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The Two Groups of Zebrafish Virus-Induced Interferons Signal via Distinct Receptors with Specific and Shared Chains

Dina Aggad,*† Martine Mazel,‡ Pierre Boudinot,‡ Knud Erik Mogensen,*‡ Ole Jensen Hamming,§ Rune Hartmann,§ Sergei Kotenko,♣ Philippe Herbomel,¶# Georges Lutfalla,¶# and Jean-Pierre Levraud2,¶#

Because the availability of fish genomic data, the number of reported sequences for fish type II helical cytokines is rapidly growing, featuring different IFNs including virus-induced IFNs (IFNβ) and IFN-γ, and IL-10 with its related cytokines (IL-20, IL-22, and IL-26). Many candidate receptors exist for these cytokines and various authors have postulated which receptor chain would be involved in which functional receptor in fish. To date, only the receptor for zebrafish IFNβ1 has been identified functionally. Three genes encoding virus-induced IFNβs have been reported in zebrafish. In addition to these genes clustered on chromosome 3, we have identified a fourth IFNβ gene on chromosome 12. All these genes possess the intron-exon organization of mammalian IFN-αs. In the zebrafish larva, all induce the expression of reporter antiviral genes; protection in a viral challenge assay was observed for types of IFN also have distinct genetic structure: type I (mainly IFN-α) and type II (IFN-β) IFNs have four exons, while type III (IFN-γ) IFNs have five. Type I and type III IFNs together constitute a distinct subgroup known as “virus-induced IFNs” as they are directly induced by viral infections while type II is not; furthermore, even though IFN-γ possesses some direct antiviral potential, its primary function appears to be activation of the adaptive immune system, notably against mycobacteria (2). In addition, although type I and III IFNs signal through different receptor complexes, both activate the same transcription factor known as the IFN-stimulated gene factor 3 and induce a similar set of downstream genes (3–5). Strikingly, most if not all species have heavily diversified their virally induced IFNs (13 α, 1 β, and 3 γ in humans). A functional type I IFN system is required by mammals to survive viral infections and most viruses have developed ways to antagonize it (reviewed in Ref. 6). The role of the more recently discovered type III IFNs is less clear, but they are crucial for preventing influenza infection via the nasal route (7) and some viral proteins also target this receptor usage. Two subgroups of fish virus-induced IFNs have been defined based on conserved cysteines, and we find that this subdivision correlates with receptor usage. Both receptor complexes include a common short chain receptor (CRFB5) and a specific long chain receptor (CRFB1 or CRFB2). The Journal of Immunology, 2009, 183: 3924–3931.

Interferons are a group of cytokines defined by their antiviral activities. In mammals, IFNs are divided into three groups according to their receptor usage (reviewed in Ref. 1). In addition to using distinct receptor complexes, the three mammalian types of IFN also have distinct genetic structure: type I (mainly α and β) IFN genes have a single exon, type II (γ) IFNs have four exons, while type III (γ) IFNs have five. Type I and type III IFNs together constitute a distinct subgroup known as “virus-induced IFNs” as they are directly induced by viral infections while type II is not; furthermore, even though IFN-γ possesses some direct antiviral potential, its primary function appears to be activation of the adaptive immune system, notably against mycobacteria (2). In addition, although type I and III IFNs signal through different receptor complexes, both activate the same transcription factor known...
but they also identified in silico two new zebrafish genes, which are not yet thought. Not only did they clone three IFNs in rainbow trout, but they also identified in silico two new zebrafish genes, which they named IFN2 and IFN3 (and which we label here IFN\textsubscript{d2} and IFN\textsubscript{d3} for the reasons stated above). Notably, they pointed out that these genes segregated into two different subsets (group I and II) characterized by the presence of two vs four conserved cysteines in the protein sequence, respectively (18). Salmon IFNs can also be subdivided in these two subgroups (13) which seem to have diverged before the main teleost radiation. In zebrafish, IFN\textsubscript{d1} belongs to group I, while IFN\textsubscript{d2} and 3 belong to group II (18). Although Zou et al. (18) could not ascertain a clear antiviral activity for the group II trout IFN, Lopez-Munoz et al. recently reported that all three zebrafish IFN\textsubscript{d}s display antiviral activity in adult fish (19).

Based upon these studies, we decided to use the zebrafish larva as a model to perform a comprehensive analysis of the IFN\textsubscript{d}s and their receptors in a teleost species. We analyzed the latest versions of the zebrafish genome assembly to identify potential new IFN genes and cloned four IFN\textsubscript{d} cDNAs. We then used different strategies to overexpress or inhibit the different IFN\textsubscript{d}s in the developing zebrafish embryo to test for in vivo biological activities including resistance to viral infections. Using loss of function and gain of function analysis, we finally identified the transmembrane components of their receptor complexes. Our experiments demonstrate that, according to the subgroup to which they belong, zebrafish IFN\textsubscript{d} bind to one of two different receptor complexes that share the same short chain but possess specific long chains.

**Materials and Methods**

**Fish**

Wild-type AB zebrafish were purchased from the Zebrafish International Resource Center (ZIRC) as embryos and raised to adulthood in our facilities. Only fish directly from ZIRC or their F\textsubscript{1} offspring were used as egg producers to avoid inbreeding effects. Depending on the desired speed of development, embryos were raised at 28°C or 24°C; all staging in the text refers to the standard 28.5°C developmental time (20).

**Viruses**

Spring viremia of carp virus (SVCV)\textsuperscript{3} was used as a rhabdovirus pathogenic for cyprinids including zebrafish (15, 21). The SVCV strain used belongs to the type I serogroup of spring viremia viruses (22). The wild-type SVCV used as a rhabdovirus pathogenic for cyprinids including zebrafish (15, 21). The SVCV strain used belongs to the type I serogroup of spring viremia viruses (22). The wild-type SVCV used in this study was isolated from infected carp. SVCV and IHNV were propagated in monolayer cultures of EPC cells. Spring viremia of carp virus (SVCV)

**Cloning the zebrafish IFN\textsubscript{d}**

The IFN\textsubscript{d} ORF were cloned in the pTol2S263C expression vector, where their expression is driven by the promoter of zebrafish ribosomal S26 gene from chromosome 23, amplified as a 1.4-kb fragment using primers S26C23E2 (TTATGGACCTTTCTTATGCTAAGT GGA) and S26C23P1 (GGTCTACATGCTTCTCCT) and cloned in the pTol2 vector from Prof. K. Kawakami (National Institute of Genetics, Shizuoka, Japan) (23). The ORF of CRFB2 was amplified by PCR using primers CRFB2.510 (TACTGCGATTGCGGACG CTTG) and CRFB2.310 (AGTTCTAGATCTGGAACACT CT), digested by XhoI plus XbaI and inserted into the PCS2+ vector, under the control of the CMV promoter. CRFB1 cloning has been described (15). Overexpression vectors are diluted to 20 ng/μl in morfolino buffer (24) containing 0.1% phenol red and 10 ng/μl pact26: mCherryF plasmid (15), and injected (1 nl) in the cytoplasm at the one cell stage. Developing embryos were observed at 24 hours post fertilization (hpf) under a fluorescence stereomicroscope and poorly fluorescent embryos were discarded.

**Morpholinos**

Morpholino oligonucleotides have already been described (15). After thawing, morpholinos are heated at 65°C for 10 min to ensure complete dissolution. Morpholinos are diluted to the desired concentration (typically, 500 μM, for a total injected amount of 4 ng) in morfolino buffer containing 0.1% phenol red. Injections are as for overexpression vectors.

**Recombinant IFNs**

The IFN\textsubscript{d}1, 2 and 4 ORFs were first amplified using the following pairs of primers (IFN\textsubscript{d}1.56: CCTGTATTTCGAGTTCTCTGCGGAAAG and IFN\textsubscript{d}1.34B: CGGATCTCATTACCTGAGGATTGA), (IFN\textsubscript{d}2.53: CCTGTATTTCGAGTTCTCTGCGGAAAG and IFN\textsubscript{d}2.310: AGCTTTTTTCTACAGGAGAATGGA) and (IFN\textsubscript{d}4.53: CCTGTATTTCGAGTTCTCTGCGGAAAG and IFN\textsubscript{d}4.31B) to produce ORFs with a TEV cleavage site replacing the leader peptide and a BarnHI site 3’ to the stop codon. The resulting fragments were re-amplified using the same 3’ primers together with the 6HISTEV 5’ primer (ATGCATCATATCATACACACGAAAACCTGATTTCGGGCT) to yield fragments encoding 6His tagged versions of the different IFN\textsubscript{d}s with a TEV cleavage site to remove the tag. The BarnHI digested fragments were cloned in PET15b digested by NcoI (blunt ended by T4 DNApol) and BarnHI. The proteins were expressed in Escherichia coli and in vitro refolded according to Dellgren et al. (25). Injections of recombinant proteins diluted in PBS containing 0.1% phenol red were performed in the caudal vein as described in Ref. 24; 2 nl were injected. Depending on the experiment, embryos/early larvae aged 36 to 60 hpf were used.

**Quantitative RT-PCR (Q-RT-PCR)**

Total RNA from 10 or more pooled zebrafish embryos or dissected spleens were prepared using Marchery-Nagel Nucleospin RNA Kit and were OligodT primed reverse transcribed using M-MLV reverse transcriptase. Quantitative RT-PCRs were performed using homemade SYBR Green mix in a LightCycler instrument (Roche), as described in (26). Results are displayed relative to GAPDH as mean values with error bars showing 95% confidence intervals in a Student’s t test. Primers are the same as in (15) except for the following ones that are used with an annealing temperature of 65°C: GAPDH (51: GCTCTTCTGCGAAGGGCATCA and 31: ATTCATCATCTGCGGAGTCAT); IFN\textsubscript{d}2 (5: see above and 3: CT GCTTTTGGTCTACCCGGGT); IFN\textsubscript{d3} (5: see above and 3: CGGTCAATGGTCTGAGGT); and IFN\textsubscript{d}4 (51: GCTCAACACAGACTTCAGGTCG and 3: CCAACTGGACTCTTGCA).

**Results**

The zebrafish genome encodes at least four related IFN\textsubscript{d}s

The sequence of zebrafish IFN\textsubscript{d}1 was used as a query sequence to screen the zebrafish genome for genes encoding IFN homologues with the use of TBlastN program at the NCBI web server (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Four IFN\textsubscript{d} genes could be identified in the V6 to V8 assemblies of the zebrafish genome. We recovered the previously described IFN\textsubscript{d}1 (initially named IFN in Ref. 11), IFN\textsubscript{d}2, and IFN\textsubscript{d}3 (named IFN2 and IFN3 in Ref. 18); conveniently, this numbering

\textsuperscript{3}Abbreviations used in this paper: SVCV, spring viremia of carp virus; IHNV, infectious haemorrhagic necrosis virus; ORF, open reading frame; hpf, hours post fertilization; hpc, hours post viral challenge; Q-RT-PCR, quantitative RT-PCR.
corresponds to their chromosomal location. As shown in Fig. 1A, IFNφ1, 2, and 3 are encoded on chromosome 3, IFNφ1 and 2 on one strand while IFNφ3 is encoded by the other strand. Exon 3 of an additional gene encoding a potential IFN homologue was also identified, and the surrounding genomic region was analyzed for exons 1, 2, 4, and 5 at the NetGene2 server (http://www.cbs.dtu.dk/services/NetGene2/). This yielded a hypothetical gene designated IFNφ4 here (or fIFN-2 in Ref. 27), encoded on another chromosome, chromosome 12, and deposited in GenBank under the accession number FJ970648 (www.ncbi.nlm.nih.gov/nuccore/238915935). Like mammalian IFNα, all four zebrafish IFNφs are encoded by genes with five exons separated by four phase zero introns. According to Zou et al.’s nomenclature (18), IFNφ1 and IFNφ4 have only one pair of conserved cysteines and belong to fish IFNφ group I while IFNφ2 and 3 with their two pairs of conserved cysteines belong to group II (Fig. 1B). A further subdivision of each of these groups in two subgroups has been recently proposed by Chang et al. (28); however, when we included the new zebrafish IFNφ4 sequence in this phylogeny, it was not possible to ascribe it with a significant bootstrap value to one or the other subgroup of 2-cysteine IFNs (Fig. 1C).

Expression of zebrafish IFNφs

IFNφ1 was previously shown to be induced in vivo by infection with the snakehead rhabdovirus in larvae and adults (29) or SVCV in larvae (15). More recently, induction of IFNφ1, 2 and 3 following intraperitoneal SVCV infection of adult zebrafish was documented (19). As embryos or early larvae are more convenient than adults to perform gain- or loss-of-functions experiments, we initially tested the expression of all four IFNφs in larvae infected by i.v. injection of either SVCV or IHNV. However, as transcripts of one of the genes could not be amplified from larvae, we also tested adults infected by immersion; only SVCV was used, as IHNV bath did not result in any obvious sign of infection (data not shown). RNAs from infected whole larvae were prepared at 24 hours post viral challenge (hpvc) (82 hpf) while RNAs from dissected adult spleens, clearly enlarged as a consequence of viral infection, were prepared at 48 hpvc. Expression levels for the different IFNφ genes were measured by Q-RT-PCR and displayed relative to GAPDH (Fig. 2).

IFNφ1 is expressed at low levels in the larvae, with a moderate induction following viral challenge. However, in the adult fish IFNφ1 is present at high constitutive levels but is still significantly induced by viral infection. Interestingly, there is no detectable expression of IFNφ2 in larvae neither before nor after viral challenge. However, the expression in adults is comparable to that of IFNφ1, indicating that the IFNφ2 gene is functional. IFNφ3 is present at relatively high constitutive levels in both larvae and adults. This high background of IFNφ3 expression, similar to that of IFNφ1 after SVCV infection, suggests that IFNφ3 expression

FIGURE 1. Virus-induced IFNs in zebrafish. A, Chromosomal localization of the zebrafish IFNφs. B, sequence alignment of the zebrafish IFNφs. Identical amino acids are shown in black outline with white lettering; similar amino acids are shown in gray outline with black lettering. Conserved cysteines important for classification are indicated by C above the alignment. Exon boundaries are indicated by arrows. C, Phylogenetic tree of various fish virus-induced IFNs obtained by the neighbor-joining method, with bootstrap values for 100 iterations. Subgroup naming according to Chang et al. (Ref. 28).
could also be regulated at a different level. We have previously shown (15) that IFN$^β$ can be transcribed from two alternative start sites, with differential splicing of the two forms giving rise to either a functional or a nonfunctional protein (note that the levels displayed in Fig. 2 correspond to the functional mRNA only); it remains to be tested if similar events, also described for trout IFN$^I$ (30), occur with the IFN$^β$ gene. IFN$^β$ is present at low constitutive levels and appears to be only moderately induced by SVCV infection in larvae and weakly in adults.

IFN$^β$1, 3, and 4 are differentially induced by IHNV and SVCV in the larva. Interestingly, while SVCV induces IFN$^β$1 and IFN$^β$4 to higher levels than IHNV in the embryo, the reverse is true for IFN$^β$3, which shows higher levels of induction with IHNV.

SVCV infection of adults induces a very high IFN$^β$ response in terms of steady-state mRNAs levels for IFN$^β$1, IFN$^β$2, and a moderate response for IFN$^β$4. Remarkably, bath SVCV infection shuts down the expression of the IFN$^β$3 gene in adult spleens.

**Biological activities of the different zebrafish IFNs**

To test for the biological activities of these newly described zebrafish IFNs, the four ORFs were cloned in the pTol2S263C expression vector. In this vector, ORF expression is driven by the promoter of zebrafish ribosomal S26 gene. The expression vectors were injected at the one-cell stage together with a similar plasmid containing a mCherry-encoding ORF, and successfully injected embryos were sorted at 24 hpf using the red fluorescence driven by the coinjected plasmid. In this system, mRNAs transcribed from the injected plasmid accumulate to a significant level starting from 12 hpf (data not shown). RNAs were prepared at either 24 or 72 hpf to quantify expression of the IFN induced reporter gene $viperin$ ($vig$-1/rsad2). Control embryos were either not injected or injected with the vector expressing mCherry alone. As shown in Fig. 3A, IFN$^β$1, 2, and 3 overexpression lead to a clear induction of $viperin$ as early as 24 hpf while IFN$^β$4 expression plasmid injected animals exhibit a less potent and less reproducible $viperin$ induction even at 72 hpf. Similar inductions of MXA were observed (data not shown).

**FIGURE 2.** Expression of zebrafish IFN$^β$ genes. Quantification by RT-PCR of IFN$^β$ mRNA, relative to GAPDH, in adults or 82 hpf larvae. Viral challenge with either SVCV or heat-adapted IHNV was performed by i.v. injection in larvae and RNA extracted from whole animals 24 hpc; SVCV challenge of adults was performed by bath infection and RNA extracted from spleens 48 hpc.

**FIGURE 3.** Induction of antiviral genes and state by zebrafish IFN$^β$s. A and C. Level of $viperin$ mRNA measured by Q-RT-PCR from whole embryos. A. Embryos at one-cell stage were either not injected, injected with a mCherry-expression plasmid, or injected with a mixture of mCherry and zIFN$^β$-expression plasmids. RNAs were tested at 24 or 72 hpf. B. Coomassie blue-stained gel of the purified recombinant IFN$^β$ proteins (1 μg/ lane); theoretical MW for rIFN$^β$1, 2, and 4 is 19.4, 18.0 and 18.6 kDa, respectively; the minor band in lane 2 is due to incomplete TEV cleavage. C. Embryos were injected i.v. at 36 hpf with recombinant IFN$^β$1, IFN$^β$2, or IFN$^β$4, or with BSA, or left un.injected, and RNA was extracted at 42 hpf. D. Larvae were i.v. injected at 54 hpf with 100 pg of recombinant proteins, and challenged 7 h later by a i.v. injection of 50 pfu of heat-adapted IHNV; mortality was monitored for 5 days. n = 14 or 15 per group.
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circulation is well established. We first tested biological response

2, or 4 were injected in the caudal vein at 36 hpf; at this time blood

challenge experiment. Early zebrafish larvae (54 hpf) were injected

a combined heat and DTT treatment and found that this totally

combinant preparations, we performed the same experiments after

inducer. As a proof of the purity and specific activity of our re-

marginal effect could be observed following IFN

hpf in A to C except for IFNα4 assays (72 hpf). D, Effects of CRFB1 and CRFB2 overexpression on viperin induction by recombinant IFNβ. Embryos were injected at the 1-cell stage with 6 pg of CRFB expression plasmid and at 36 hpf with ~100 pg of protein iv; RNA was extracted at 40 hpf. E, Effects of CRFB-specific morpholinos on viperin induction by recombinant IFNβ. Embryos were injected at the 1-cell stage with 4 ng of morpholino and at 36 hpf with ~100 pg of protein iv; RNA was extracted at 42 hpf. F, Survival curves in IHNV challenge experiments. Four nanograms of morpholino were injected at the 1-cell stage, ~100 pg recombinant IFNβ (or BSA) injected i.v. at 49 hpf, and 50 pfu of 25.70 IHNV injected i.v. at 54 hpf. n = 9 to 14 per group.

To confirm that the observed induction of reporter genes was

indeed mediated by IFNs, we decided to produce recombinant

proteins. Initially, the production all 4 IFNαs was attempted;

IFNα1, 2, and 4 were produced in E. coli and extensively purified

(Fig. 3B), but we failed to produce IFNα3. Recombinant IFNα1,

2, or 4 were injected in the caudal vein at 36 hpf; at this time blood
circulation is well established. We first tested biological response

at 4, 6, 24, 48, and 72 h post injection and observed reporter gene

inductions as soon as 4 h, peaking at 6 h, lower at 24 h and un-
detectable at 48 and 72 h (data not shown). Fig. 3C shows the

viperin inductions at 6 h post injection. These data confirm that

IFNα1 and 2 are potent inducers of viperin while IFNα4 is a poor

inducer. As a proof of the purity and specific activity of our re-

combinant preparations, we performed the same experiments after

a combined heat and DTT treatment and found that this totally

abrogated its activity (data not shown).

The activity of zebrafish IFNαs was also assessed in a viral

challenge experiment. Early zebrafish larvae (54 hpf) were injected

in the caudal vein with recombinant IFNα1, 2, or 4 at different

concentrations. Six or 7 hours later, they were challenged by i.v.

injection of ~50 pfu of the heat-adapted 25–70 IHNV virus, and

incubated at 24°C. At this dose, all larvae normally die between 48

and 72 hpc (M. Ludwig, P. Boudinot, E. Colucci-Guyon, M. Bré-

mont, P. Herbold, and J.-P. Levraud, manuscript in preparation),
as was observed in the control group injected with BSA instead of

IFN (Fig. 3D). In contrast, a significant (and dose-dependent, data

not shown) increase in survival time was observed following IFNα1 or 2 injection; IFNα1 was the most potent protein. Only a

marginal effect could be observed following IFNα4 injection at its

highest dose of 100 pg per larva.

Group I and II zebrafish IFNα signal through different receptor complexes

We have already shown that IFNα1 signals through a receptor

complex that includes CRFB1 and CRFB5 (15). To check whether

the other IFNαs would signal through the same receptor complex,

we measured viperin induction upon overexpressing the different

IFNαs in embryos in which either CRFB1 or CRFB5 had been

knocked down using morpholinos (Fig. 4A). The CRFB5 morphol-

ino abolished viperin induction by all IFNαs. Knock-down of

CRFB1 clearly suppressed the induction by IFNα1, and also

reduced the already moderate induction by IFNα4. In contrast, it

barely affected induction by IFNα2 and 3, and far less than CRFB5

knock-down. This suggests that CRFB5 is a shared component

required for all IFNαs and implies that IFNα2 and 3 signal

through receptors that include CRFB5 together with a chain different

from CRFB1.

To identify the second transmembrane component of the IFNα2

and 3 receptors, morpholinos targeting the other members of the

class II cytokine receptor family in zebrafish were tested for their

capacity to interfere with IFNα2 and IFNα3 signaling. Of all the

tested morpholinos, only the one targeting CRFB2 had an effect on

IFNα2 signaling as measured by viperin induction (Fig. 4A and data not shown).

We then performed gain of function analysis of the receptors. As

previously reported in the case of the IFNα1 receptor complex

(15), we did not observe that CRFB5 would be limiting in medi-

ating the biological effects of IFNα2, 3, and 4. We therefore report

only results for CRFB1 and CRFB2 overexpression. As shown in

Fig. 4C, overexpression of CRFB1 leads to overinduction of
viperin in the case of IFNφ1 and 4 overexpressing larvae. In the case of IFNφ2 and 3, it is CRFB2 over-expression that leads to viperin overinduction. These gain of function analyses thus confirm the results of the morpholino loss of function analysis and shows that IFNφ1 and 4 use a receptor complex including CRFB1 while IFNφ2 and 3 use a receptor complex including CRFB2.

Similar loss of function and gain of function experiments were performed using i.v. injection of recombinant proteins. For gain of function analysis with recombinant proteins, total RNAs were prepared 4 h after i.v. injections of recombinant proteins to stress the over-induction. Results are depicted in Fig. 4D: as inductions with the less potent IFNφ4 did not yield statistically significant results, they have not been included. As expected, overexpression of CRFB1 led to an overinduction of viperin following IFNφ1 injection while overexpression of CRFB2 led to an overinduction of viperin following IFNφ2 injection. Unexpectedly, overexpression of CRFB2 also leads to a weak overinduction of viperin following IFNφ1 injection, and a similar effect is observed for CRFB1 with IFNφ2. This was not observed in the experiments where the embryos were overexpressing the IFNs, which could suggest that bac-
terially produced recombinant IFNs could signal on heterologous receptors at high concentration. In Fig. 4E, embryos injected with morpholinos against either CRFB1 or 2 were injected with either IFNφ1, 2, or 4 recombinant proteins. Again, knock-down of CRFB1 negatively affected IFNφ1 and 4 signaling whereas knock-
down of CRFB2 negatively affected IFNφ2 signaling, thus confirm-
ing our previous findings. Intriguingly, knocking down CRFB2 led to over-induction of viperin by IFNφ1 and vice versa with moCRFB1 and IFNφ2. This is observed only when injecting recombinant proteins and probably reveals kinetical differences due to the burst of injected IFN as opposed to the constant perfu-
sion of IFN in the case of overexpressing larvae. It suggests that the receptors may be competing for downstream signaling ele-
ments (kinases?) present in limiting amounts.

To further clarify the roles played by CRFB1 and CRFB2 in IFNφ1 and IFNφ2 signaling, we tested their influence in viral challenge experiments. We generated morphant larvae, injected them i.v. at 49 hpf with recombinant IFNφ, challenged them 5 h later with an injection of IHNV, and monitored their survival (Fig. 4F). As observed previously, in control animals, IFNφ1 exerts a substantially better protection than IFNφ2 (Fig. 4F, left). The knock-down of CRFB1 almost completely abolishes the protective effect provided by IFNφ1, similarly to what had been observed in SVCV infections (15), while that of CRFB2 only has a very marginal effect. The reciprocal experiment, however, was less conclusive, with both CRFB1 and CRFB2 apparently involved in medi-
ating the antiviral effect of IFNφ2. This is probably due to the fact that the overall IFNφ mediated effect results not only from the injected recombinant IFN, but also from the endogenous produc-
tion of antiviral cytokines induced by the viral infection. As shown in Fig. 2, IHNV infection induces both IFNφ1 and IFNφ2 expres-
sion. Thus, especially when taking into account the apparently greater antiviral activity of IFNφ1 over the other IFNφs, these results are still compatible with a signaling of IFNφ1 through CRFB1 and IFNφ2 through CRFB2.

**Discussion**

Mammals have three types of IFNs: Type I (IFNα/β) in hu-
mans and type III (IFNA) are induced by viral infection, while type II (IFNγ) is mainly a Th1 cytokine involved in both acquired and innate immunity (1). Although most species have a single type II IFN, all known species have numerous virally induced IFNs. Mammals have used both gene duplications and gene conversions to increase the number of copies of useful genes and create extra material from which new IFN genes may arise (31). Since the first repor-
t of a cloned IFN gene in fish, many new genes have been reported suggesting that this diversification of the virus-induced IFNs is a widespread phenomenon in vertebrates. We have tried to reach a comprehensive description of the virally induced IFNs in zebrfish based upon analysis of the reported genome sequence. These analyses have yielded four candidate genes that we have named according to their chromosomal locations: IFNφ1 to IFNφ4. They all have the same intron/exon structure, but can be classified in two groups according to their conserved cysteines as proposed by Zou et al. (18). The new zebrfish IFNφ4 sequence belongs to group I with only one pair of conserved cysteines; however, it does not fall neatly into one of the subgroups recently proposed by Chang et al. (28). The classification of teleost virus-
induced IFN genes is thus set for further refinements in the future, especially with a wider sampling of species given the overrepre-
sentation of genes from salmonids in the current list.

Interestingly, the expression and induction patterns of the ze-
brafish IFNφs differ. In larvae, we found no conditions where IFNφ2 expression could be detected, while the other subtypes show a detectable constitutive expression and a significant induc-
tion with two rhabdoviruses, a vesiculovirus (SVCV) and a no-
virhabdovirus (IHNV). High expression of IFNφ2 in adult spleens suggests that the expression pattern of this IFN is restricted to one or more cell population that is absent from the embryo. We are currently engineering reporter fish lines to characterize such IFNφ2 producing cells.

Bath SVCV infections of adults revealed a very interesting sit-
uation where the levels of IFNφ1, IFNφ2, and IFNφ4 mRNAs in the spleen are increased over the constitutive expression while that of IFNφ3 is decreased. This decrease of IFNφ3 mRNA following SVCV infection of adults is in stark contrast with a recent report of IFNφ3 induction in the head of i.p. SVCV infected zebrafish (19), again suggesting the involvement of responsive cell popula-
tions with very specific tissue distributions.

The induction pattern also depends on the virus: in the embryo, while SVCV mainly induces IFNφ1 and 4 (group I), IHNV mainly induces IFNφ3 (group II). The very high level of uninduced IFNφ3 mRNA in the embryo suggests that like IFNφ1 or trout IFN1, the regulation of the expression of this subtype could include alternative promoters and/or posttranscriptional events. We will clone the full length mRNA from infected and uninfected larvae to test this possibility. Our findings generally support the idea of a selective advantage given by a diverse array of virus-
induced IFNs differentially up-regulated in specific contexts and cell types.

In mammals, several signaling pathways are used for the induc-
tion of the virus-induced IFNs; the differences between IFNα- and IFNβ-inducing cascades are now well established (32). Elucida-
tion of the equivalent pathways that lead to the induction of spe-
cific fish IFNφ genes will be of great interest, and may also explain the divergent patterns of expression that we have observed be-
tween larvae and adults. The studies published so far have shown that TLR3 and TLR22 (33), TRIF (34), and MAVS and RIG-I (35) can participate in fish IFN induction; and two studies started to address the question of the specificity of these pathways for the various IFNs of salmonids by promoter analysis and measure of induction by different stimuli (13, 30).

We tested the biological activities by measuring the transcrip-
tional induction of known IFN responsive genes in response to different IFNφ subtypes by two methods: overexpression in the developing embryo or i.v. injection of the recombinant protein. Recombinant IFNφ2 could not be produced; we have very recently
found that its purification requires different conditions, which will be described in a future paper. Both methods indicate that IFN-α1 and 2 have a high biological activity while IFN-α4 appears much less potent; IFN-α6 was only tested by over-expression but appeared as potent as IFN-α1 and 2. We also tested the biological activity of IFN-βs in terms of resistance to viral infection and found that they were well correlated with the reporter gene induction results. IFN-α1 and, to a lesser extent, IFN-α2 effectively slowed down the course of an experimental IHNV infection, while IFN-α4 did not, at least using a single-dose protocol. This suggests that IFN-α4 has a lower specific activity or is much less stable in the embryo. This is reminiscent of the human situation where IFN-α1 displays a very low specific activity as compared with other IFN-α subtypes (36). In this regard, however, a difference between the human and zebrafish is that human IFN-α1 is one of the most abundant IFNs synthesized during viral challenges probably as a decoy against viruses that produce anti-IFN proteins (37). In our tests, IFN-α4 was the subtype with the lowest expression level, but it is possible that under different conditions (other pathogens?) this subtype might be expressed at a much higher level. It must be noted that we have not analyzed a complete array of virus/IFN-induced reporter genes in this work, and that we used only rhabdoviruses; thus, the specific regulation and activity of the IFN-βs may be different in the context of infection by viruses belonging to other families and inducing different pathogenesis.

Although checking contribution of specific transmembrane components in the signaling pathway of the different zebrafish IFN-βs, we have shown that IFN-β1 and 4 (group I) and IFN-β2 and 3 (group II) do not bind to the same receptor complexes. Both complexes include CRFB5 as a common chain with a short intracellular domain. The receptor complex for group I is made up of CRFB1 plus CRFB5 while the receptor complex for group II is made up of CRFB2 plus CRFB5. In the line of our recent paper (15), and provided that the “IFN-β” designation is accepted for virus-induced fish IFNs, we suggest that CRFB1 be named IFN-βR1, and that CRFB2 be named IFN-βR1a. Fig. 5 summarizes the structure of the virally induced IFNs receptors, with a given IFN-β binding to a specific receptor complex according to the group to which it belongs. This raises the question of the pattern of expression of these receptors. Of CRFB1, CRFB2, and CRFB5, only the latter is highly expressed and we have successfully used in situ hybridization to show that it is widely expressed in all tissues of the developing zebrafish embryo (15). In contrast, CRFB1 and CRFB2 are very weakly expressed; this allows gain of function analysis but has so far prevented our attempts of in situ hybridization. This suggests that we are not in the situation where only a small subset of cells would express these receptors at high levels, but rather a situation where many cells express low levels of mRNAs.

Another question raised by the presence of multiple receptors for virus-induced IFNs is that of the transduction pathways. Some overlap of the pathways downstream of the two receptor complexes is to be expected, because some genes, like viperin that we have used as a reporter, are induced by both. We can reasonably postulate that Tyk2 is the kinase associated with CRFB5 while Jak1 would be associated with CRFB1 and 2. Because Jak1 does not seem duplicated in zebrafish, both receptors would use the same kinases. Concerning the STATs, the situation is more complicated because the zebrafish has two STAT1 proteins: STAT1a and STAT1b (16). We are designing experiments to test for the contribution of each in the transduction pathways for both receptor systems.

Interestingly, a phylogenetic analysis of the fish CRFBs reveals that CRFB1 and CRFB2 are most closely related and probably derived from a relatively recent duplication (12, 16, 38). The fact that Tetraodon nigroviridis has a third gene (CRFB3) highly related to CRFB2 and probably derived by an even more recent gene duplication (12), suggests that the diversification of the virus-induced IFN family in fish is still active. It would be very interesting to analyze the full repertoire of IFN-β genes in Tetraodon and to determine the corresponding receptors. Would CRFB3 define a specific receptor for a new IFN subset, or simply play a redundant role? The next question will be to determine whether fish from the various teleost subgroups stick to the zebrafish IFN-β scheme with two receptor systems or to the tetraodon scheme with three.

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Disclosures
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