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

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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 (IFNα) 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 IFNα1 has been identified functionally. Three genes encoding virus-induced IFNαs have been reported in zebrafish. In addition to these genes clustered on chromosome 3, we have identified a fourth IFNα gene on chromosome 12. All these genes possess the intron-exon organization of mammalian λ IFNs. In the zebrafish larva, all induce the expression of reporter antiviral genes; protection in a viral challenge assay was observed for IFNα1 and IFNα2. Using a combination of gain- and loss-of-function experiments, we also show that all zebrafish IFNαs do not bind to the same receptor. 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) IFN genes have four exons, while type III (λ) 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 λ 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 subgroup (8).

Although IFN-like activities have been recognized in fish for decades (9), it is only through recent progress in genome sequencing that fish IFNs could be identified. The first antiviral activities attributed to cloned fish IFNs were reported in 2003 (10–12). Virus-induced fish IFNs have now been reported in all deeply studied fish species; most, if not all, teleost species possess several genes encoding virally induced IFNs, the current record being held by the salmon with 11 members (13). The classification of fish virus-induced IFNs remains controversial. Based on putative structural features, several authors consider them as type I IFNs (reviewed in Ref. 14). In contrast, on the basis of the exon-intron organization of their genes (12) and the structure of their receptor (15), we previously suggested that they should be rather considered as counterparts of the mammalian type III IFNs; these arguments probably apply to all fish virus-induced IFNs described to date, because no fish IFN gene without introns has been reported, nor a putative receptor chain with the structural features of IFNAR1 (see Fig. 5). Because fish virus-induced IFNs show a combination of features observed in mammalian type I and type III IFNs, Stein et al. (16) have proposed that they should be named IFNα. We adopt this nomenclature in this study.

Because IFNs play critical roles at the crossroads of innate and acquired immunity, it is difficult to sort out their respective contributions to either. The zebrafish embryo or larva is an interesting vertebrate model for the study of innate immunity because acquired immunity is not fully functional before 4 to 6 wk of development (17). Furthermore, at such an early stage, zebrafish make a convenient experimental system, allowing for a number of easy

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genetic manipulations. Performing gain and loss of function experiments makes it possible to dissect the contribution of individual cytokines to dedicated innate immunity pathways.

After the report of a first functional zebrafish IFN\(\delta\), we identified CRFB1 and CRFB5 as the two transmembrane components of its receptor, dubbing it the receptor of “the” zebrafish IFN (15). However, shortly thereafter, a report by Zou et al. (18) made it clear that fish virus-induced IFNs were more diverse than previously thought. Not only did they clone three IFNs in rainbow trout, but they also identified in silico two new zebrafish genes, which they named IFN\(2\) and IFN\(3\) (and which we label here IFN\(\delta2\) and IFN\(\delta3\) 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\(\phi1\) belongs to group I, while IFN\(\delta2\) 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\(\delta\)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\(\delta\) 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\(\delta\) cDNAs. We then used different strategies to overexpress or inject the different IFN\(\delta\)s in 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\(\delta\) 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 F1 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)\(^3\) was used as a rhabdovirus pathogen 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 typically infecting salmonids. An American strain of SVCV was used to infect zebrafish larvae. Both strains, which grows at 25°C and was used to infect zebrafish larvae. Both SVCV and IHNV were propagated in monolayer cultures of EPC cells. Viruses were prepared using Marcherey-Nagel Nucleospin RNA Kit and were quantitated by spectrophotometry. Total RNA from 10 or more pooled zebrafish embryos or dissected spleens 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 larval stages aged 36 to 60 hpf were used.

**Cloning the zebrafish IFN\(\delta2\), 3, and 4**

IFN\(\delta3\) and 4 open reading frame (ORFs) were amplified from infected larvae by RT-PCR using the following primers: IFN\(\delta3\) (GATTCCT TAGGACATGTCCA) and IFN\(\delta4\) (GAATCGATCAGTCAGTCA) and IFN\(\delta4\) (TTACTCGAGTTACGTTGGCAAG). Due to the lack of expression of IFN\(\delta2\) in the larva, the whole gene was amplified from genomic DNA and cloned in the expression vector using IFN\(\delta2\) (GACAGCTCTAAACANATGG AATTTTGG) and IFN\(\delta3\) (CGGATCTTATATATTAGGTGAA).

**Overexpression plasmids**

The four IFN\(\delta\) ORFs were cloned in the pTOl2S263C expression vector, where their expression is driven by the promotor of zebrafish ribosomal S26 gene from chromosome 23, amplified as a 6.3-kb fragment using primers S26C23E2b (TTATGAGCTCTCTTCTTAGCTAACAGTCTGA) and S26C23P1 (GATTCTACAGCATTGCCCT) 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 zCRFB2.510 (TTACTCGAGTTACGTTGGCAAG) and zCRFB2.310 (AGCTCTAGATACTTGGAACACTAT). Morpholinos have already been described (15). After thawing, morpholinos were heated at 65°C for 10 min to ensure complete dissolution. Morpholinos are diluted to the desired concentration (typically, 500 \(\mu\)M, for a total injected amount of 4 ng) in morpholino buffer containing 0.1% phenol red. Injections are as for overexpression vectors.

**Recombinant IFNs**

The IFN\(\delta1\), 2, and 4 ORFs were first amplified using the following pairs of primers (IFN\(\delta1\)56: CCGTGATTTTACTTTCCGGGAAATGC and IFN\(\delta1\)34B: CCGGATCTCAGCTTTCCGGAGTTGA), (IFN\(\delta2\)53: CCTTTTTCCTTCCGGGAACATGGATCTTC and IFN\(\delta2\)31B) and (IFN\(\delta4\)53: CCTTTTTCCTTCCGGGAACATGGATCTTC and IFN\(\delta4\)31B) to produce ORFs with a TEV cleavage site replacing the leader peptides and a BamHI site 3' to the stop codon. The resulting fragments were reamplified using the same 3' primers together with the 6HISTEV 5' primer (ATGATCATATCATACACACGAAAACCTGTATTTTCAGGGTGTAAC) and IFN\(\delta4\)31B) to yield fragments encoding 6HIS tagged versions of the different IFN\(\delta\)s with a TEV cleavage site to remove the tag. The BamHI digested fragments were cloned in PET15b digested by NcoI ( blunt ended by T4 DNA pol) and BamHII. 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 larval stages 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 Marcherey-Nagel Nucleospin RNA Kit and were OligoT 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 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: GCCCTTTGGGAAAGGTCATCA and 31: ATTCATCATACTTGGGGGATT); IFN\(\delta2\) (5: see above and 3: CT GCTTTTGGCTACCCGGGT); IFN\(\delta3\) (5: see above and 3: CGGTCGAAT GTTTCGGAGGTT); and IFN\(\delta4\) (5: GTCAAAACAGACATCGTCATCA and 3: CGAAGCTGCATGTCTTC).

**Results**

The zebrafish genome encodes at least four related IFN\(\delta\)s

The sequence of zebrafish IFN\(\phi1\) 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\(\delta\) genes could be identified in the V6 to V8 assemblies of the zebrafish genome. We recovered the previously described IFN\(\phi1\) (initially named IFN in Ref. 11), IFN\(\delta2\), and IFN\(\delta3\) (named IFN2 and IFN3 in Ref. 18); consequently, this numbering

\(^3\) 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, IFN\(_{H9278}\) 1, 2, and 3 are encoded on chromosome 3, IFN\(_{H9278}\) 1 and 2 on one strand while IFN\(_{H9278}\) 3 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 IFN\(_{H9278}\) 4 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 IFN\(_{H9261}\), all four zebrafish IFN\(_{H9278}\) s are encoded by genes with five exons separated by four phase zero introns. According to Zou et al.’s nomenclature (18), IFN\(_{H9278}\) 1 and IFN\(_{H9278}\) 4 have only one pair of conserved cysteines and belong to fish IFN\(_{H9278}\) group I while IFN\(_{H9278}\) 2 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 IFN\(_{H9278}\) 4 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 IFN\(_{H9278}\)s
IFN\(_{H9278}\) 1 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 IFN\(_{H9278}\) 1, 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 IFN\(_{H9278}\)s in larvae 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 IFN\(_{H9278}\) genes were measured by Q-RT-PCR and displayed relative to GAPDH (Fig. 2).

IFN\(_{H9278}\) 1 is expressed at low levels in the larvae, with a moderate induction following viral challenge. However, in the adult fish IFN\(_{H9278}\) 1 is present at high constitutive levels but is still significantly induced by viral infection. Interestingly, there is no detectable expression of IFN\(_{H9278}\) 2 in larvae neither before nor after viral challenge. However, the expression in adults is comparable to that of IFN\(_{H9278}\) 1, indicating that the IFN\(_{H9278}\) 2 gene is functional. IFN\(_{H9278}\) 3 is present at relatively high constitutive levels in both larvae and adults. This high background of IFN\(_{H9278}\) 3 expression, similar to that of IFN\(_{H9278}\) 1 after SVCV infection, suggests that IFN\(_{H9278}\) 3 expression...
could also be regulated at a different level. We have previously shown (15) that IFN/H9278 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 (30), occur with the IFN/3 gene. IFN/4 is present at low constitutive levels and appears to be only moderately induced by SVCV infection in larvae and weakly in adults.

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

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

Biological activities of the different zebrafish IFN

To test for the biological activities of these newly described zebrafish IFNs, the four ORFs were cloned in the p Tol2S263C 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/1, 2, and 3 overexpression lead to a clear induction of viperin as early as 24 hpf while IFN/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).
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 IFNβs was attempted; IFNβ1, 2, and 4 were produced in E. coli and extensively purified (Fig. 3B), but we failed to produce IFNβ3. Recombinant IFNβ1, 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 induction as soon as 4 h, peaking at 6 h, lower at 24 h and undetectable at 48 and 72 h (data not shown). Fig. 3C shows the \textit{viperin} induction at 6 h post injection. These data confirm that IFNβ1 and 2 are potent inducers of \textit{viperin} while IFNβ4 is a poor inducer. As a proof of the purity and specific activity of our recombinant 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 IFNβs was also assessed in a viral challenge experiment. Early zebrafish larvae (54 hpf) were injected in the caudal vein with recombinant IFNβ1, 2, or 4 at different concentrations. Six or 7 hours later, they were challenged by i.v. injection of ~30 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. Bougniot, E. Colucci-Guyon, M. Brémont, 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 IFNβ1 or 2 injection; IFNβ1 was the most potent protein. Only a marginal effect could be observed following IFNβ4 injection at its highest dose of 100 pg per larva.

**Group I and II zebrafish IFNβ signal through different receptor complexes**

We have already shown that IFNβ1 signals through a receptor complex that includes CRFB1 and CRFB5 (15). To check whether the other IFNβs would signal through the same receptor complex, we measured viperin induction upon overexpressing the different IFNβs in embryos in which either CRFB1 or CRFB5 had been knocked down using morpholinos (Fig. 4A). The CRFB5 morpholino abolished \textit{viperin} induction by all IFNβs. Knock-down of CRFB1 clearly suppressed the induction by IFNβ1, and also reduced the already moderate induction by IFNβ4. In contrast, it barely affected induction by IFNβ2 and 3, and far less than CRFB5 knock-down. This suggests that CRFB5 is a shared component required for all IFNβs and implies that IFNβ2 and 3 signal through receptors that include CRFB5 together with a chain different from CRFB1.

To identify the second transmembrane component of the IFNβ2 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 IFNβ2 and IFNβ3 signaling. Of all the tested morpholinos, only the one targeting CRFB2 had an effect on IFNβ2 or IFNβ3 signaling as measured by \textit{viperin} induction (Fig. 4B and data not shown).

We then performed gain of function analysis of the receptors. As previously reported in the case of the IFNβ1 receptor complex (15), we did not observe that CRFB5 would be limiting in mediating the biological effects of IFNβ2, 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 IFNβ1 and 4 overexpressing larvae. In the case of IFNβ2 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 IFNβ1 and 4 use a receptor complex including CRFB1 while IFNβ2 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 IFNβ4 did not yield statistically significant results, they have not been included. As expected, overexpression of CRFB1 led to an overinduction of viperin following IFNβ1 injection while overexpression of CRFB2 led to an overinduction of viperin following IFNβ2 injection. Unexpectedly, overexpression of CRFB2 also leads to a weak overinduction of viperin following IFNβ1 injection, and a similar effect is observed for CRFB1 with IFNβ2. This was not observed in the experiments where the embryos were overexpressing the IFNs, which could suggest that bacterially 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 IFNβ1, 2, or 4 recombinant proteins. Again, knock-down of CRFB1 negatively affected IFNβ1 and 4 signaling whereas knock-down of CRFB2 negatively affected IFNβ2 signaling, thus confirming our previous findings. Intriguingly, knocking down CRFB2 led to over-induction of viperin by IFNβ1 and vice versa with moCRFB1 and IFNβ2. 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 perfusion of IFN in the case of overexpressing larvae. It suggests that the receptors may be competing for downstream signaling elements (kinases?) present in limiting amounts.

To further clarify the roles played by CRFB1 and CRFB2 in IFNβ1 and IFNβ2 signaling, we tested their influence in viral challenge experiments. We generated morphant larvae, injected them i.v. at 49 hpf with recombinant IFNβ, challenged them 5 h later with an injection of IHNV, and monitored their survival (Fig. 4F). As observed previously, in control animals, IFNβ1 exerts a substantially better protection than IFNβ2 (Fig. 4F, left). The knock-down of CRFB1 almost completely abolishes the protective effect provided by IFNβ1, 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 mediating the antiviral effect of IFNβ2. This is probably due to the fact that the overall IFNβ mediated effect results not only from the injected recombinant IFN, but also from the endogenous production of antiviral cytokines induced by the viral infection. As shown in Fig. 2, IHNV infection induces both IFNβ1 and IFNβ2 expression. Thus, especially when taking into account the apparently greater antiviral activity of IFNβ1 over the other IFNs, these results are still compatible with a signaling of IFNβ1 through CRFB1 and IFNβ2 through CRFB2.

**Discussion**

Mammals have three types of IFNs: Type I (IFNα/β/ω/κ in humans) 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 report 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 zebrafish based upon analysis of the reported genome sequence. These analyses have yielded four candidate genes that we have named according to their chromosomal locations: IFNβ1 to IFNβ4. 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 zebrafish IFNβ4 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 overrepresentation of genes from salmonids in the current list.

Interestingly, the expression and induction patterns of the zebrafish IFNβs differ. In larvae, we found no conditions where IFNβ2 expression could be detected, while the other subtypes show a detectable constitutive expression and a significant induction with two rhadoviruses, a vesiculovirus (SVCV) and a no-virhabdovirus (IHNV). High expression of IFNβ2 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 IFNβ2 producing cells.

Bath SVCV infections of adults revealed a very interesting situation where the levels of IFNβ1, IFNβ2, and IFNβ4 mRNAs in the spleen are increased over the constitutive expression while that of IFNβ3 is decreased. This decrease of IFNβ3 mRNA following SVCV infection of adults is in stark contrast with a recent report of IFNβ3 induction in the head of i.p. SVCV infected zebrafish (19), again suggesting the involvement of responsive cell populations with very specific tissue distributions.

The induction pattern also depends on the virus: in the embryo, while SVCV mainly induces IFNβ1 and 4 (group I), IHNV mainly induces IFNβ3 (group II). The very high level of uninduced IFNβ3 mRNA in the embryo suggests that like IFNβ1 or trout IFN1, the regulation of the expression of this subtype could include alternative promoters and/or postranscriptional 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 induction of the virus-induced IFNs; the differences between IFNα- and IFNβ-inducing cascades are now well established (32). Elucidation of the equivalent pathways that lead to the induction of specific fish IFNβ genes will be of great interest, and may also explain the divergent patterns of expression that we have observed between 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 transcriptional induction of known IFN responsive genes in response to different IFNβ subtypes by two methods: overexpression in the developing embryo or i.v. injection of the recombinant protein. Recombinant IFNβ3 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-γ1
and 2 have a high biological activity while IFN-δ4 appears much less potent; IFN-δ6 was only tested by over-expression but appeared as potent as IFN-γ1 and 2. We also tested the biological activity of IFN-δs in terms of resistance to viral infection and found that they were well correlated with the reporter gene induction results. IFN-γ1 and, to a lesser extent, IFN-δ2 effectively slowed down the course of an experimental IHNV infection, while IFN-δ4 did not, at least using a single-dose protocol. This suggests that IFN-δ4 has a lower specific activity or is much less stable in the embryo. This is reminiscent of the human situation where IFNα1 displays a very low specific activity as compared with other IFNα subtypes (36). In this regard, however, a difference between the human and zebrafish is that human IFNα1 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-δ4 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 rhadoviruses; thus, the specific regulation and activity of the IFN-δs may be different in the context of infection by viruses belonging to other families and inducing different pathogens.

Although checking contribution of specific transmembrane components in the signaling pathway of the different zebrafish IFN-δs, we have shown that IFN-γ1 and 4 (group I) and IFN-δ2 and 3 (group II) do not bind to the same receptor complexes. Both complexes include CRFB5 as a common chain with a short intra-cellular 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-δ” designation is accepted for virus-induced fish IFNs, we suggest that CRFB1 be named IFN-δR1, and that CRFB2 be named IFN-δR1. Fig. 5 summarizes the structure of the virally induced IFNs receptors, with a given IFN-δ 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: STAT1α 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-δ genes in Tetraodon 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-δ scheme with two receptor systems or to the tetraodon scheme with three.

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mice against influenza A virus but not against hepatotropic viruses. PLoS Pathog. 4: e1000151.