Extraordinary variation in a diversified family of immune-type receptor genes

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Immune inhibitory receptor genes that encode a variable (V) region, a unique V-like C2 (V/C2) domain, a transmembrane region, and a cytoplasmic tail containing immunoreceptor tyrosine-based inhibition motifs (ITIMs) have been described previously in two lineages of bony fish. In the present study, eleven related genes encoding distinct structural forms have been identified in *Ictalurus punctatus* (channel catfish), a well characterized immunological model system that represents a third independent bony fish lineage. Each of the different genes encodes an N-terminal V region but differs in the number of extracellular Ig domains, number and location of joining (J) region-like motifs, presence of transmembrane regions, presence of charged residues in transmembrane regions, presence of cytoplasmic tails, and/or distribution of ITIM(s) within the cytoplasmic tails. Variation in the numbers of genomic copies of the different gene types, their patterns of expression, and relative levels of expression in mixed leukocyte cultures (MLC) is reported. V region-containing immune-type genes constitute a far more complex family than recognized originally and include individual members that might function in inhibitory or, potentially activatory manners.

Extended multigene families belonging to the Ig gene superfamily (IgSF) account for a diverse range of immunological functions including recognition of antigens and antigenic peptides by both somatically rearranging Ig and T cell antigen receptor (TCR) genes, as well as by major histocompatibility complex (MHC) molecules. The origins of the three diverse systems of effector molecules can be traced through analyses of these genes in extant species of representative early, jawed vertebrates (1). Recently, multigene families which encode novel immune-type receptors (*NITR/nitr*) have been described in *Spheroides nephelus* (Southern pufferfish; ref. 2) and *Danio rerio* (zebrafish; ref. 3). The *NITR* genes described in these species encode two extracellular Ig domains [a variable (V) domain and a V-like C2 (V/C2) domain], a transmembrane region, and most often, immunoreceptor tyrosine-based inhibition motifs (ITIMs) in the cytoplasmic tail. The general structural characteristics of the NITR V domain are common to the corresponding regions of both Ig and TCR (4); whereas ITIMs are found in several inhibitory receptors, which are encoded at the leukocyte receptor cluster (LRC) on human chromosome 19q13.3–13.4 and at a corresponding location on mouse chromosome 7 and include natural killer (NK) receptors, such as killer cell Ig-type receptors (KIRs) (5). Unlike *NITR* genes, LRC genes do not encode V regions. A number of questions arise regarding the distribution of the *NITR* genes in vertebrate phylogeny, their function, and the relatedness of *NITR* genes to other families of genes that are involved in immune function, specifically, the immune inhibitory receptors of the mammalian LRC.

The lack of immunologically relevant *in vitro* culture systems in pufferfish and zebrafish severely limits functional assessments. In contrast, *in vitro* immunological phenomena can be studied in *Ictalurus punctatus* (channel catfish), and specific functions have been assigned to cells possessing defined cell surface phenotypes

(6). Furthermore, functionally distinct clonal macrophage, B, T and NK-like cell lines (7–10) are available. In this study, genes related to *NITR*s have been identified in *Ictalurus* and are shown to encode for an extraordinary continuum of structural variation, including gene products that lack ITIMs and possess a positively charged residue in the transmembrane region, reminiscent of activating receptors of the LRC as well as TCRα/β (11–13). Different *NITR* genes exhibit both tissue- and lineage-specific expression patterns, and their expression is differentially regulated during the course of *in vitro* immune stimulation.

Materials and Methods

General Methods. For RNA isolation, poly(A)$^+$ selection, and construction of λDASH II (Stratagene) genomic, λZAP II (Stratagene), and λ Trpl-Ex2 (CLONTECH) cDNA libraries, we used standard technology (2). Libraries were screened with probes complementing *NITR* sequences from zebrafish, pufferfish, trout (J.A.Y., unpublished observations), and *Ictalurus*. Rapid amplification of cDNA ends (RACE) was performed by using *Ictalurus* head kidney mRNA and the GeneRacer kit (Invitrogen). The methods used for automated DNA sequencing, directional deletion of genomic clones, sequence alignments, identity searches, and assignment of protein domains have been described (2, 3).

RNA and DNA Blot Analyses. RNA blots were prepared as described by using Zetaprobe-GT (Bio-Rad; ref. 2). Probes complementing the V regions of *IpNITR*s 1–5 (labeled to equivalent specific activity) were hybridized in Expresshyb (CLONTECH) at 68°C and washed according to the manufacturer's specifications.

Genomic DNA (5 μg) was digested to completion with *Eco*RI or *Hin*dIII and subjected to electrophoresis in 1% LE agarose, transferred to Zetaprobe-GT under denaturing conditions, and UV crosslinked. Blots were hybridized either in Expresshyb (see above) or in 0.6 M NaCl/0.2 M Tris/0.02 M EDTA/0.5% SDS

Fig. 1. (*A*) CLUSTALW alignment of the predicted translation products of *IpNITR*s 1–4. The prototypic pufferfish (SN6A) and zebrafish (Nitr3.1) are shown for comparison. Identical residues are reverse image (black), functionally equivalent residues are in reverse image (gray). Leader (L), V, V/C2, J (FGXG), transmembrane (TM), CDR1 (111111), and CDR2 (222222) are indicated. Conserved cysteines are shaded with blue. Positively charged residues within the TM are shaded with red. Consensus ITIMs are shaded with yellow and ITIM-related sequences are shaded with orange. (*B*) CLUSTALW alignment of the predicted translation products of the single Ig domain-containing *IpNITR*s 5–11. The zebrafish Nitr3r.1 and portions of IpNITR1 in which the V/C2 region has been removed (circles) are shown for comparison; note the high level of identity of the C-terminal regions of IpNITRs 1, 6, 8, and 9. Labeling is as in *A*.

(conventional conditions) at 65°C and washed in 0.15 M NaCl/0.015 M Na citrate at 52°C.

***In Vitro* Allogeneic Stimulation.** *In vitro* allogeneic stimulations were performed by using peripheral blood leukocytes (PBLs) from outbred *Ictalurus* as effectors and γ-irradiated 1G8 clonal B cells as stimulators, at a 5:2 effector to stimulator ratio (14). Allogeneic stimulation was monitored both by [³H]thymidine uptake and allospecific cytotoxicity as measured by ⁵¹Cr release from 5×10^4 labeled 1G8 (as homologous targets) or 3B11 (as heterologous targets) B cells exposed to varying numbers of mixed leukocyte culture (MLC) effectors. MLC cytotoxic effectors were cloned subsequently by limiting dilutions in the presence of homologous target cells and yielded the 3H9 NK-like cell line, among others (9, 15).

Results and Discussion

Identification of Ictalurus NITRs. Heterologous screening of *Ictalurus* cDNA libraries (activated PBL, normal spleen, and head kidney) by using pufferfish *NITR* probes resulted in the identification of *IpNITR*2 and *IpNITR*4 (Fig. 1*A*; designations are based on the predicted level of structural complexity; see below). *IpNITR*3 was identified by heterologous screening of a second, higher representation *Ictalurus* head kidney cDNA library by using a probe complementing a V/C2 domain of a trout *NITR*. In addition, an *IpNITR*3-like genomic clone also was identified

and a two-round RACE strategy was used to identify the corresponding cDNA; however, multiple, closely related cDNA products were identified. Only cDNAs encoding an entire ORF were characterized further and include: *IpNITR*1 (Fig. 1*A*) as well as four additional transcripts (*IpNITR*s 5, 6, 8, and 9; Fig. 1*B*). *IpNITR*s 5, 6, 8, and 9 are distinguished by the presence of only a single (V) Ig domain. A fifth gene containing a single V domain (*IpNITR*7; Fig. 1*B*) was identified in a cDNA library screen by using an *IpNITR*3 probe. *IpNITR*10 and *IpNITR*11 were identified in the course of screening a 3H9 (an NK-like cell line) cDNA library with *IpNITR*3 and *IpNITR*5 V domain probes. Notwithstanding the variation that has been recognized in the overall organization of *NITR* genes, the particular methods used in studies to date with *Ictalurus* are not exhaustive in terms of defining all related genes, nor have been the strategies used previously to identify related genes in pufferfish (2) and zebrafish (3). Finally, although the degree of structural variation described herein is extreme, the designation *NITR* is used throughout to describe the *Ictalurus* genes based on the similarities of their Ig domains to the prototypic genes that were identified in the two other species of bony fish.

IpNITRs Exhibit Structural Diversity. Comparisons of predicted peptide structures of *Ictalurus* NITRs show considerable variation (Fig. 2). Specifically, mammalian Ig J motifs (FGXG), resembling those described previously in the V and V/C2
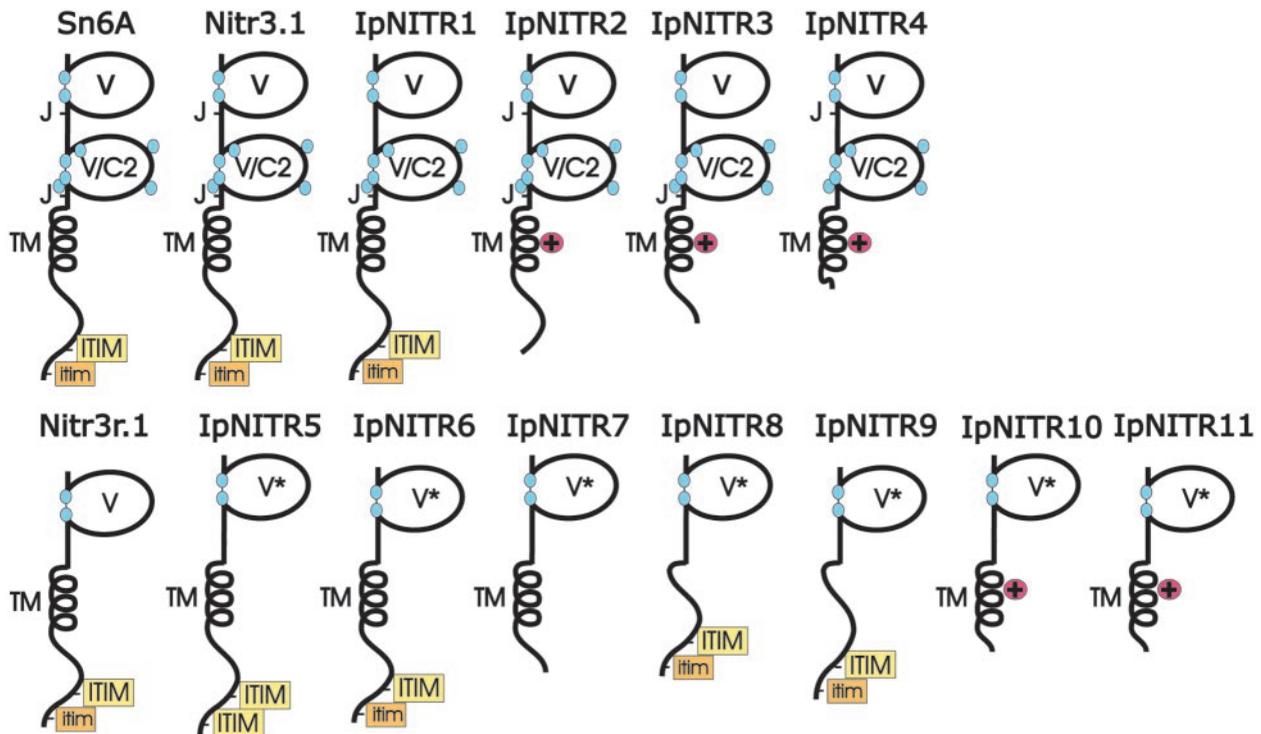
Fig. 2. Diversity in IpNITR structure. V, V/C2, joining (J; FGXG), and transmembrane (TM) regions are indicated. Consensus ITIMs are highlighted in yellow, ITIM-like sequences are highlighted in orange. Conserved cysteines are indicated with a blue oval. Conserved positively charged residues (+) are highlighted in red. The sequences of the Ig domains of IpNITRs 5–11 are related closely and indicated by ∗. Pufferfish SN6A (2) and zebrafish Nitr3.1 and Nitr3r.1 (3) are shown for comparison.

domains of NITRs found in other species of bony fish (2, 3), are present in the V regions of IpNITR2 and IpNITR4, as well as in the V/C2 domains of IpNITRs 1–3 (Fig. 1A). Cysteines C^{25} and C^{109} (assignment based on IMGT V region nomenclature; http://imgt.cines.fr) are predicted to provide a second disulfide bridge between the β-pleated sheets of the V/C2 domain and are conserved in IpNITRs 1–4 as well as in nearly all NITRs from other species (refs. 2 and 3; Fig. 1A).

The use of a charged group within a hydrophobic transmembrane region to stabilize interactions with adaptor proteins and/or signaling complexes is a common mechanism in certain activatory members of the LRC, as well as TCR and Ig (12). The ITIM-containing NITRs described here, as well as all of the other NITRs characterized to date, lack intramembranous charged groups. However, IpNITRs 2–4 contain an arginine in their transmembrane region (Fig. 1A). By analogy to the activatory signaling complexes that form between certain NK receptors and DAP family members (12), this charged group could potentially interact with an as yet unidentified adaptor protein. Overall, IpNITRs 1–4 vary in the (i) number of J-like sequences, (ii) presence of a positively charged residue in the transmembrane region, (iii) length of the cytoplasmic tail, and (iv) presence of ITIM and ITIM-like sequences (Figs. 1A and 2). It is likely that these structural differences are associated with functional variation (see below).

In contrast, IpNITRs 5–11 possess single V domains, which are ≈85–100% related. The V domain of IpNITR6 (selected as the reference) and the corresponding portions of IpNITRs 5 and 7–11 are from ≈91–99% related at the nucleotide sequence level. Variation is limited largely to two highly similar patterns in the second complementarity determining region (CDR2); one includes IpNITRs 6, 8, and 9 and the other includes IpNITRs 5, 7, 10, and 11. The single V-containing IpNITRs are most

structurally similar to the zebrafish receptor Nitr3r.1(3) (Figs. 1B and 2).

The sequences of the transmembrane regions of IpNITRs 5–7, 10, and 11 are very similar; however, the transmembrane region of both IpNITR10 and IpNITR11 possess a single, positively charged residue. Although IpNITR10 and IpNITR11 lack extended C-terminal sequence, several positively charged residues are immediately C-terminal of their transmembrane regions. These charged residues are conserved in IpNITRs 5–7, as well as IpNITRs 1–3; positively charged residues also are present in roughly corresponding positions in IpNITR8 and IpNITR9, which lack transmembrane regions. In addition, C-terminal sequences of IpNITR8 and IpNITR9 are nearly identical to IpNITR6 and IpNITR1. The ITIM and ITIM-like sequences in IpNITRs 6, 8, and 9, as well as IpNITR1, are identical, as is the more N-terminal ITIM in IpNITR5. IpNITR5 is the only NITR-related gene characterized thus far that possesses two consensus ITIMs (Fig. 1).

Genomic Analyses. Owing to the variation in the overall structure of *IpNITRs*, single exon probes (V-domain) were used in Southern blot analyses. Relatively few fragments hybridized with the *IpNITR* 1, 2, and 4 probes. Differences in the numbers of hybridizing elements in the *Eco*RI and *Hin*dIII digests can be accounted for by *Eco*RI sites in the V regions of *IpNITR*1 and *IpNITR*2 and a *Hin*dIII site in the V region of *IpNITR*4 (Fig. 3). It is likely that only one or two copies of *IpNITRs* 1, 2, and 4 are present. In contrast, multiple fragments hybridize with the *IpNITR*3 and *IpNITR*5 V probes (which are ≈67% identical at the nucleotide level). Although similarities in hybridization patterns are evident when using Expresshyb (Fig. 3A), there also are unique features—e.g., an ≈9-kb band in the *Eco*RI-digest probed with *IpNITR*3 is not prominent with the *IpNITR*5 probe. Under conventional conditions, only the IpNITR5 "family"

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A.

IpNITR1 IpNITR2 IpNITR3 IpNITR4 IpNITR5

E H E H E H E H E H

kb

23
9.4
6.7

4.4

2.3
2.0

0.6

B.

IpNITR3 IpNITR5
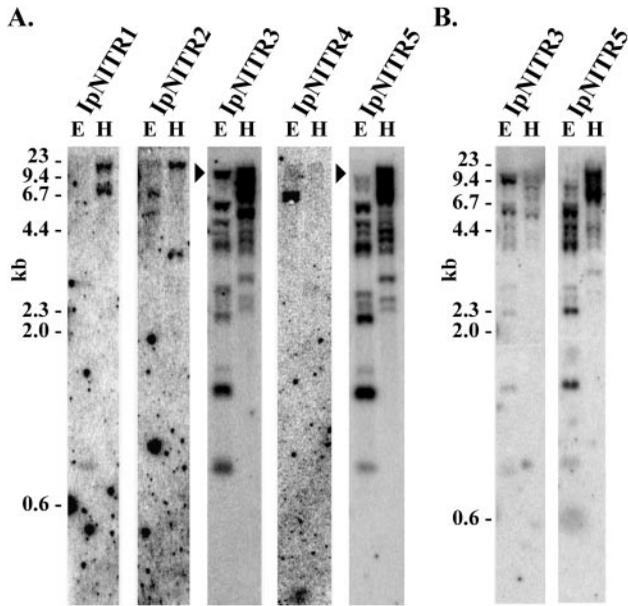
E H E H

kb

23
9.4
6.7

4.4

2.3
2.0

0.6

Fig. 3. Genomic complexity of *IpNITR*s. Southern blot transfers were hybridized in Expresshyb (*A*) or under conventional conditions (*B*) with probes complementing the V domains of IpNITRs 1–5 labeled to equivalent specific activity. Arrowheads are used to emphasize certain positions; size standards are indicated. Restriction sites for *Eco*RI and *Hin*dIII within V domains account for the differences in hybridization intensity for individual probes.

appears to be highly complex (Fig. 3*B*). This observation is consistent with the documented nucleotide sequence identities between the structurally distinct *IpNITR*s 5–11 and the likelihood that there are additional related types of genes.

The genomic organization of several IpNITRs has been determined (Fig. 4). The predicted exon structures (splice donor and acceptor sites, termination codons, and polyadenylation signal sequences) are entirely consistent with the cDNA sequences. The overall genomic organization of *IpNITR*s 1–4 resembles that of *NITR* genes that have been identified in pufferfish (2) and zebrafish (3), with the exception of cytoplasmic exons in IpNITRs 2–4. The possibility remains that variation in the structure of NITRs could derive in part through differential RNA processing, which has been proposed for the *nitr4* genes in zebrafish (3); however, there is no other evidence for alternative RNA processing contributing to the variability of *NITR*s. *IpNITR*2 and *IpNITR*4 are separated by an ≈3-kb
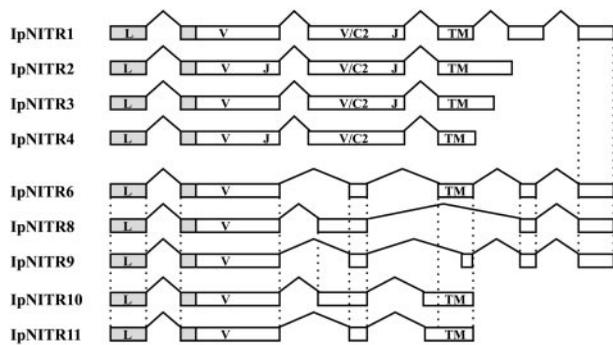
Fig. 4. Exon organization of IpNITRs. Exons are boxed, intergenic sequence similarity is indicated by vertical dotted lines; leader (L), V, V/C2, joining (J), and transmembrane (TM) regions are indicated. Some components of the genomic organization of IpNITRs 1, 6, 8, and 9 are inferred from highly related genes.
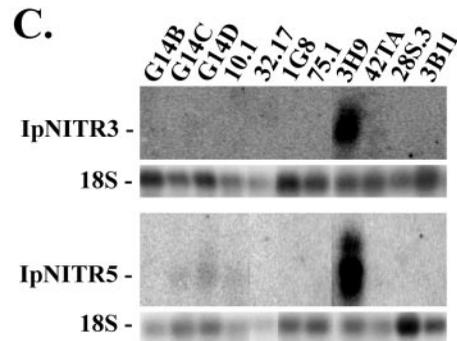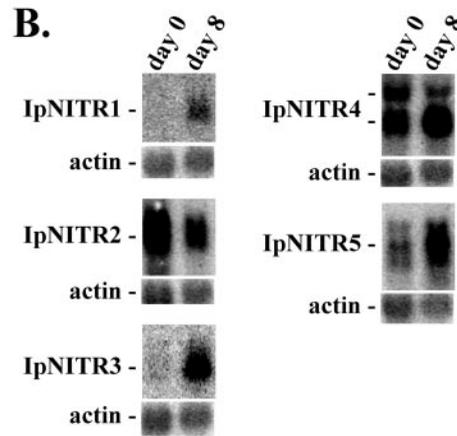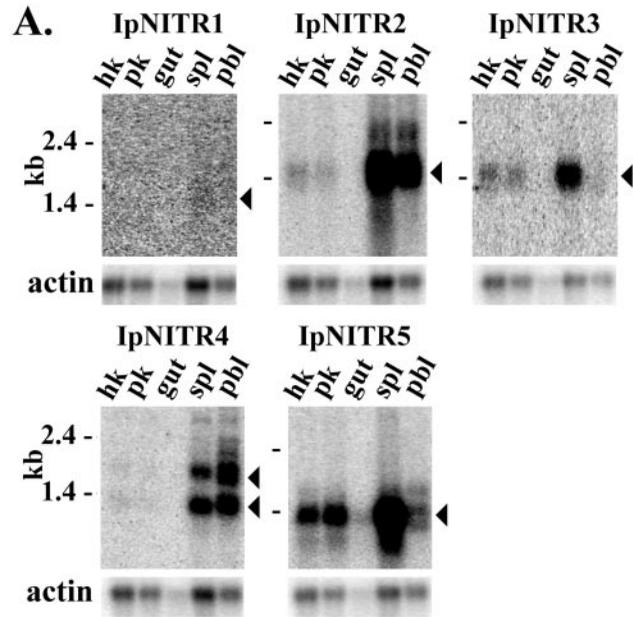
A.

IpNITR1   IpNITR2   IpNITR3

hk pk gut spl pbl   hk pk gut spl pbl   hk pk gut spl pbl

kb

2.4

1.4

actin

IpNITR4   IpNITR5

hk pk gut spl pbl   hk pk gut spl pbl

kb

2.4

1.4

actin

B.

day 0 day 8

IpNITR1
actin

IpNITR2
actin

IpNITR3
actin

day 0 day 8

IpNITR4
actin

IpNITR5
actin

C.

G14B G14C G14D 10.1 32.17 1G8 75.1 3H9 42TA 28S.3 3B11

IpNITR3
18S

IpNITR5
18S

Fig. 5. Expression patterns of *IpNITR*s. (*A*) RNA blot [≈1 μg/track of poly(A)+ mRNA] of head kidney (hk), posterior kidney (pk), gut, spleen (spl), and peripheral blood leukocytes (pbl). Probes complementing the V region of *IpNITR*s 1–5 were labeled to equivalent specific activity. Predominant transcripts are indicated with arrowheads. β-actin was used as a loading control; size standards are indicated. (*B*) RNA blot comparing expression of *IpNITR*s 1–5 at time 0 and after day 8 of one-way MLC. Total RNA (10 μg) was loaded into each track. Probes, hybridization, and loading controls are as in *A*. (*C*) RNA blot comparing expression of *IpNITR*s 1–5 in *Ictalurus* cell lines derived from peripheral blood leukocytes. Cell lines used include TCRα/β+ T cells (G14B, G14C, G14D, 32.17, and 28S.3), IgM+IgD− B cells (1G8), IgM+IgD+ B cells (3B11), TCRαβ−, Ig− cytotoxic cells (10.1, 75.1, and 3H9), and macrophages (42TA). Total RNA (2 μg) was loaded into each track. Probes and hybridization are as in *A*. 18S RNA served as a loading control.

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intervening sequence; no evidence has been found for physical linkage between additional NITR elements. Extensive genomic sequencing such as that conducted previously in pufferfish (2) and which currently is in progress through the Zebrafish Genome Project likely will be needed to fully interpret the overall organization of *IpNITRs*.

IpNITR Genes Exhibit Tissue-Specific Expression. RNA blots are consistent with differential expression of *IpNITR* genes (Fig. 5*A*). Several conclusions can be drawn: (*i*) strong hybridization signals can be detected with the *IpNITR* 2–5 probes; (*ii*) *IpNITR*1 is detected only in spleen and at very reduced levels relative to the other *IpNITR* genes; (*iii*) different hybridization patterns occur with each of the aforementioned probes (based on genomic analysis, similarities in the *IpNITR*3 and *IpNITR*5 hybridization patterns are likely probe-related); (*iv*) two different RNA populations consistently hybridize with the *IpNITR*2 and *IpNITR*4 probes and this result could be reproduced by using RNA extracted from different animals; and (*v*) the broadest expression pattern is achieved with the *IpNITR*5 probe, which also produces the most complex genomic hybridization pattern in the Southern blot analyses.

Expression of IpNITR Genes in the MLC. One-way MLC using PBL responders and γ-irradiated clonal (1G8) B cell stimulators was performed and the kinetics of [³H]thymidine uptake were quantitated. Thymidine uptake predictably increased between one and six days, with a plateau achieved by day 9 (14). A biphasic increase in *TCRα* expression was observed relative to β-actin and likely is due to activation in primary allospecific T cells and T helper cells, with a later apparent increase as those T cells expand clonally in the effector population (data not shown). RNA analyses were carried out on mRNA isolated from an 8-day MLC as well as from an unstimulated day 0 control employing probes complementing *IpNITR*s 1–5 (Fig. 5*B*). The increase in expression of *IpNITR*s 1, 3, and 5 is consistent with an expansion of the lineage(s) expressing these genes through the course of the MLC. Significant levels of basal expression are evident for *IpNITR*2 and *IpNITR*4, which are expressed abundantly in PBLs (Fig. 5*A*). The expression of *IpNITR*2 decreases and the expression of *IpNITR*4 remains roughly equivalent in the 8-day MLC. These expression patterns reflect the kinetics of the

MLC reaction; *IpNITR*2 and *IpNITR*4 are likely expressed in several different cell types, of which some die off and others expand. Con A-stimulation failed to increase expression of *IpNITR*s (data not shown), which is inconsistent with expression of these receptors on T lymphocytes.

RNA from different *Ictalurus* cell lines also was hybridized with probes complementing the five different *NITR* families (Fig. 5*C*). Although expression of *IpNITR*s 1, 2, and 4 was not detected in any of the cell lines tested (data not shown), a high level of expression of *IpNITR*3 and *IpNITR*5 was noted for cell line 3H9, which was derived from PBLs through repeated allogeneic stimulation by using irradiated 1G8 cells and cloned by limiting dilution; considerably weaker hybridization was noted for cell lines G14C and G14D (both T cell lines derived from a gynogenetically derived channel catfish), as well as for 10.1 (NK-like cells; ref. 9). Functionally, 3H9 is cytotoxic toward a number of allogeneic cell lines (15). Because 3H9 is negative for both *TCRα/β* and IgM messages, and expresses the ligand for mAb CC41 (15), which is hypothesized to correspond to CD56, a mammalian NK marker, it might represent a NK equivalent or alternatively a γδT cell. Although 3H9 cells are negative for IgM mRNA, they show positive immunofluorescence to mAbs specific to the IgM heavy chain (Igμ) and both isotypes of Ictalurus Ig light chains (F and G) (15). Simultaneous expression of two light chain isotypes and the ability to modulate IgM off the cell surface by using anti-*Ictalurus* Igμ and replace it with affinity-purified *Ictalurus* antitrinitrophenol IgM antibodies are consistent with surface IgM being bound to a putative Fc receptor.

Limited screening of a 3H9 cDNA library resulted in the identification of *IpNITR*10, *IpNITR*11, several copies of *IpNITR*5, and three cDNAs corresponding to partially processed *IpNITR*3 and *IpNITR*5. The length variation in these various forms explains, in part, the disperse band observed in RNA blot analyses (Fig. 5*C*). Whereas function cannot be inferred from the expression patterns of *NITR*s, their expression in cytotoxic effectors and likely in other cell types further supports their possible role(s) in regulating various immune effector functions, as well as other recognition processes.

Relatedness of the NITRs and Relationships to the LRC. Various NITRs described to date possess characteristics of the inhibitory receptors encoded at the mammalian LRC, including Ig domains
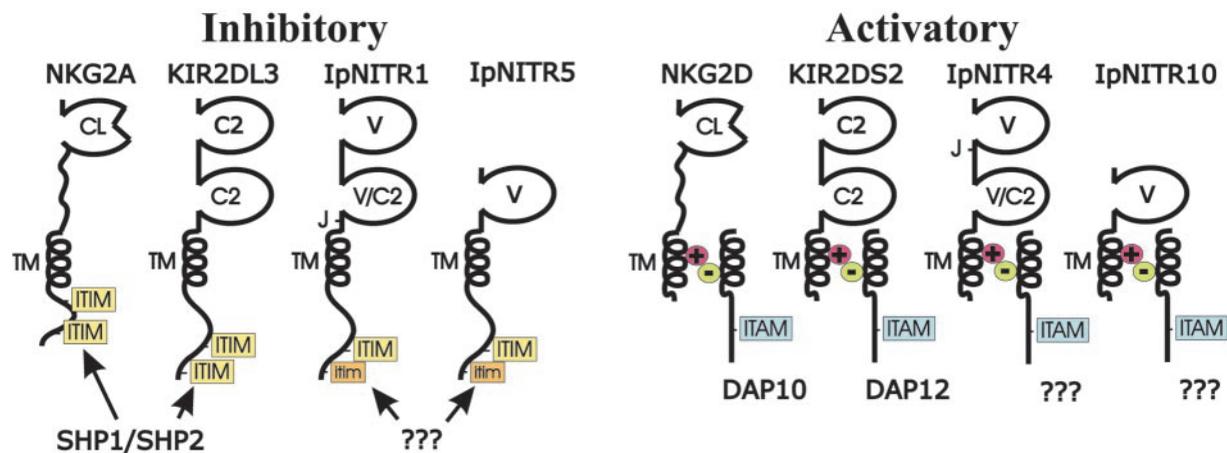

Fig. 6. Hypothetical signaling relationships of V domain-containing IpNITRs to LRC gene products. IpNITR1 and IpNITR5 are depicted as immune inhibitory receptors, which use two potential ITIMs and might signal through interactions with SHP1 and/or SHP2 as demonstrated previously for the mammalian receptors NKG2A and KIR2DL3. In contrast, IpNITR4 and IpNITR10 are depicted as immune activatory receptors lacking an ITIM and possessing an intramembranous positively charged residue and might functionally correspond to the mammalian receptors NKG2D and KIR2DS2 (13), which are associated with the negative charge-containing adaptors DAP10 and DAP12, respectively. Abbreviations are as in Fig. 2. C-type lectin domains (CL) and immunoreceptor tyrosine-based activation motifs (ITAM) also are shown.

and ITIM-mediated inhibition of MAPK (3) as well as NK killing (transfected mammalian cell lines) *in vitro* (J. Djeu, S. Wei, J. A. Y., and G. H. L., unpublished observations). The NITR variants described here expand the range of related gene products. IpNITRs 1–4 possess two extracellular Ig domains, a transmembrane region, and cytoplasmic tails of varying lengths (with or without ITIMs), and resemble most closely the NITRs described in pufferfish and zebrafish. IpNITRs 2–4, which possess charged intramembranous residues and for which equivalents have not yet been identified in the two other teleost fish systems, could potentially associate with adaptor molecules and function in an activatory pathway rather than in the postulated inhibitory pathway of the ITIM-containing NITR genes (Fig. 6). The identification of single Ig domain receptors that also possess charged intramembranous residues (IpNITR10 and IpNITR11) suggests that such pairings might be a basic feature of the NITRs. Identification of suitable adaptor molecules in this species would strengthen such proposals. As a family, NITRs exhibit potential for the type(s) of highly regulated positive and negative signals associated with the LRC gene products (ref. 4; see below).

IpNITRs 5–11 possess a single V domain and can be grouped into four potential functionally significant categories: transmembrane-containing with and without inhibitory motifs, transmembrane-containing with positively charged residues, and transmembrane-lacking. Structural comparisons can be made to several recently described mammalian single Ig domain-containing receptors, PD-1 (16) and NKP44 (17), or pairs of receptors, CMRF-35 and CMRF-35-H9 (18), as well as PILRa/FDF03/FDF03-ΔTM and PILRb (19, 20). These receptors are encoded outside the LRC and each receptor or pair of receptors is on a different chromosome. Of these, PILRa and PILRb appear most related to IpNITRs 5–11; PILRa is a single Ig domain receptor with a cytoplasmic tail containing two ITIM motifs (a putative inhibitory form). PILRb is a very similar receptor (90% amino acid identity through the first three exons) containing both a short highly charged cytoplasmic tail and a charged lysine in the transmembrane (a putative activatory form). Two alternatively spliced variants of the membrane-bound PILRa containing identical leader sequences and lacking TM regions are likely secreted in a manner similar to IpNITR8 and IpNITR9. However, unlike mammalian forms, all of the IpNITRs encode V regions, which are related closely.

Evolution of the NITR Genes. Collectively, the results reported here define a continuum of variation in form and presumably function for the NITRs. At this stage in the analysis of NITRs it is not possible to determine whether the V region serves a primary recognition capacity similar to that seen in Ig and TCR, or whether these receptors recognize a common ligand—e.g., major histocompatibility complex (MHC)—as do several products of the LRC. What relationships, if any, the single V domain receptors have to the more typical diversified NITRs also remains to be determined.

Ictalurus and *Danio* (zebrafish) are representative members of a large assemblage the Ostariophysans, which encompass one of the four phylogenetic infrastructures of bony fish. *Spheroides* (pufferfish) represents a highly divergent lineage within that group. The studies reported here both confirm the presence of *NITR* genes in a third lineage of bony fish and expand the range of genes that possess an *NITR* character to now include eleven different members. Even in the absence of large-scale genomic scanning or a more comprehensive cDNA cloning strategy, a remarkable continuum of structural variation is defined. High levels of relatedness between exons that have been identified in different genes appear to be a common feature of the *IpNITR* genes and are consistent with gene duplication and exon exchange (2, 4). Whether the single Ig domain-containing genes found in *Ictalurus* diverged from or potentially gave rise to the genes possessing two extracellular domains is not apparent; however, extensive genomic sequencing available for the zebrafish *nitr* gene locus suggests that the single Ig domain gene *nitr3r.1* derived from a two-Ig-domain *nitr3* precursor (J.A.Y., unpublished observations).

The introduction of the *IpNITR* data provides additional possible precursor gene structures that might have factored in the evolution of rearranging immune receptors. Defining hierarchical relationships of the divergent forms of *NITR*s seen in extant bony fish would benefit from information about related genes present in species that diverged earlier in vertebrate phylogeny. This being the case, efforts are underway in our laboratory to identify gene structures related to the *NITR*s in cartilaginous fish, jawless vertebrates, and protochordates that are requiring the development of novel technologies to exploit the limited sequence identities between the highly diverse *NITR* genes.

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