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The Antigen Receptor (NCCRP-1) on Catfish and Zebrafish Nonspecific Cytotoxic Cells Belongs to a New Gene Family Characterized by an F-Box-Associated Domain

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Abstract. The catfish nonspecific cytotoxic cell receptor protein (NCCRP-1) provides an important function in target cell recognition and activation of cytotoxicity. This report identifies and characterizes a zebrafish orthologue of the catfish NCCRP-1. The zebrafish NCCRP-1 cDNA contains an open reading frame that encodes a predicted protein of 237 amino acids with a MW of 27 kDa and a p*I* of 5.5. Sequence similarities comparisons show that the NCCRP-1 receptors from these two phylogenetically distant species share a high degree of identity. These results suggested that NCCRP-1 performs a crucial function in innate immunity in teleosts. Further, a zebrafish 17-mer peptide corresponding to the catfish NCCRP-1 antigen-binding domain inhibited (catfish) cytotoxicity toward conventional tumor target cells (HL-60). These data appeared to indicate that the zebrafish NCCRP-1 protein may function as an antigen recognition molecule and, as such, may participate in innate immunity in teleosts. A homology search of the zebrafish NCCRP-1 protein revealed that it 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 FBA is currently of unknown function. A new gene family is proposed in this work, based on similarities in the FBA sequences with the catfish and zebrafish NCCRP-1 peptides. This new gene family includes several F-box domain-containing proteins and a predicted *C. elegans* protein.

Key words: Zebrafish — Nonspecific cytotoxic cells — Antigen-binding domain — Catfish — expressed sequence tags — F-box — F-box-associated domain

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Introduction

Nonspecific cytotoxic cells (NCC) are the "lower" vertebrate counterparts of mammalian natural killer (NK) cells. NCC lyse a wide variety of tumor targets, protozoan parasites, and virus-infected cells and probably participate in antibacterial immunity by elicitation and secretion of cytokines (Evans et al. 1984; Graves et al. 1985a, b; Evans and Friedmann 1992). NCC collaborate with other nonspecific effectors (phagocytes, etc.) to provide innate resistance during acute stress responses induced by opportunistic organisms, pathogens, and protozoan parasites (Zuznetsov 1996; Ross and Vetvicka 1993). Several anuran species (Watson and Horton 1996; Ghoneum and Cooper 1990) as well as many teleosts, e.g., tilapia (Faisal et al. 1989), damselfish (McKinney and Schmale 1994), rainbow trout (Greenlee et al. 1991), and carp (Suzumura et al. 1994), have been shown to have NCC or similar cytotoxic cells. In these species, NCC may also participate as cellular effectors of innate immunity.

NCC and mammalian IL-2-activated NK cells (lym-

phokine activated/adherent lymphokine activated; LAK/ALAK) either have a promiscuous antigen recognition capability or recognize the same antigen on most target cells. All NCC-sensitive target cells are also targets of human ALAKs (Evans and Friedmann 1992). LAK and ALAK cells from mice, rats, and humans "acquire" an ability to lyse numerous histological types of target cells, whereas NCC innately have this ability.

While mammalian NK cells undergo negative regulation following recognition and binding of class I histocompatibility alloantigen (Moretta et al. 1990a, b), no such inhibitory receptors have yet been described for teleost NCC. Unlike the consequence of alloantigen recognition, NK/ALAK/NCC binding to a conventional antigen on a target cell may be required to initiate the lytic cycle. Not a single hypothesis has been brought forward to explain the mechanism of mammalian NK recognition of protozoan parasite antigens, and it is not clear how certain bacteria activate NK cells to secrete cytokines such as γ-interferon. In teleosts, the antigen recognition receptor for NCC, NCCRP-1, has been sequenced (Jaso-Friedmann et al. 1997a; Evans et al. 1998, 1999). Binding of the target cell ligand NKTag (natural killer target antigen/NKTag) (Evans et al. 1996; Jaso-Friedmann et al. 1996, 1997b) to the receptor NCCRP-1 leads to target cell killing. NKTag is found on many histological types of tumor cells (Evans et al. 1996) and on protozoan parasites (Leary et al. 1994). We have obtained a complete cDNA-derived amino acid sequence of NKTag (Jaso-Friedmann et al. 1997a) and propose that NKTag may be the only conventional antigen recognized by NCC.

In the present study we have cloned and characterized the zebrafish orthologue of the catfish NCCRP-1 from expressed sequence tags (ESTs). A homology search comparison shows that NCCRP-1 from at least three phylogenetically distant species (catfish, tilapia, and zebrafish) share a high degree of identity. These results suggested that NCCRP-1 performs a crucial function in innate immunity in teleosts. Further homology analysis yielded a significant level of identity with another group of proteins belonging to an F-box subfamily (Winston et al. 1999; Kipreos and Pagano 2000). 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 FBA, currently of unknown function, is present in the NCCRP-1 DNA in all the teleost species. Based on these results, a new gene family is proposed containing the C-terminal FBA domain but carrying distinct functional domains at the N terminus.

Materials and Methods

Enzymes. Restriction enzymes were purchased from Roche Molecular Biochemicals (Indianapolis, IN) and from Promega (Madison, WI). The Expand Long PCR reagent kit was obtained from Roche Molecular Biochemicals. Plasmid purification columns were purchased from Qiagen (Valencia, CA). All other chemicals were molecular biology grade and were obtained from standard suppliers.

DNA Sequencing and Primer Preparation. DNA sequencing was done using Taq polymerase in the dideoxy dye termination reaction (Sanger et al. 1977). ESTs were cloned into plasmids and both strands of at least two clones were sequenced. Sequences were analyzed using a 373A DNA Sequencer (Applied Biosystems) at the Molecular Genetics Instrumentation Facility, University of Georgia, following the standard protocol as described by the manufacturer. Primers for PCR and DNA sequencing were synthesized by the Molecular Genetics Instrumentation Facility (University of Georgia, Athens).

Expressed Sequence Tag Identification and Computer Analyses. The zebrafish NCCRP-1 sequence was identified by a BLAST (Altschul et al. 1990) search of the zebrafish EST database at the National Center for Biotechnology Information (NCBI). ESTs were purchased from Genome Systems, Inc. (St. Louis, MO). The confirmed sequence of the zebrafish NCCRP-1 orthologue has been submitted to Genbank under accession number AAF19642. Multiple sequence alignments were performed using ClustalW (Thompson et al. 1994). The alignment was manually edited to minimize insertions and deletions and includes just the homologous regions, which comprised the C-terminal F-box-associated domain for each sequence. Phylogenetic analyses were performed using MEGA version 2.0 (Kumar et al. 2001). The aligned data set was analyzed by the criteria of maximum parsimony using the branch-and-bound algorithm. The reliability of the trees was tested using 500 bootstrap replicates. The alignment was also analyzed by the neighbor-joining (NJ) method (Saitou and Nei 1987), as implemented by Mega. For NJ, Poisson correction was used with the complete deletion option, and again, 500 bootstrap replicates were analyzed. Finally, the data were analyzed with PAUP version 4.0b8 using an exhaustive search with the maximum parsimony algorithm and the *C. elegans* sequence as an outgroup.

Inhibition of Catfish NCC Activity with NCCRP-1 Peptides from Zebrafish. The University of Georgia Molecular Genetics Facility synthesized the 17-mer peptide corresponding to the antigen-binding site on catfish NCCRP-1. The zebrafish orthologue peptide corresponding to amino acids (aa) 105–120 (Fig. 2), as well as a negative control peptide (same aa composition in a "scrambled" sequence), also was tested. Lyophilized peptides were resuspended in distilled water until dissolved. An equal volume of (2×) PBS/0.5% BSA/20 m*M* glucose was then added to obtain the indicated protein concentrations. Peptides were incubated with labeled target cells for 0.5–1 h at room temperature. The synthetic peptides were tested on target cells and were shown not to produce nonspecific toxic effects on any target cell (data not shown). Cytotoxicity assays were conducted as described previously (Jaso-Friedmann et al. 1997a).

Results

Identification of Zebrafish NCCRP-1. We expected that the zebrafish NCCRP-1 protein would share a high degree of similarity with its catfish orthologue. In an attempt to identify zebrafish ESTs encoding NCCRP-1, BLAST searches were conducted of the zebrafish EST data bank using the catfish NCCRP-1 protein as a query. This search identified only four ESTs, all of which were found in a fin regeneration library. EST AI331491 was characterized further by sequencing (Fig. 1). Comparison of the EST AI331491 translation product with the se-

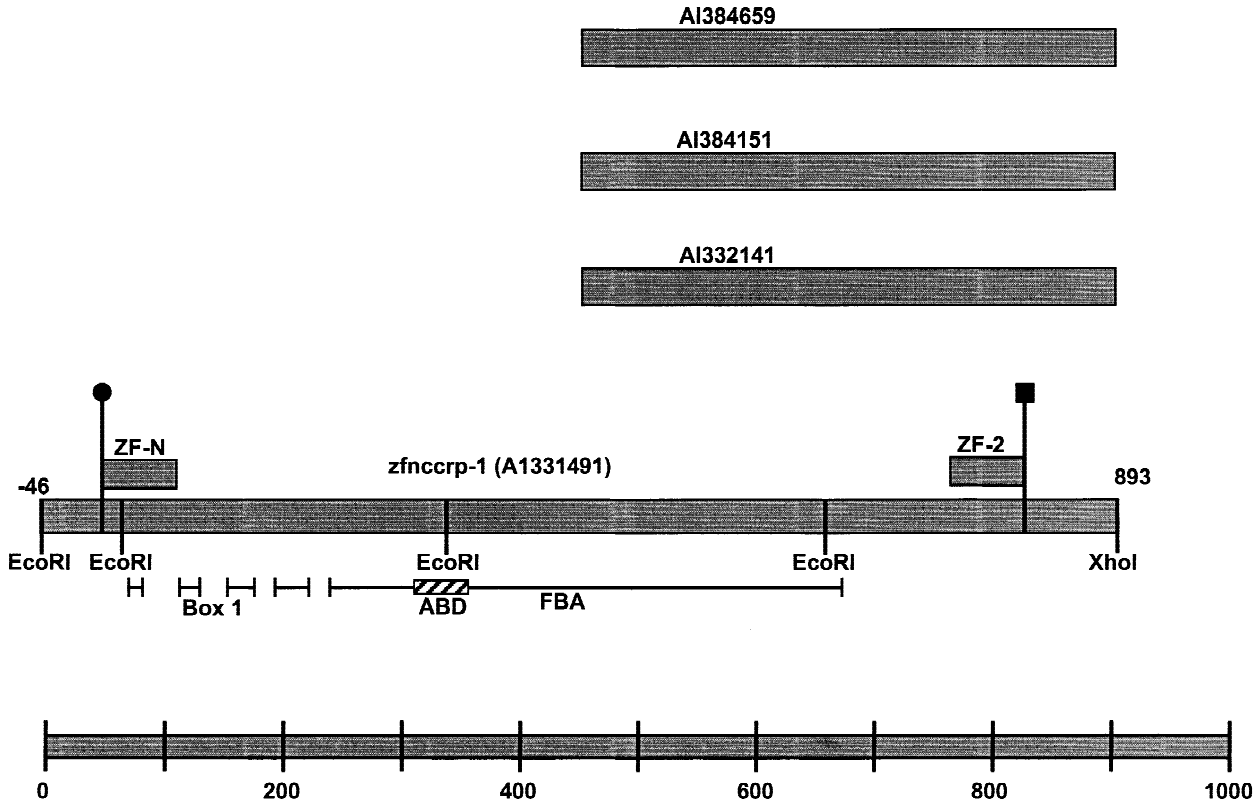

Fig. 1. Schematic representation of the zebrafish NCCRP-1 EST. A BLAST search of the zebrafish EST database using the catfish NCCRP-1 amino acid sequence as a query identified four highly similar EST sequences (AI331491, AI384659, AI384151, and AI332141). Sequencing of EST AI331491 allowed characterization of the entire zebrafish NCCRP-1 ORF. In addition, RT-PCR with primers ZF-N (+1 to +30 bp; Fig. 2) and ZF-2 (+685 to +715 bp; Fig. 2) was used to amplify the complete NCCRP-1 ORF from liver cDNA. A *line with a filled circle* indicates the initiation codon (position +1) of the ORF. A *line with a filled square* indicates the position of the stop codon of the ORF. The *open box* indicates the ORF (nucleotide sequence and translation shown in Fig. 2). A scale (bp) is shown at the bottom.

quence of the catfish NCCRP-1 protein indicated that this clone contained the complete ORF of the zebrafish NCCRP-1 orthologue. To confirm the sequence of this ORF, an RT-PCR reaction was performed using zebrafish liver RNA as a template and primers at positions +1 to +30 (ZF-N) and +685 to +715 (ZF-2) (Fig. 1). A product of the expected size was obtained. This product was sequenced and the translation of that sequence showed 100% identity to the translated sequence from the AI331491 clone.

Characteristics of the Zebrafish EST Sequence. Computer analysis of the sequence of clone AI331491 predicted a 46-bp 5′-untranslated region, an 837-bp coding region, and 129 bp of a 3′-untranslated region followed by a poly(A) tail (Fig. 2). Although the putative 5′-untranslated region does not contain an upstream in-frame stop codon, several lines of evidence suggested that the indicated ATG is the correct initiation site. First, the sequence surrounding this ATG codon conforms to the consensus sequence for eukaryotic translation initiation sequences with a purine at position −3 (Kozak 1989), which is the most critical residue for translation initiation (Fig. 2). Second, the position of the initiation codon of the zebrafish is in agreement with the location of the initiation codon of the gene of the catfish and tilapia orthologues. The catfish NCCRP-1 gene has been cloned and sequenced, and it has an upstream stop codon in-frame with the initiation codon (accession number AF159718). The zebrafish cDNA is predicted to encode a 237-aa protein with a MW of 27 kDa, a p*I* of 5.5, and two putative Asn-linked glycosylation sites in aa positions 52–55 and 80–83. The polyadenylation signal (AATAAA) used by the zebrafish *nccrp-1* gene is identical to the catfish and tilapia NCCRP-1 transcript polyadenylation signals.

Functional Domain Conservation in the Two Predicted NCCRP-1 Proteins. The zebrafish NCCRP-1 protein is 66.2% identical to the catfish and tilapia homologues. As no information is available about the zebrafish innate immune system, it was of interest to study the possibility that the proteins serve a similar function in all three species. We have reported previously that activation of catfish NCC leads to the physical association of the receptor NCCRP-1 with Janus-family kinases (JAK), before phosphorylation of the transcriptional activator STAT, a step that is required for its

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                CGGCACGAGAGTGAGCGGACCACGGGGGAAACAGTCTGACTCTGCA  -46
ATGGCGACTGACTGGAAGCAGAAGTGTGACTCGGAATGGCAGCTCGGGGCTCACGGTGTC   60
  M  A  T  D  W  K  Q  K  C  D  S  E  W  Q  L  G  A  H  G  V   20

CCAATGCCTGACACCGTGGACTGGAAATCGGTGTTCGAGACGAAGCCGTTCGAGCGCAAT  120
  P  M  P  D  T  V  D  W  K  S  V  F  E  T  K  P  F  E  R  N   40

CTACTGCAAAACCCCTCGCCTTACGGTGTGAACCACACTGTTCCGCCACCTGAACCCCAT  180
  L  L  Q  N  P  S  P  Y  G  V  N  H  T  V  P  P  P  E  P  H   60

CGGTCAGGAATACCACCTCCTTCAGACCGACCACCTCAGTTGGAGCCTGAAGGTAATTTC  240
  R  S  G  I  P  P  P  S  D  R  P  P  Q  L  E  P  E  G  N  F   80

TCTGGCTGGAAAACTAATACAGAAGTTTTGCCCTATGACACTAGTGGAATTCCTCCCGGT  300
  S  G  W  K  T  N  T  E  V  L  P  Y  D  T  S  G  I  P  P  G  100

GTTGTGATCTGCCAGCTTCCTCAGCACAGGTGGTTCACTCTAGAGCAGTGTGTGGACCTG  360
  V  V  I  C  Q  L  P  Q  H  R  W  F  T  L  E  Q  C  V  D  L  120

AAGGCAGCAGGTCTGTGGGACCAACTGCTGGACGACTTTCAGCCAGAGATTGTCATTGAA  420
  K  A  A  G  L  W  D  Q  L  L  D  D  F  Q  P  E  I  V  I  E  140

GACTGGTATGAGGAAAGCCAGCTTCATAAATGCATCTATCAGCTTGATGTGAAGCTCCTG  480
  D  W  Y  E  E  S  Q  L  H  K  C  I  Y  Q  L  D  V  K  L  L  160

GGTGCTGATGGTGAGACTGTTATCAAGCAGCACACCTATAACCCTGAAGAGGACCTGGAG  540
  G  A  D  G  E  T  V  I  K  Q  H  T  Y  N  P  E  E  D  L  E  180

TGCTACTCACACAACTGGAAAAAGGTCTCCCATGTGTTCTCCAAGTATGGGCCGGGGGTG  600
  C  Y  S  H  N  W  K  K  V  S  H  V  F  S  K  Y  G  P  G  V  200

CGGTACATTCACTTCCTCCACAGACTGAAGAACCAGTTCATGGTTGAATTCTTTAATACC  660
  R  Y  I  H  F  L  H  R  L  K  N  Q  F  M  V  E  F  F  N  T  220

AAAGTCACAGACAGCTCAGTTATTGTCAAGACCAGCAAACCCAGTGTGAAATAATAATCC  720
  K  V  T  D  S  S  V  I  V  K  T  S  K  P  S  V  K  *  *      237
ATCAGTCAAATTAATTTGATAAAGTCAGCAATAATATTACAAGTATTCAACTTGCATAGT  780
TCTTAAAGTAACTGCATTGACAGTAATATAATAAAACATTCCTTTCTCAAAAAAAAAAAA  840
AAAAAAAAAAAAAAA                                               954
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Fig. 2. Nucleotide sequence of the zebrafish NCCRP-1 cDNA **(top)** and amino acid translation **(bottom)**. Both nucleotide and amino acid sequences are numbered from the beginning of the ORF. Nucleotides upstream of the first in-frame ATG are assigned negative numbers. The peptide sequence used in the cytotoxicity assay is in *boldface.* The putative polyadenylation signal is indicated by the *underlined* 14 bp upstream of the poly(A) tail. The sequence data are available from GenBank (accession number AF207707) for zebrafish NCCRP-1.

nuclear translocation (Evans et al. 1999; Evans and Jaso-Friedmann 2000). Analysis of the predicted amino acid sequence of the zebrafish NCCRP-1 protein revealed that it shares these proposed physiologically relevant proline-rich motif domains (Box-1 motifs) characteristic of the N-terminus region of the catfish and tilapia orthologues. Box-1 motifs were first described in gp130 heavy chains associated with the IL-6 receptor (Marakami et al. 1991). The truncated form of this motif (PXP) is considered to be the high-affinity docking site for JAK, and it is present in the zebrafish orthologue at least three times: aa 21–23, 45–47, and 57–59. Interestingly, the full-length Box-1 motif that is defined by a proline-rich region is also present in the zebrafish NCCRP-1 orthologue in the inverted direction (Fig. 3). Furthermore, at least one additional PPP motif (aa 55–57) is also shared by the three NCCRP-1 orthologues.

Inhibition of Catfish NCC Target Cell Lysis with Synthetic Peptides of the Zebrafish NCCRP-1 Orthologue. Further evidence of functional similarity between the two NCCRP-1 proteins was obtained in the next experiments. The catfish NCCRP-1 target recognition sequence has been mapped to amino acids corresponding to positions 105 to 120 (Evans et al. 1998). Peptides corresponding to the antigen recognition sequence of zebrafish and catfish NCCRP-1 (aa 105–120) (Fig. 2) were synthesized, as well as a negative control peptide (same aa composition in a "scrambled" sequence). The peptides were tested in a catfish NCC cytotoxicity assay, and their ability to compete for target cell binding with the native receptor on catfish NCC was assessed. Results show that the zebrafish NCCRP-1 peptide corresponding to the aa 105–120 competes for binding to an antigen on target cells recognized by catfish NCCRP-1 (Table 1). A comparison between these two peptides defined a consensus sequence that may indicate the amino acids involved in target–ligand interactions and that may act as "anchors" to bind to NKTag on the target cell (Fig. 4).

Homology Analysis and Definition of a New Gene Family. To identify sequences related to the zebrafish NCCRP-1 we performed a PSI-BLAST search of Genbank (Altschul et al. 1997). Two iterations of PSI-BLAST identified all sequences related to NCCRP-1. The search yielded 10 mammalian sequences and one from the nematode *C. elegans* with significant levels of similarity to NCCRP-1 (Fig. 5). All of the identified

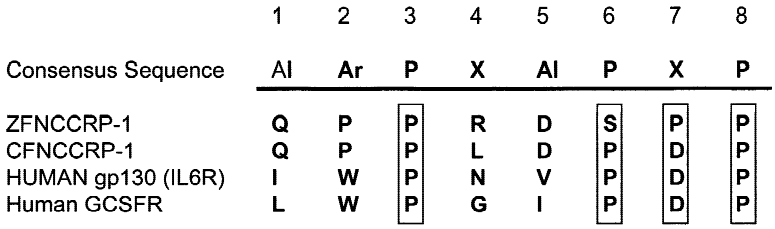

Fig. 3. Sequence alignment of the Box-1 motif from zebrafish and catfish NCCRP-1. The two top sequences correspond to the NCCRP-1 amino acids that are part of the Box-1 motif. Published sequences from mammalian receptors are compared. The amino acids at positions 1 and 5 are often aliphatic, while the one at position 2 can have an aromatic side chain. The X at positions 4 and 7 indicates a highly variable residue. The *boxed* amino acids indicate the positions where proline residues must be placed to constitute a Box-1 motif.

Table 1. Competitive inhibition of NCC cytotoxicity with the NCCRP-1 synthetic peptide designed based on the zebrafish receptor orthologue[a]

Peptides:	Y L P T F R W F S L E Q R V D L Q L P Q H R W F T L E Q C V D L L Q L F D W V R C H Q Q P E L T	Catfish Zebrafish Control (scrambled Zf sequence)
		Percentage specific release
Treatment	Concentration (mg/ml)	HL-60 IM-9
Media (no protein)	—	20 31
Control peptide	1.0	23 41
	0.5	19 32
	0.25	16 29
Zebrafish peptide	1.0	3 18
	0.5	2 20
	0.25	10 20
Catfish peptide	1.0	3 13
	0.5	5 17
	0.25	6 19

[a] Target cells were incubated with the indicated synthetic peptide for 30 min, purified NCC from catfish anterior kidney were added, and the mixture was cultivated for a 3-h killing assay (E:T ratio, 160:1). Supernatants were harvested and ^{51}Cr release was determined.

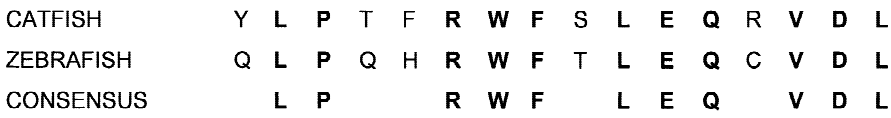

Fig. 4. Consensus sequence of the NCCRP-1 antigen-binding site. A comparison of the synthetic peptides that showed cytotoxicity inhibition generates a consensus sequence (residues in *black*) that may indicate critical residues in target antigen recognition.

sequences belong to the F-box family of proteins, and specifically to a subgroup of this family that encodes an F-box-associated (FBA) domain at the C terminus. The similarity of the zebrafish NCCRP-1 to these proteins does not include the N-terminal F-box domain, but is restricted to the carboxy terminus consisting of approximately 150 aa of the proteins, which comprises the F-box-associated domain (FBA) (Winston et al. 1999) (Fig. 5). The first 90 aa of the F-box proteins contains the F-box domain (Winston et al. 1999; Erhardt et al. 1998) and PEST domains (Rogers et al. 1986) that are not found in either of the teleost NCCRP-1. This argues that although NCCRP-1 and the F-box proteins share some structural similarity, their functions are likely to be distinct. Phylogenetic analysis of the aligned proteins suggests that the NCCRP-1 proteins are more closely related to the mammalian proteins than is the single *C. elegans* sequence (Fig. 6). The phylogram presented in Fig. 6 shows that the teleost proteins cluster in a single clade, while the mammalian F-box proteins are grouped in another. For these analyses the *C. elegans* protein was chosen as the outgroup. A new protein family is therefore proposed which includes the NCCRP-1 proteins and FBA domain-containing F-box proteins (Fig. 6) (Winston et al. 1999; Erhardt et al. 1998). The difference in the N terminus of the proteins defines at least two subfamilies in this new family of proteins. The NCCRP-1 subfamily, which does not contain the F-box motif, and the subfamily made of F-box-containing peptides (Fig. 5). Both share the common FBA domain.

An interesting observation was the discovery of a distinct motif (RNLLXNP, aa 39–44 in the zebrafish protein) in all the aligned proteins of the proposed family members (Table 2). This motif was scanned against the

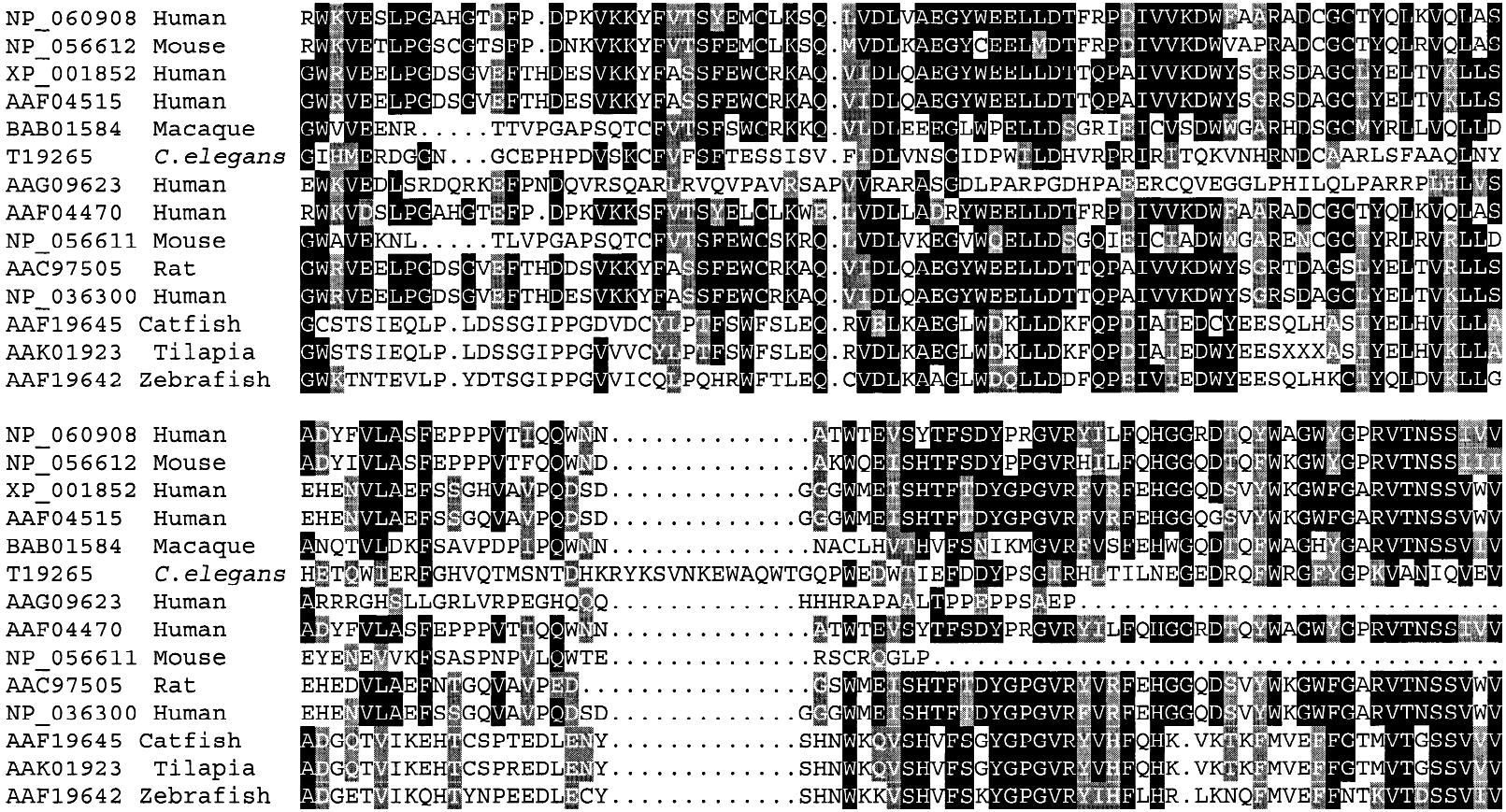

Fig. 5. Sequence alignment of the F-box-associated domain proteins and teleost NCCRP-1 proteins. The aligned region comprises the F-box-associated domain of each protein and excludes the N-terminal 110 aa (approximately) of the F-box proteins and the first 80 aa of the NCCRP-1 orthologues. For each sequence the accession number is followed by the common name of the organism. Two sequences (accession numbers NP_056611 and AAG09623) identified in the PSI Blast search are included in the alignment but were excluded from the phylogenetic analysis. These sequences were truncated at the C terminus and therefore lacked a complete FBA domain. The *shading* indicates the relative conservation of amino acids in each aligned column, with darker shading indicating a more strongly conserved position.

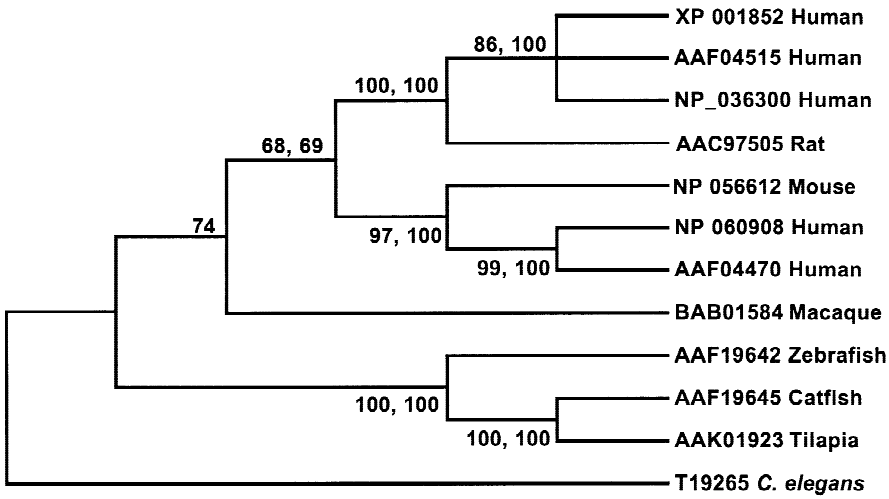

Fig. 6. Phylogram showing relationships of the F-box-associated domains of the NCCRP-1 and F-box proteins. The tree was derived by parsimony analysis, with both PAUP version 4.0b8 and Mega version 2. Numbers shown above the branches are bootstrap values based upon 500 replicates for parsimony and neighbor joining (NJ), respectively. A separate analysis using maximum likelihood (ML) produced a tree with similar topology. The only difference between the analyses was the placement of the macaque F-box protein (accession number BAB01584). While ML has it in its own group with a mammalian clade, NJ does not indicate its order of divergence.

Table 2. Comparison of the conserved motif found in the different proteins in the F-box family

NP_060908	Human	RNLLRNPCAE
NP_056612	Mouse	RNLLRNPCAE
XP_001852	Human	RNLLRNPCGE
AAF04515	Human	RNLLRNPCGE
BAB01584	Macaque	RPIGRNPCGQ
AAG09623	Human	RNLLHNPCAE
AAF04470	Human	RNLLRNPCAE
NP_056611	Mouse	RNLIHNSCGE
AAC97505	Rat	RNLLRNPCGE
NP_036300	Human	RNLLRNPCGE
AAF19645	Catfish	RNLLKNPSPH
AAK01923	Tilapia	RNLLKNPSPH
AAF19642	Zebrafish	RNLLQNPSPY

Swiss-Prot and TrEMBL databases using ScanProsite (Hofmann et al. 1999), however, no other proteins containing this motif were identified. Curiously, the motif was found to be present on the C-terminal side of the FBA domain in the teleost NCCRP-1 proteins, while it is on the N-terminal side of the same domain in all the mammalian members. This motif is absent from the *C. elegans* protein. The significance of this motif is presently not known. It appears to have first evolved after the speciation of nematodes but before that of teleosts from mammals. It has been conserved through evolution all the way to the human proteins.

Discussion

Natural killer (NK) cells recognize and lyse a wide variety of targets including transformed cells, parasites, and virus-infected cells (Zunino and Huding 1998; Petkus and Baum 1987; Jimenez and Murphy 1984). Receptor proteins that are localized in the cell membrane mediate recognition of these targets. A monoclonal antibody (mab) 5C6 was derived against the catfish equivalent of NK cells (i.e., NCC) that inhibits the interaction of catfish and tilapia NCC with their targets (Jaso-Friedmann et al. 1997a; Jaso-Friedmann and Evans 1999). A receptor on NCC responsible for ligand recognition, NCCRP-1 was identified with the mab 5C6. Catfish and tilapia NCCRP-1 have an apparent MW of 32 kDa and have been sequenced at both cDNA (Jaso-Friedmann et al. 1997a) and genomic levels (accession numbers AF 159718 for catfish and AF 318073 for tilapia).

In an attempt to identify the zebrafish equivalent of catfish and tilapia NCCRP-1, zebrafish EST, and the Swissprot databases were searched using the amino acid sequence of catfish NCCRP-1 as a query. These searches identified four zebrafish ESTs encoding approximately 400 bp of the 3′ region of the NCCRP-1 zebrafish orthologue. One of the zebrafish ESTs (No. AI331491) was chosen for further characterization and was found to have the entire zebrafish NCCRP-1 ORF. AI331491 encoded a 237-aa ORF with a MW of 27 kDa and a p*I* of 5.5. This putative ORF has 66.2% sequence identity with the catfish NCCRP-1 protein. The sequence identity of the zebrafish NCCRP-1 with the protein from catfish and tilapia appears to be sufficient for this molecule to have maintained the same function in these species.

The signature motifs that have been implicated in the signaling properties of this protein are the Box-1 motifs. Both forms of these motifs (truncated and full-length) at the N-terminus end of the NCCRP-1 proteins were first reported to be essential in the function of the catfish protein and are also present in the tilapia and zebrafish orthologues. As reported, these motifs serve as docking sites for JAK kinases, which then become activated by phosphorylation of their tyrosine residues and initiate STAT translocation into the nucleus. We have shown these events to take place in activated NCC (Evans et al. 1999). Furthermore, the promoter region of the NCCRP-1 gene in catfish and zebrafish contains several

putative STAT-binding sites. It is unknown at this time whether these sites function in the transcriptional activation of NCCRP-1. In addition to the Box-1 motifs, there is at least one PPP motif that is present in all the orthologues. These polyproline repeats have previously been shown to provide docking sites for proteins containing SH3 domains. Their function in NCCRP-1 is presently not known.

The other functionally essential region of the catfish and tilapia NCCRP-1 is the antigen recognition domain, which is composed of approximately 16 aa. This region has been implicated in the formation of conjugates with target cells, a necessary step in killing (Jaso-Friedmann et al. 1997a; Evans et al. 1998, 1999; Evans and Jaso-Friedmann 2000). In the present study, cytotoxicity inhibition experiments showed that zebrafish-specific peptides compete for the binding of catfish NCC to tumor target cells (Table 1). These results are in agreement with the conservation of a similar function for the zebrafish orthologue.

Homology searches identified a subset of the proposed F-box superfamily of proteins (Winston et al. 1999; Kipreos and Pagano 2000) as having significant similarity to NCCRP-1. The zebrafish and catfish NCCRP-1 proteins share approximately 30% identity with the members of the F-box superfamily of proteins. The homology between the F-box proteins and the NCCRP-1 is restricted to what was termed as the F-box-associated domain (FBA) (Winston et al. 1999) localized in the C terminus of the proteins. The N terminus of NCCRP-1 has a deletion of 40 aa compared with the Fbx proteins. That deletion encompassed all of the function-associated F-box domain that is characteristic of the F-box superfamily of proteins (Winston et al. 1999; Erhardt et al. 1998).

The best-studied function for F-box proteins has been reported to be the binding of substrates for ubiquitin-mediated proteolysis (Kipreos and Pagano 2000). In this pathway, ubiquinated proteins are targeted for proteolysis by the proteosome. The ubiquitine ligase complex, which is also termed SCF^{Cdc4p}, catalyzes the ubiquination of the proteins and it is made by assembly of the peptides Skp1p, Cdc4p, and Cdc53p (Bai et al. 1996; Kipreos and Pagano 2000). Skp1p mediates the degradation of cell cycle regulators through a 40-aa motif present in the N-terminal region of several proteins, which is termed the F-box motif. There is evidence that NFB42, a rat neural F-box protein, is regulated by being targeted to the proteosome through its interaction with Skp1p (Ng et al. 1998). Deletion of the NFB42 F-box domain inhibits this interaction (Erhardt et al. 1998).

The motifs associated with most F-box proteins at the carboxyl-terminal end of the proteins have not been assigned a defined function, although they are believed to be important in protein–protein interactions, as well as to have many phosphokinase sites (Erhardt et al. 1998; Kipreos and Pagano 2000). The high degree of identity that the NCCRP-1 proteins share at the C-terminal region with the F-box proteins suggests that this subfamily may interact with similar cytoplasm proteins and therefore may have comparable metabolic roles. The NFB42 protein is expressed at a very high level in neurons (Erhardt et al. 1998). NCCRP-1 is expressed in fish NCC cells playing a pivotal role in the fish innate immune response (Jaso-Friedmann et al. 1997a; Jaso-Friedmann and Evans 1999). The interaction of the immune system with the nervous system has been documented previously (Hiramoto et al. 1999). Stress and even the perception of stress can alter NK cell activity (Hiramoto et al. 1999). Also, stress-inducible major compatibility complex class I chain-related genes (MICA) can activate NK cells (Bauer et al. 1999; Wu et al. 1999). Although F-box proteins and NCCRP-1 could provide a link between these two well-connected systems, a most likely explanation for the shared domain (FBA) would be that they perform a similar function in both systems. It is important to note that the antigen recognition domain of NCCRP-1, proven crucial to the role of the NCCRP-1 proteins (Evans et al. 1999), is, as expected, poorly represented in the F-box proteins.

The inclusion of the F-box proteins with the NCCRP-1 proteins allows the definition of a new gene family, based on the similarities in the FBA domain. The members of the F-box family were previously grouped in a phylogenetically heterologous family which share the F-box domain and encompassed approximately 40 to 50 aa at the N-terminal region of the F-box proteins (Winston et al. 1999; Cenciarelli et al. 1999; Regan-Reimann et al. 1999; Kipreos and Pagano 2000). The F-box superfamily is not as phylogenetically robust as the new family that we propose in this study. We have defined the new family by the F-box-associated domain or FBA. This domain includes approximately 150 aa of the C-terminal region.

We propose the inclusion of two subfamilies within the FBA superfamily. One subfamily is the NCCRP-1 subfamily of proteins that includes the catfish, tilapia, and zebrafish NCCRP-1 proteins. The other subfamily is the F-box subfamily of proteins that includes the mammalian proteins. The difference between these two families is that they carry distinct functional domains at the N terminus. While NCCRP-1 proteins do not have the F-box motif, they have the FBA motif. On the other hand, the members of the F-box subfamily have both motifs but do not share the NCCRP-1 functional domains. This indicates that it is possible to have an FBA domain without the F-box motif. Therefore, the F-box-associated domain or FBA is a misnomer. There are antecedents to this type of partial homology due to exon shuffling (Morganstern and Atchley 1999). The gene that codes for the tissue plasminogen activator in mammals is made up of several exons from different genes (Lewine 1997).

We have placed the NCCRP-1 within a novel family of proteins that encompasses members that apparently have diverse functions. Currently, the function of the FBA domain present in the mammalian F-box proteins is unknown. In the NCCRP-1 proteins the antigen-binding site is within the FBA domain. Therefore the teleost NCCRP-1 proteins are the first members of the FBA superfamily for which a function has been defined for the FBA domain. We are confident that when more characterization work is done with the different protein members of this family, functional commonality will emerge.

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