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In Vivo Analysis of Ifn-γ1 and Ifn-γ2 Signaling in Zebrafish

Dina Aggad,*†,1 Cornelia Stein,‡,1 Dirk Sieger,‡,2 Martine Mazel,*† Pierre Boudinot,§ Philippe Herbomel,*,‡ Jean-Pierre Levraud,*‡ Georges Lutfalla,*,‡ and Maria Leptin‡

The zebrafish genome contains a large number of genes encoding potential cytokine receptor genes as judged by homology to mammalian receptors. The sequences are too divergent to allow unambiguous assignments of all receptors to specific cytokines, and only a few have been assigned functions by functional studies. Among receptors for class II helical cytokines—i.e., IFNs that include virus-induced Ifns (Ifn-ω) and type II Ifns (Ifn-γ), together with II-10 and its related cytokines (II-20, II-22, and II-26)—only the Ifn-ω-specific complexes have been functionally identified, whereas the receptors for the two Ifn-γ (Ifn-γ1 and Ifn-γ2) are unknown. In this work, we identify conditions in which Ifn-γ1 and Ifn-γ2 (also called IFNG or IFN-γ and IFN-gammarel) are induced in fish larvae and adults. We use morpholino-mediated loss-of-function analysis to screen candidate receptors and identify the components of their receptor complexes. We find that Ifn-γ1 and Ifn-γ2 bind to different receptor complexes. The receptor complex for Ifn-γ2 includes cytokine receptor family B (Crfb6) together with Crfb13 and Crfb17, whereas the receptor complex for Ifn-γ1 does not include Crfb6 or Crfb13 but includes Crfb17. We also show that of the two Jak2 paralogues present in the zebrafish Jak2a but not Jak2b is involved in the intracellular transmission of the Ifn-γ signal. These results shed new light on the evolution of the Ifn-γ signaling in fish and tetrapods and contribute toward an integrated view of the innate immune regulation in vertebrates. The Journal of Immunology, 2010, 185: 000–000.

Interferons are members of the large family of helical cytokines. These cytokines are structured around a four α helix bundle and are divided into class I cytokines, which include hormones of the prolactin and growth hormone family together with erythropoietin, leptin, and the majority of ILs (1), and class II cytokines. Class II cytokines are represented by the IFNs together with IL-10 and its relatives (IL-20, IL-22, IL-24, and IL-26). In mammals, the IFN-subclass is divided into three types: type I (IFN-α/β/ω/e/κ) and type III (IFN-λ) are the main players in innate immunity against viral infections. Type II, with its only representative IFN-γ, has clear antiviral activity but is also an immune regulator required for resistance to a wide range of bacterial and protozoan pathogens (e.g., Salmonella, leishmaniasis, and trypanosomes) in mice. In man, it appears to be instrumental in resistance to mycobacterial diseases (2, 3) but also is involved in Ag presentation (4), cancer immunoediting (5), leukocyte–endothelium interactions (2), and osteoclastogenesis suppression (6, 7).

In mammals, IFN-γ predominantly signals as an extracellular homodimer through the activation of its hetero-tetrameric receptor composed of two copies of IFN-γ receptor 1 (IFNGR1) and two copies of IFNGR2, which in turn leads to the activation of JAK1 and JAK2 and subsequent phosphorylation of STAT1. The activated STAT1 homodimer migrates to the nucleus where it activates the transcription of IFN-γ-stimulated genes.

IFN-like proteins, like other class II helical cytokines, also have been described in nonmammalian vertebrates, including many teleosts such as rainbow trout (8), common carp (9), zebrafish (10), and grass carp (11). The presence of two different ifn-γ genes (ifn-γ1 and ifn-γ2) seems to be the rule in teleosts, and they may have distinct expression ranges and functions in some fish species (9). By sequence and also by the intron-exon structure of their genes, both are good candidates to be homologous to the mammalian Ifn-γ (12). The zebrafish has become an interesting biological model for the analysis of immunity, and it is therefore important to understand the role of IFN signaling in this species. The early developmental stages are of particular interest, because the adaptive immune system begins to develop only at 4–6 wk of age (13), and up to this point the larva relies entirely on innate immune mechanisms. Later stages, by contrast, can be used to assay adaptive immune functions.

Studies using adult zebrafish tissues challenged in vitro by LPS or polyriboinosinic-polyribocytidilic acid (poly-IC) suggested that ifn-γ1 and ifn-γ2 both can be induced by these stimuli (10). Recent in vivo studies on Ifn-γ target gene induction showed that the response elicited by Ifn-γ1 and Ifn-γ2, as measured by target gene induction, was identical for both Ifn-γ.
knockdown of either one of the ifn-γ genes does not render the embryos susceptible to bacterial infection. However, simultaneous knockdown of both ifn-γ genes leads to a dramatic decrease in survival postinfection. These experiments imply (at least partial) redundancy in the elicited response (14). However, in vitro crosslinking experiments with recombinant extracellular domains of potential goldfish receptors have suggested that they do not bind to the same receptors (15).

As class II helical cytokines, Ifn-γ1 and Ifn-γ2 are expected to bind receptor complexes made up of two different transmembrane chains belonging to the helical class II cytokine receptor (hCRII) family. Analysis of the zebrasfish genome has revealed the presence of 15 such hCRII genes (12, 16–18). Phylogenetic comparisons have identified clear orthologues of the mammalian IL-20R chains 1 and 2, named cytokine receptor family B (Crbβ8 and Crbβ16 in fish, and IL-22BP (Crbβ9 in fish)) (12, 18). There are genes encoding proteins with high similarity to tissue factor in the zebrafish and other teleosts, and these have been duplicated in most fish (Crbβ10 and Crbβ11). The other receptor chains in this family cannot be matched unambiguously between fish and mammals. However, functional studies can be used to test which receptors are required for the response to individual ligands. Such studies have shown three receptor chains, Crb1β, Crb2β, and Crb5β, to be necessary for responses to different Ifn-γ (17, 19) [which resemble both human type I and type III IFNs in sequence and functions and were previously also named zIFN, IFN-α, or IFN-λ (12, 17, 20)].

In contrast to the cytokines and the receptors, the intracellular molecules acting in IFN signaling downstream of the activated receptor complexes are much more highly conserved in mammals, Jak1 and Jak2 bind to the activated IFNγR and are required for IFN-γ signaling. The zebrafish has one gene encoding the homologue of Jak1, whereas the Jak2-encoding gene has been duplicated. Previous reports have shown that the two encoded proteins, Jak2A and Jak2B, are not involved in the same signaling pathways in zebrafish (21, 22). Their implication in IFN-γ signaling still has to be checked. The IFN system of the zebrafish thus is beginning to be understood, but the results so far provide only a patchwork of parts of the signaling where these IFNs are induced. We also present in vivo infection experiments using either 30 or 100 CFU, which show that the results obtained in vitro can be matched unambiguously between fish and mammals.

Materials and Methods

Fish

Wild-type AB zebrasfish or zebrasfish of the Cologne strain were used during the study. Wild-type AB zebrasfish were purchased from the Zebrafish International Resource Center (Eugene, OR) as embryos and raised to adulthood in our facilities. Only fish directly from the Zebrafish International Resource Center or their F1 offspring were used as egg producers to avoid inbreeding effects.

Zebrafish were bred at 26.5°C on a 14 h light/10 h dark cycle. Embryos were collected by natural spawning and were staged according to Kimmel et al. (23).

Cloning the hCRII (Crbβ) open reading frames

3' and 5' RACE were performed using the GeneRacer kit from Invitrogen (Carlsbad, CA). Amplified products were tested for the presence of specific products using internal oligonucleotides and cloned using the Topo TA cloning kit from Invitrogen. Bacterial colonies were screened using the internal oligonucleotides, and the plasmids were sequenced entirely. The open reading frames (ORFs) of tested crbβ were cloned in the pTol2S263C or pCS2+ expression vectors (19, 24).

Expression vectors for Ifn-γ

The ORF of ifn-γ1 was amplified from reverse transcription products using Ifnγ1.50 and Ifnγ1.30 primers and NotI cloned into the pCT2S263C expression vector (17). For Ifn-γ2, the gene was amplified from genomic DNA. Due to the large size of intron 3, exons 1–3 have been amplified with primers Ifnγ2.1.50 and Ifnγ2.1.30R, and exon 4 with primers Ifnγ2.1.3D and Ifnγ2.30. Both PCR fragments then were ligated and inserted using NotI into the pTol2S263C expression vector. All of the oligonucleotides used in this study are listed in Table I.

Quantitative RT-PCR analysis of reporter gene induction

To determine the effects of crfb-specific morpholinos on irgfl expression induced by Ifn-γ1 or Ifn-γ2, total RNA was prepared at 30 h postfertilization (hpf) from pools of ∼10 larvae. Total RNA from infected larvae and controls (10 larveae each) were reverse transcribed by immersion in a bath containing the virus, and spleens were dissected to prepare RNA at 48 h postinfection (hpi). Larvae were infected with SIVNV via injection of 30 PFU into the caudal vein, and total RNA from the whole larvae was prepared at 24 hpi. Infection with infectious hematopoietic necrosis virus (IHNV) was made by injection into the caudal vein (100 PFU) of 60 hpf larvae, and total RNA from the whole larvae was prepared at 48 hpi.

Zebrasfish larvae (5 d postfertilization) were exposed to poly-IC by bathing in a 100 µg/ml solution, and RNA from whole larvae was prepared after 24 h. Induction of ifn-γ1 was used to control the efficacy of the treatment.

For bacterial infections, we used Escherichia coli (3000 CFU; strain XL-10 with a dsRed expression plasmid pRTZ3), Mycobacterium marinum (60–100 CFU; strain M) (27), and Y. ruckeri [30–100 CFU; strain M (27), with pMyco-GFP plasmid] and Y. ruckeri [30–100 CFU; strain M] with a dsRed expression plasmid pRZT3), and Zebrafish larvae were infected by injection into the caudal vein of zebrafish larvae (aged 30–32 hpf), and RNA from these larvae was isolated using the GeneRacer kit from Invitrogen (Carlsbad, CA). Amplified products were tested for the presence of specific products using the GeneRacer kit from Invitrogen.

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In the case of Y. ruckeri, we had determined that 30 CFU gives the most reliable difference in the survival curves of untreated and IFN-knockdown larvae. However, the response of the fish to pathogens is variable, depending either on the health or stress state of the fish or perhaps on unknown parameters of the pathogen cultures. We therefore sometimes used 100 CFU instead of 30 CFU. Fig. 3 and Supplemental Fig. 3 give examples of experiments using either 30 or 100 CFU, which show that the results obtained are the same in principle.

For statistics on the survival of larvae infected with Y. ruckeri, the survival rate of each of the populations of morpholino-injected embryos was compared with that of the un.injected embryos using the log-rank (Mantel-Cox) test.

Cloning and mutagenesis of Jak2a and Jak2b

The ORF of jak2β was amplified from reverse transcription RNA using Jak2β.501 and Jak2β.301N as primers. The amplified fragment was treated with T4PNK and NotI and then introduced into the SmaI + NotI digested pCS2+ expression vector, and the resulting plasmid was used as a template for site-directed mutagenesis using Jak2β.50 and Jak2β.30N as primers. To obtain constitutively active versions of Jak2a and Jak2b (Jak2aCA and Jak2bCA), we created point mutants that correspond to the constitutively active Drosophila hopT42
of triplicate infections. We amplified by PCR two fragments with the following pairs of primers: Jak2b.501 + Jak2bCAR and Jak2bCAD + Jak2b.301N. Primers Jak2bCAR and Jak2bCAD overlap and contain the mutation that will be introduced in the sequence Jak2b to create the E648K substitution. Both fragments then were used as templates for amplification using Jak2b.501 and Jak2b.301N as primers. The amplified fragment was cloned into the vector pExpress-1 following the same strategy as previously. A pGEM-T vector harboring Jak2aCA (21) has been provided by A.Y. Leung (Hong Kong, China) and A.C. Ward (Victoria, Australia). The Jak2aCA ORF was amplified from this vector using Jak2a.50 and Jak2a.30N as primers, treated with T4PNK and NotI, and cloned in Smal + NotI digested pExpress-1. The p-Express-1-derived expression vectors were injected into embryos at the one-cell stage, and mRNA was prepared at 30 hpf.

Morpholinos

Morpholino oligonucleotides were purchased from Gene Tools (Philomath, OR). They were dissolved and kept frozen in morpholino buffer [120 mM KCl 120 mM and HEPES 20 mM (pH7.2)]. After being thawed, morpholinos were heated at 65˚C for 10 min to ensure complete dissolution. Morpholinos were diluted to a concentration of 1 mM in morpholino buffer containing 0.1% phenol red and co-injected with the Act26mCherry reporter plasmid (17). Developing embryos were observed at 24 hpf under a fluorescence stereomicroscope. Only larvae that were judged to have been injected successfully, as judged by expression of mCherryF, were kept and used for analysis. The typical injected volume was 1–2 nl. The morpholino sequences are listed in Table II.

Results

Induction of zebrafish Ifn-γ1 and Ifn-γ2 in larvae and adults

Discriminating components of innate and acquired immunity is facilitated in zebrafish because larvae are endowed with only innate immune defenses, whereas adults also possess adaptive immunity. We began the current study by comparing the expression levels of ifn-γ1 and ifn-γ2 after infecting larvae and adults with either bacteria or viruses. We used quantitative RT-PCR, with the expression of GAPDH mRNA serving as an internal control for each RNA sample. Larvae and adults were challenged by infection with SVCV, IHNV, poly-IC, or bacteria (E. coli, M. marinum, or Y. ruckeri). We have shown previously that ifn-γ expression levels in the larvae do not change upon infection with E. coli or Y. ruckeri (14), which is confirmed in this study (Fig. 1). We find in addition that they also do not react to infection with virus or challenge with poly-IC. However, M. marinum infection leads to a significant increase in ifn-γ expression in the larvae, showing that there are conditions under which ifn-γ1 and ifn-γ2 can be induced. Although both ifn-γ genes are not induced during SVCV infections of zebrafish larvae, they are induced when adults are challenged with the virus. One possible explanation for this difference is that during such challenges in adults Ifn-γs are produced by cellular populations that are not present in larvae. An alternative interpretation, that the molecules that sense the viral infection are absent, is unlikely, because other responses to viral infections (e.g., induction of Ifn-α1 and Tnf-α) are intact (17). Induction of ifn-γ2, both in larvae and in adults, is more pronounced than ifn-γ1 induction.

The helical cytokine receptor repertoire in zebrafish

Fifteen genes encoding members of the hCRII family have been predicted or experimentally identified in the zebrafish. Our previous searches for these genes, which had led to the identification of 10 different cDNAs for expressed receptor chains (Crfb1, Crfb2, and Crfb4–11) (17) and a further five that had been predicted but not experimentally verified (Crfb12–16) (18), were based on releases 6 and 7 of the zebrafish genome assembly. We have now reanalyzed the genome using the latest release (Zv8) to look for further hCRII genes and have identified an additional gene that we have named crfb17. We used 5’ and 3’ RACE to clone cDNAs for Crfb12–17. As shown in Fig. 2A, there is now a repertoire of 16 hCRII chains. Three of these can be excluded as candidates for Ifn receptors: Crfb9, which is highly similar to IL-22BP and has no transmembrane domain, and Crfb10 and Crfb11 [Zfin, ef3a and ef3b, respectively (30)] that encode two paralogous copies of tissue factor, which also are found in Tetraodon, where they are expressed in a complementary fashion in the brain (Crfb11) and the other tissues (Crfb10) (12). This leaves 13 receptor chains as candidates for Ifn-γ receptors. Three of these, Crfb1, Crfb2, and Crfb5, were shown previously to participate in Ifn-γ signaling (17, 19).

Do Ifn-γ1 and Ifn-γ2 use the same receptor complexes for signaling?

To test which of the receptor chains are involved in Ifn-γ signal reception, we designed antisense morpholino oligonucleotides to knock them down (Table I). The efficacy of the morpholinos was tested by their ability to suppress expression in zebrafish embryos of GFP constructs that contained the target site of the AUG morpholinos and by RT-PCR analysis of receptor chain transcripts in the case of splice morpholinos (Supplemental Figs. 1, 2). As an assay for the biological effects of ifn-γ overexpression, we measured the expression of genes that we have found previously to respond to Ifn-γ signaling. These include the genes for the IFN-regulated GTPase F1 (irgf1) (14) and for the low molecular mass protein 2 (lmp2; also known as psmb9a) (14, 31). One-cell stage embryos were injected with an expression vector or mRNA encoding either ifn-γ1 or ifn-γ2 together with morpholinos blocking specific receptor chains. Morpholinos for all 13 candidates were tested. Experimental overexpression of Ifn-γ1 or Ifn-γ2 leads to a strong upregulation of irgf1 mRNA (Fig. 2). Coinjection of a morpholino

Figure 1. Expression of zebrafish ifn genes. Quantification by quantitative RT-PCR of ifn-γ mRNA relative to GAPDH in adult or in larvae. Challenge with either IHNV, E. coli, Y. ruckeri, or M. marinum was performed by i.v. injection in 60 hpf larvae (SVCV and IHNV) and 30 hpf larvae (E. coli, Y. ruckeri, and M. marinum); RNA was extracted from whole animals (10 larvae per sample) at 24 hpi (E. coli and Y. ruckeri) or 7 dpi (M. marinum). Poly-IC challenge of 8 dpf larvae was performed by bathing; RNA was extracted from whole animals (10 animals per experiment) after 24 h bathing. SVCV challenge of adults was performed by bath infection, and RNA was extracted from spleens from 10 adults per experiment 48 h later. The data are the results of triplicate infections. ifn-γ1 expression is shown as a control.
that has been shown previously to interfere with Ifn-γ1 signal transduction (17, 19) does not interfere with induction of irgf1 by Ifn-γ. However, we observe a significant change in irgf1 induction with morpholinos directed against Crfb6, Crfb13, and Crfb17 (Fig. 2).

The Crfb17 morpholino is the only one that interferes with Ifn-γ1 signal transduction (Fig. 2B). Crfb6- and Crfb13-specific morpholinos completely block Ifn-γ2 signal transduction (Fig. 2C) compared with the incomplete block by a Crfb17-specific morpholino. To confirm these results, both AUG and splice morpholinos for Crfb6, Crfb13, and Crfb17 were used. In each case, both morpholinos gave similar results (Fig. 2). Similar results were also obtained when measuring imp2 induction as a reporter (data not shown). These results suggest that Ifn-γ2 mainly transduces via a receptor complex containing Crfb6, Crfb13, and Crfb17. The fact that the Crfb6 and Crfb13 morpholinos are not active against Ifn-γ1 signal transduction shows that Ifn-γ1 does not signal through the same receptor complexes as Ifn-γ2 (Table II).

Because the failure to identify a second receptor chain for Ifn-γ1 could be due to the fact that Ifn-γ1 transduces through two different complexes, we also tested the effect of simultaneous knockdown of different combinations of two receptor chains by coinjection of pairs of morpholinos. The following pairs were tested: 4 + 6, 4 + 13, 4 + 15, 4 + 16, 6 + 13, 6 + 15, 6 + 16, 13 + 15, 13 + 16, and 15 + 16. None of these had an effect on Ifn-γ1 signaling (data not shown).

**In vivo evidence for the role of the receptor chains in survival of infected larvae**

The experiments described above assessed the effects of knocking down the receptors in an artificial, overexpression situation. We wanted to see whether loss of these receptor chains also affected natural physiological responses and therefore tested their role in responses to infection with bacteria. A shown in Fig. 1, M. marinum is the only tested pathogen that leads to strong Ifn-γ induction in the larvae. However, the response to Mycobacterium is slow (reaction detectable at 7 dpi) and therefore is not a suitable assay to be used with a morpholino approach. We have shown previously that fish embryos have the ability to respond to Y. ruckeri infections and that Ifn-γ1 and Ifn-γ2 are involved in protection against Y. ruckeri (31). We used resistance to this pathogen to assay the functions of the receptors. Because Ifn-γ1 and Ifn-γ2 act redundantly, interfering with only one of them does not impair the capacity of the larvae to respond to bacterial infection. Accordingly, knocking down a receptor specific for only one Ifn-γ should not have a significant effect on the survival of infected fish. However, the impact of knocking down a Crfb participating in a given Ifn-γ receptor becomes apparent when the ligand of the other Ifn-γ receptor is downregulated simultaneously. Thus, we investigated the importance of the receptor for one of the Ifn-γ for the outcome of the bacterial infection when the other Ifn-γ was blocked.

We assayed the survival rates of embryos infected at 30–32 hpf with 30–100 CPU of Y. ruckeri after blocking Crfb6, Crfb13, Crfb17, Ifn-γ1, or Ifn-γ2 with morpholinos alone or in combinations. Embryos in which only one of these genes has been blocked showed survival rates comparable to those of untreated embryos (Fig. 3). This confirms that knockdown of a single gene, whether it encodes the ligand or a receptor chain, does not render the embryos more susceptible to Y. ruckeri infection. However, when embryos were injected simultaneously with crfb13 and ifn-γ1 morpholinos, the survival period postinfection with Y. ruckeri was reduced significantly to a level comparable to that of embryos injected with ifn-γ1 plus ifn-γ2 morpholinos. Simultaneous knockdown of crfb13 and (ifn-γ2 did not result in a higher susceptibility to Y. ruckeri infection than that observed for the infected controls. These findings are consistent with Crfb13 acting as part of receptors specific for Ifn-γ2. By contrast, in the case of crfb17, combination of knockdown with either ifn-γ1 or ifn-γ2 both led to a reduction in survival. Surprisingly, knocking down crfb17 alone did not lead to susceptibility, although Crfb17 is indeed capable of transducing both the Ifn-γ1 and the Ifn-γ2 signal (see previous section, Fig. 2). We believe that part of the explanation is that the morpholino against crfb17 is not fully effective [we see in both RT-PCR experiments that target gene induction is not fully suppressed (Fig. 2)]. We know from our Ifn-γ1 and Ifn-γ2 knockdown experiments that approximately half of the dose of Ifn signaling is still sufficient for survival (Fig. 3). We suggest that, if in addition to reducing signaling through Crfb17 by a large extent we now also completely remove either Ifn-γ1 or Ifn-γ2, then the re-
resulting signaling level falls below a critical threshold that makes the difference between survival and death.

If both crfb17 and crfb13 morpholinos are used simultaneously, then survival is reduced, which is not the case if either one is used alone. This shows that even if Ifn-γ activity is only inhibited partially (by the crfb17 morpholino), then the complete blockage of Ifn-γ2 activity (by the crfb13 morpholino) leads to enhanced sensitivity to Y. ruckeri infection. Finally, we tested crfb6 morpholinos in combination with other morpholinos. Knockdown of crfb6 enhances the effect of ifn-γ1 but not ifn-γ2 knockdown, consistent with a role for Crfb6 as part of the Ifn-γ2 receptor.

Distinct functions of the Jak2 paralogues

The fact that both Ifn-γ1 and Ifn-γ2 induce the same set of genes while not inducing each other (14) indicates that the signaling pathways downstream of their different receptor complexes must overlap. As shown in Fig. 4A, zebrafish Crfb6, Crfb13, and Crfb17 harbor the typical sequences for IFNGR JAKs and STAT1 binding sites. The mammalian IFNGR1 contains three critical intracellular motifs (Fig. 4). A Leu-Pro-Lys-Ser (LPKS) sequence acts as the binding site for JAK1 (32, 33), with the proline having a dominant function in JAK1 recruitment (34, 35). The zebrafish Crfb13 and Crfb17 contain the essential proline residue but differ in the sur-

Table I. Oligonucleotides

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UTR, untranslated region.
replacing the histidine (Fig. 4A). A Leu-Ile dipeptide adjacent to the JAK1 binding motif is important for receptor-mediated ligand internalization and degradation (32, 36). The conserved leucine residue also is found in Crfb13 and Crfb17. Of the mammalian STAT1-binding motif Tyr-Asp-Lys-Pro-His (YDKPH) in IFNGR1 residue also is found in Crfb13 and Crfb17. Of the mammalian internalization and degradation (32, 36). The conserved leucine binding motif is important for receptor-mediated ligand rounding sequence (Fig. 4A). A JAK2 binding motif in the mammalian STAT1-binding motif Tyr-Asp-Lys-Pro-His (YDKPH) in IFNGR1

is involved in Ifn-γ target genes, suggesting that only this protein is involved in Ifn-γ signaling (Fig. 5).

Discussion

Mammals have three types of IFNs: type I (IFN-α/β/ω/e/κ in humans) and type III (IFN-λ) are induced by viral infection, whereas type II (IFN-γ) is mainly a Th1 cytokine involved in both acquired and innate immunity. Although the virally induced IFN genes have been amplified in many species by gene duplications, with gene conversions contributing to the creation of new specificities in the resulting large gene families, the number of type II IFN genes is much more limited. Most mammalian species have a single IFN-γ gene, and most fish species have two, raising the question of potential redundancy of these genes in fish. This has been looked at in terms of in vivo function and, more widely, in terms of their induction by different stimuli. In various fish species, they both respond to the same stimuli, as we also find for the two zebrafish ifn-g genes. We have used a variety of pathogens and molecules to induce an

response in zebrafish. In these experiments, we have relied on previously established protocols that were developed independently in hu-

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response in zebrafish. In these experiments, we have relied on previously established protocols that were developed independently in hu-

Table II. Morpholinos targeting either splices of the precursor mRNAs or 5’ UTRs of the mRNAs

<table>
<thead>
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<th>Gene</th>
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<td>5’ UTR</td>
<td>AAAAGAATACGTCATAGATAGT</td>
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</table>

Splice, splice site; UTR, untranslated region.

Splice, splice site; UTR, untranslated region.
What influence might this have on our results? Immunity develops progressively during zebrafish ontogeny (38). Because zebrafish hatch and thus become exposed to pathogens within 1 d of fertilization, an effective immune system has to be in place very early. By 30 hpf, early macrophages already patrol the tissues and engulf bacteria (39); neutrophils arise by 2 dpf (40) and are followed by other myeloid populations, such as eosinophils by 6 dpf (38). Mature lymphocytes require weeks to appear, even though lymphoid progenitors start to colonize the thymus by 4 dpf (13). The development of cytokine responses during the first days and weeks of zebrafish development has been less well studied. It is reasonable to assume that as the fish develops the magnitude of cytokine production increases, and this may partially explain why the Ifn-γ production that we measured in response to M. marinum at Day 8 was greater than that for the pathogens assayed at Day 3. Nevertheless, induction of other cytokines can occur early: we found clear induction of ifn-γ1 by Y. ruckeri in fish aged 54 hpf. This implies that pattern recognition receptors and the associated signaling cascades are present at an early stage. In addition, zebrafish embryos are able to respond to changes in Ifn-γ levels at an early age (14). For these reasons, we believe that the differential inductions of ifn-γ genes by the various stimuli that we used reflect mostly specificity of the host response toward certain pathogen-associated molecular patterns.

![Diagram](Image 337x631 to 358x661)

**FIGURE 3.** Survival of crfb knockdown embryos after bacterial infection. A, Survival of crfb13 and ifn-γ knockdown embryos infected with 30 CFU Y. ruckeri. Embryos were left untreated (control) or injected at the one-cell stage with morpholinos specific for ifn-γ1, ifn-γ2, and crfb13 alone or in combination as indicated (three independent experiments; n = 25 for each treatment). x-axis, hours postinfection; y-axis, percentage of surviving embryos. The left panel shows the survival rates for embryos that were not infected; the right panel shows the survival rates for embryos that were infected. B, Survival of crfb17 and ifn-γ knockdown embryos infected with 100 CFU Y. ruckeri (two independent experiments; n = 40 for each treatment). C, Survival of crfb17 and crfb13 knockdown embryos infected with 30 CFU Y. ruckeri (two independent experiments; n = 40 for each treatment). D, Survival of various crfb and ifn-γ knockdown embryos infected with 30 CFU Y. ruckeri (one experiment; n = 40 for each treatment). The survival rate of each of the populations of morpholino-injected embryos was compared with that of the noninjected embryos using the log-rank (Mantel-Cox) test. The χ² values for the comparisons were as follows (χ² < 3.84 is not statistically significant; significant values with p < 0.05 [95%] are underlined): (A) ifn-γ1 Mo = 0.25, ifn-γ2 Mo = 0.27, ifn-γ1 + crfb13 Mo = 4.19, ifn-γ2 + crfb13 Mo = 0.14, ifn-γ1 + ifn-γ2 Mo = 5.13; (B) crfb17 Mo = 0.31, ifn-γ1 + crfb17 Mo = 15.46, ifn-γ1 + ifn-γ2 Mo = 10.99, ifn-γ1 + ifn-γ2 Mo = 21.52; (C) crfb13 Mo = 1.42, crfb17 Mo = 0.27, crfb13 + crfb17 Mo = 26.99, ifn-γ1 + ifn-γ2 Mo = 53.54; (D) ifn-γ1 + crfb6 Mo = 5.63, ifn-γ2 + crfb6 Mo = 0.13, crfb6 + crfb13 Mo = 0.11, crfb6 + crfb17 Mo = 0.00, ifn-γ1 + ifn-γ2 Mo = 14.02, crfb13 + crfb17 Mo = 8.90.

![Diagram](Image 337x672 to 363x702)

**FIGURE 4.** Jak2a and Jak2b contribution to the Ifn-γ signaling pathway. A. The JAK and STAT interaction sites in the IFN-γ receptor chains. Schematic representation of a mammalian IFN-γ receptor with the binding motifs for JAK and STAT. For each motif, the diagram shows an alignment of the corresponding sequences in man, mouse, and zebrafish to illustrate the conservation of these motifs. The conserved sequences include the ligand internalization and degradation motifs. B and C. Effect of expressing wild-type and CA forms of Jak2a and Jak2b in early zebrafish embryos on the expression of Ifn-γ target genes. Quantitative RT-PCR measurements of irg1 and il8 relative to gapdh mRNA in larvae (prepared from 10 animals per experiment) at 30 hpf. Dr, Danio rerio; Hs, Homo sapiens; Mm, Mus musculus.

![Diagram](Image 469x684 to 526x702)

**FIGURE 5.** Model for the Ifn-γ receptors in man and zebrafish. In man, the active IFN-γ receptor consists of two copies each of IFNGR1 and IFNGR2. We propose a model for the zebrafish where the Ifn-γ1 receptor consists of Crfb17 and an unknown additional chain, whereas the receptor for Ifn-γ2 contains two identical short chains (Crfb6) and two different long chains (Crfb17 and Crfb13). For simplicity, this model shows zebrafish Ifn-γ1 and Ifn-γ2 as homodimers. We cannot exclude the possibility that there might also be Ifn-γ1/Ifn-γ2 heterodimers.
Structural studies have shown that two molecules intertwine to produce the active IFN-γ homodimer (41). The functional receptor for IFN-γ is a tetramer with two IFNGR1 and two IFNGR2 chains. We have no data on whether zebrafish Ifn-γ1 and Ifn-γ2 act as monomers or as dimers, and our experiments have not addressed the stoichiometry of the complexes either at the ligand level or at the receptor level. If the two IFNs act as dimers, then this opens the possibility that they might produce heterodimers, in addition to the two types of homodimers. It is not known whether it is in fact possible for intertwined dimers to be formed from polypeptides produced from different mRNAs or whether such dimers can only be made cotranslationally from one single mRNA.

In addition, our earlier results (14) on the function of the two IFNs argue against heterodimers. Our results showed that both IFNs need to be knocked down to block signaling. If all or a major fraction of the IFN needed to be heterodimeric, then reducing one IFN alone should reduce the available active IFN and affect signaling, but this was not the case. In which combinations the fish Ifn-γ polypeptides act remains an interesting open question that will necessitate biochemical analysis.

The finding that Ifn-γ1 and Ifn-γ2 appear to induce the accumulation of the RNAs of the same set of target genes (14) may mean that they use the same receptor complexes, but it is equally possible that they use different receptors but that their signaling pathways converge intracellularly. The strategy that we have adopted to address this issue has been to identify the complete repertoire of the candidate receptors in zebrafish and to systematically knock them down using morpholinos targeting either the AUG or a splice site. Our results, summarized in Fig. 5, show that Ifn-γ1 and Ifn-γ2 do not use the same receptor complexes. Crfb6, Crfb13, and Crfb17 are necessary components of the Ifn-γ2 receptor complex, although neither Crfb6 nor Crfb13 is involved in Ifn-γ1 signal transduction. Out of the candidates that we have tested, only Crfb17 is involved in an Ifn-γ1 receptor complex. In our search for the Ifn-γ receptors (19), we had shown that for those receptor chains that were expressed at rate-limiting levels (Crfb1 and Crfb2) the simultaneous overexpression of a ligand and its receptor chains could be used to confirm the morpholino loss-of-function data for the identification of the receptor chains. However, such gain-of-function experiments did not work for highly expressed receptor chains such as Crfb5. Similarly, in the case of Ifn-γ1 and Ifn-γ2, the candidate receptors are highly expressed, and when we conducted gain-of-function experiments to confirm the morpholino loss-of-function experiments, we did not obtain conclusive results. This suggests that these receptor chains are not limiting in Ifn-γ signal transduction in the zebrafish larvae. While this article was in preparation, two groups reported the identification of Ifn-γ receptor chains in different fish species. Gao et al. (42) reported that tagged recombinant rainbow trout Ifn-γ can be cross-linked to cells expressing a tagged version of the trout Crfb13, whereas experiments in CHO cells suggested the involvement of both Crfb13 and Crfb6 in Ifn-γ binding. Grayfer and Belosevic (15) published in vitro cross-linking experiments suggesting that the two recombinant Ifn-γ molecules from goldfish do not bind the same receptors. Their data suggest that goldfish Ifn-γ1 binds to a homologue of Crfb17, whereas goldfish Ifn-γ2 binds to a homologue of Crfb13. These results are in complete agreement with our in vivo experiments and suggest that this scheme is common to these fishes.

One possibility is that Ifn-γ2 binds to a receptor complex containing two Crfb6 chains, one Crfb13 chain, and one Crfb17 chain. We draw these conclusions from work in the larvae. It is possible that the situation in adults is more complicated with differential expression patterns for some of these receptors. Thus, cell populations with a $2 \times$ Crfb6 + 2 Crfb13 receptor composition might coexist with populations with a $2 \times$ Crfb6 + 2 Crfb17 composition or mixed receptors. Different receptor chain combinations could result in different binding affinities of Ifn-γ2 for different cell populations and also differences in induced genes dependent on the differentiation states of these cell populations. Analysis of this issue will await the study of Ifn-γ receptor chains and Ifn-γ-induced genes in purified subpopulations of cells. We will start these analyses using spleens from infected fishes.

The situation for Ifn-γ1 is more difficult to interpret, with our experiments excluding Crfb6 and Crfb13 and pointing only at Crfb17. It is not clear why we have not been able to identify another receptor chain involved in Ifn-γ1 signaling, but we can envisage several explanations.

First, we may have reached the limits of the strategy of using morpholinos to knock down gene function in the developing zebrafish. The morpholinos have been tested for their efficacy either as splice morpholinos (RT-PCR) or AUG morpholinos (GFP constructs), and the majority have been found to reduce mRNA levels by >90% (Supplemental Figs. 1, 2). In mammals, the IFNGR1 is in excess on the membrane, whereas IFNGR2 is the limiting receptor chain (43, 44). We cannot exclude the possibility that the remaining activity may be sufficient for the observed biological activity because we know from our studies on Ifn-γ that very low levels of Crfb expression can mediate signaling. Furthermore, morpholinos are not able to remove existing, stable proteins, and we know that many of the Crfb mRNA are maternally supplied, so there may be receptors present on the cell surface. Thus, a less-than-complete knockdown of the postulated second receptor chain may not have a measurable effect on signaling, and we will need to study true loss-of-function mutants.

Second, despite many efforts to extend it, the repertoire of receptors that we have identified so far may still be incomplete. A missing gene or genes may not be recognizable by bioinformatic methods, or they may lie in one of the remaining gaps in the genome assembly.

Third, a receptor composed of Crfb17 alone might be sufficient for binding extracellular Ifn-γ1 and transduce its signal to the inside of the cells. There is no precedent for this, and it is far more difficult to test.

Although Ifn-γ1 and Ifn-γ2 use different receptor complexes, they appear to regulate the same range of target genes. In mammals, IFN-γ signaling involves the kinases JAK1 and JAK2 and the downstream molecule STAT1 (45). In zebrafish, the genes for both Jak2 and Stat1 are duplicated (18, 21, 22, 46). Our results using constitutively active mutant constructs of Jak2 suggest that Jak2a but not Jak2b is able to induce genes that we know are Ifn-γ targets. Because there is no straightforward way of generating constitutively active versions of the Stat proteins, it still remains to be determined which of the two Stat1 paralogues in the zebrafish (18) is involved in transducing the Ifn-γ signal.

The emerging picture for the Ifn-γ system in fish is that of a more complex system than that in mammals. This complexity is obvious at the level of ligands and receptors and probably also at the level of different cell populations showing different responses according to the receptor complexes that they express. The present work lays the groundwork for future studies on the structure of cytokine networks in vertebrates and their evolution.

Acknowledgments

We thank Koichi Kawakami for the gift of the Tol2 transposon system, Alister Ward and Anskar Leung for the gift of the constitutively active Jak2a kinase, Donald F. Amend for the IHNV strain, and Röbbe Wünschiers for help with the statistical analyses. D.A., M.M., and G.L. are grateful to Alain Ghysen, Christine Dambly-Chaudiere, and Nicolas Cubedo for...
their help and access to their fish facilities, Caty Gonzalez for her technical help, Adeline BurguiÈre and Laurent Kremer for help with M. marinum, and theindsight to help with the’re analysis. K. J. Dorian, S. L. Johnson, J. H. Postlethwait, L. I. Zon, and A. F. Wilks. 1999. Gene duplication of zebrafish JAK2 homologs is accompanied by divergent embryonic expression patterns: only jak2a is expressed during erythropoiesis. Blood 94: 2622–2636.


References

The authors have no financial conflicts of interest.

Disclosures


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Corrections


Two errors appeared in the published morpholino sequences (Table II).

In the sequence for the crfb1 splice morpholino, a “G” was omitted from position 22. The correct sequence for this morpholino is:

5′-CGCCAAGATCATACCTGTAAAGTAA-3′.

The sequence of the ifn-γ1-1 (NM_001020793) 5′ UTR morpholino was not listed correctly. The correct sequence for the ifn-γ1-1 5′ UTR morpholino is:

5′-TTTCTGTGCTGAACCAAGTGATG-3′.

The corrected sequences have been implemented in The Zebrafish Model Organism database. The authors are grateful to H.L. Paddock of the Zebrafish International Resource Center, http://zfin.org/ (University of Oregon, Eugene, OR) for drawing their attention to the mistakes.

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**Figure legends for supplementary figures**

**Supplementary figure 1**

Effect of splice Morpholinos on the mRNAs of *crfb* genes.

mRNA was prepared from untreated larvae (c) and larvae that had been injected with the indicated Morpholinos (MO). RT-PCR with primer pairs flanking the targeted splice junctions (Table 1) was used to assess the effect of the Morpholinos on splicing.

RT-PCR with Finnzymes’ Phusion Hot Start High-Fidelity DNA Polymerase was carried out under the following conditions: Initial denaturation 30s at 98°C, denaturation 10s at 98°C, annealing 20s at 65°C, extension 30s (*crfb*1, 2, 5, 6, 12, 13, 14, 15 and 17) and 1min (*crfb*4, 7, 8 and 16) at 72°C and final extension 10 min at 72°C. Molecular weight marker is the Quick-Load 100 bp DNA Ladder.

Predicted sizes of the PCR products in untreated larvae:

- *crfb*1: 250bp
- *crfb*2: 525bp
- *crfb*4: 1300bp
- *crfb*5: 300bp
- *crfb*6: 300bp
- *crfb*7: 1321bp
- *crfb*8: 1520bp
- *crfb*12: 501bp
- *crfb*13: 492bp
- *crfb*14: 495bp
- *crfb*15: 320bp
- *crfb*16: 1000bp
- *crfb*17: 350bp

**Supplementary figure 2**

Control experiments to verify the morpholino-mediated knockdown of *crfb*1, 6, and 7.

Bright field and eGFP fluorescence images of embryos at 28-32 hpf. The embryos were injected with *crfb*1, 6, or 7 UTR-GFP RNA alone or together with *crfb*1, 6, or 7 morpholino.

(A)- (D’) *crfb*6 and untreated control, (E)- (H’) *crfb*7 and untreated control, (I)- (L’) *crfb*1 and untreated control; (A), (A’) *crfb*6-5’UTR-GFP RNA injected embryos, (B), (B’) *crfb*6-5’UTR-GFP RNA + *crfb*6 Mo (0.6mM) injected embryos; (C), (C’) *crfb*6-5’UTR-GFP RNA + *crfb*1 Mo (0.6mM) injected embryos, (D), (D’) untreated sibling embryos; (E), (E’) *crfb*7-5’UTR-GFP RNA injected embryos, (F), (F’) *crfb*7-5’UTR-GFP RNA + *crfb*7 Mo (0.9mM) injected embryos; (G), (G’) *crfb*7-5’UTR-GFP RNA
Injections were repeated at least two times with similar results. 91-97% of the crfb-5’UTR-GFP RNA injected embryos showed fluorescence. The embryos injected with crfb6-5’UTR-GFP RNA + crfb6 Mo and those injected with crfb1-5’UTR-GFP RNA + crfb1 Mo did not show fluorescence; 78% of the embryos injected with crfb7-5’UTR-GFP RNA + crfb7 Mo showed decreased fluorescence, the remaining 22% showed fluorescence. (A)-(L’) whole mount embryos (28hpf).

To test the binding of the different morpholinos, the 5’ UTR sequences of the respective genes were amplified with primers that contained suitable restriction sites (Table 1). Digested and purified PCR products were cloned into the pCS2+/eGFP. Capped RNA was synthesized in vitro using the Message Machine kit (Ambion), according to the supplier’s manual. RNA was injected at 100 ng/ml, with and without the respective morpholino into 1–2-cell stage embryos. On average, the injected volume was 2 nl. GFP fluorescence was visualized using a Leica MZ 16FA stereomicroscope.

**Supplementary figure 3**

Further examples of the experiments shown in figure 3.

**A. Survival of crfb13 and ifn-γ knockdown embryos infected with 30 cfu *Y. ruckeri*.** Embryos were left untreated (control) or injected at the one-cell stage with morpholinos specific for *ifn-γ1*, *ifn-γ2*, and crfb13 alone or in combination as indicated (n = 25 for each treatment). X-axis: hours post infection, y-axis: percentage of surviving embryos. The left panel shows the survival rates for embryos that were not infected; in the right panel the embryos were infected.

**B. Survival of crfb17 and ifn-γ knockdown embryos infected with 30 cfu *Y. ruckeri* (n = 40 for each treatment).**

**C. Survival of crfb17 and ifn-γ knockdown embryos infected with 100 cfu *Y. ruckeri* (n = 40 for each treatment).**
D. Survival of crfb17 and crfb13 knockdown embryos infected with 30 cfu *Y. ruckeri* (n = 40 for each treatment).

The log-rank (Mantel-Cox) test was used to compare each of the populations of treated embryos to that of untreated embryos. The $\chi^2$ values for the comparisons were as follows ($\chi^2<3.84$ is not statistically significant; significant values with $p<0.05$ (95%) are underlined): (A). $\text{ifn-}\gamma 1 \text{ Mo} = 0.00$, $\text{ifn-}\gamma 2 \text{ Mo} = 0.01$, $\text{ifn-}\gamma 1+\text{crfb13 Mo} = 9.04$, $\text{ifn-}\gamma 2+\text{crfb13 Mo} = 0.31$, $\text{ifn-}\gamma 1+\text{ifn-}\gamma 2 \text{ Mo} = 12.36$. (B). $\text{ifn-}\gamma 1+\text{crfb17 Mo} = 8.34$, $\text{ifn-}\gamma 2+\text{crfb17 Mo} = 6.67$, $\text{ifn-}\gamma 1+\text{ifn-}\gamma 2 \text{ Mo} = 12.77$. (C). $\text{ifn-}\gamma 1+\text{crfb17 Mo} = 18.06$, $\text{ifn-}\gamma 2+\text{crfb17 Mo} = 10.24$, $\text{ifn-}\gamma 1+\text{ifn-}\gamma 2 \text{ Mo} = 18.53$. (D). $\text{crfb13 Mo} = 0.01$, $\text{crfb17 Mo} = 0.08$, $\text{crfb13+crfb17 Mo} = 9.22$, $\text{ifn-}\gamma 1+\text{ifn-}\gamma 2 \text{ Mo} = 18.61$. 