using the *NILT1* and *NILT2* sequences identified several homologs on Chr 1 in a telomeric region of 270 kb and a single homolog on Chr 18. Surprisingly, no positive hits were obtained when the *Fugu* genome was probed with *NILT1* and *NILT2* sequences, while zebrafish and *Fugu* share orthologous Nitrs. This could be due to the fact that the role of NILT receptors have been taken over by other divergent receptors similar to the Ly-49 C-type lectin receptors in mice, which act as the functional homolog of the KIRs (McQueen and Parham 2002).

The unambiguous assignment the carp *NILT* sequences as orthologs or paralogs of other known immune receptors is at this point difficult. It is tempting to speculate that there is an orthologous relation between the *NILTs* and genes encoded in the LRC. In this respect, the Nitrs fulfill more of the criteria based on conserved synteny (Yoder et al. 2001), although validation awaits identification of Nitr orthologs in mammals (Litman et al. 2001). Comparing the structure and chromosomal localisation of the *NILTs* and *Nitrs* clearly demonstrates that these are distinct clusters of immune receptor genes. The *NILT* sequences resemble the human genes *CMRF35* and *NKp44*. These genes are located on Chrs 17 and 6, respectively, and are found in regions that are composed of clusters of related genes. The *NKp44* (HUGO ID *NCR2*) gene is clustered

with *TREM* genes (Allcock et al. 2003), which are activating receptors of myeloid cells. *CMRF35* belongs to a family of activating and inhibitory gene members, including *IREM1* (accession no. AF375480), which are expressed on both lymphoid and myeloid cells (Clark et al. 2000, 2001). Taken together, it seems likely that the *NILT* genes could be orthologous with one of these human gene clusters. However, this should be confirmed by the fact that the clusters are located in syntenic regions. The only indication in favour of this proposition is the fact that the human Chr 6 also contains the MHC at position p21.31, which is approximately 10 Mb upstream of the *TREM/NKp44* cluster. This positional relationship is very reminiscent of that in zebrafish, where the zebrafish *NILT* homologs detected by blast search on Chr 1 are located 7 Mb downstream of four major histocompatibility class I *Dare-ZE* sequences originally described by Kruiswijk et al. (2002).

In conclusion, we have identified a novel group of receptors, which are highly polymorphic and have different signalling abilities based on the presence of an ITAM (Cyca-NILT1) or ITIMs (Cyca-NILT2) in their cytoplasmic regions. The immunoglobulin domain is structurally similar to that of NKp44 and is a new V-type domain. Genome scans revealed the presence of similar genes in zebrafish and homologs in human such as *CMRF35*, *TREM*, and *NKp44*. These genes are found in small clusters containing a limited number of isoforms located on different chromosomes. The NILT1 and NILT2 receptors are highly polymorphic and show extensive haplotypic variation. These characteristics challenge the paradigm that the repertoire of receptors involved in innate immunity is, with the notable exception of the KIRs, restricted. The *NILT* genes form a second complex cluster of activating and inhibitory receptors together with the *Nitr* genes. We are currently annotating in detail the *NILT* region on zebrafish Chr 1 to further our understanding of these receptors.

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