We constructed chimeric receptors wherein the extracellular domain of the erythropoietin receptor (EpoR) was fused to the transmembrane and intracellular domains of the interferon (IFN) type I receptor subunits, IFNaR1 or IFNaR2–2. Transfection into 2TgGH and Tyk2-deficient 11,1 cells showed that EpoR/IFNaR2–2 alone was able to transduce a signal upon stimulation with erythropoietin (Epo), as judged by induction of the interferon type I-inducible 6-16 promoter. In contrast, protection against infection with encephalomyocarditis virus or vesicular stomatitis virus was reduced or absent, respectively. To further investigate the role of IFNaR1 in the induction of an antiviral state, we analyzed the Epo-versus IFNo-induced transcription of a set of genes, involved in antiviral protection. Up to 24 h after stimulation with Epo or IFNo, comparable transcription of the p56, dsRNA-dependent protein kinase, 2′-5′A synthetase, and MxA genes was seen. However, at later time points, only in the case of Epo induction, a sharp decrease of mRNA levels was observed. Western blotting analysis of dsRNA-dependent protein kinase showed a similar pattern at the protein level. Taken together, our results imply a role for IFNaR1 in the induction of sustained mRNA and protein levels that are likely required for optimal antiviral activity.

It is generally accepted that activation of a cell by a cytokine is initiated by ligand-induced clustering of receptor subunits, which can occur as di-, tri-, or higher order oligomers, involving identical or related subunits. With the exception of the heptamembrane-spanning receptors, members from the hematopoietin/IFN, tumor necrosis factor/nerve growth factor, and tyrosine kinase receptor families are all activated by this mechanism of cytokine-driven multimerization. Ligand binding to the first receptor component induces the association with additional receptor subunits eventually resulting in an increase of affinity for the ligand (1, 2). Alternatively, preformed receptor complexes may also exist on the cell membrane (3).

Interferons belong to the class I cytokine family and are divided into type I interferons (at least 14 IFNa subtypes, IFNb, IFNo, and the bovine embryonic IFNg) that have antiviral, cytostatic, and hematopoietic activities on many cell types, and the type II interferon or IFNg that is also involved in many immune functions. Both types of interferons bind to distinct receptors that belong to the class I cytokine receptors. The type I interferon receptor (IFNaR) is composed of two subunits, IFNaR1 and IFNaR2–2. As a result of alternative splicing, subtypes of the latter subunit do also exist: a cytoplasmic truncated transmembrane form (IFNaR2–1) and a soluble form (IFNaR2–3) (4–8).

A large body of evidence has shown that the class I cytokine receptors make use of associated kinases (JAKs) to start intracellular tyrosine phosphorylation, resulting in the activation of the so-called Stat proteins. This JAK/Stat pathway is essential for transcription of many of the cytokine-inducible genes (9, 10, 11). In particular, interferon-induced clustering of the IFNaR1 and IFNaR2–2 subunits results in activation of JAK1 and Tyk2 by reciprocal transphosphorylation, and in phosphorylation of the receptor intracellular domains. Stat1 and Stat2 subsequently bind to the phosphorylated receptors and form heterodimers after being phosphorylated themselves. The activated Stat1/Stat2 heterodimer then associates with a DNA binding subunit, p48, to form the multisubunit complex, ISGF3, that binds with high affinity to the interferon-stimulated response element enhancer sequences, located in the promoters of the interferon-stimulated genes (9, 12–15).

One of the enigmas in IFN type I signal transduction is the specificity by which the IFNaR receptor is activated. Specificity of a cytokine can be determined at the intracellular level, where cell-specific signal transduction components can bind the cytoplasmic portions of the receptor subunits, thereby causing distinct effects of the same cytokine on different cells. At the extracellular level, however, many cytokines share a common receptor subunit (such as, for instance, the common cβ subunit for the IL-3, granulocyte-macrophage colony-stimulating factor, and IL-5 receptor complexes), and ligand-specific alkaline phosphatase; Stat, signal transducer and activator of transcription; VSV, vesicular stomatitis virus; RT, reverse transcription; PCR, polymerase chain reaction; IRTAM, interferon receptor tyrosine activation motif; TRBP, TAR RNA-binding protein; PKR, dsRNA-dependent protein kinase.

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signaling is at least in part derived from association with cytokine-specific additional receptor components (such as IL-3Rα, granulocyte-macrophage colony-stimulating factor receptor α chain, and IL-5Rα) (1). The interferon type I receptor seems to be an exception to this rule. Although IFNα and IFNβ share the same receptor components, differences in specificity in antiviral, antiproliferative, and clinical effects have been reported (16–21). Moreover, differences between IFNα and IFNβ have also been characterized at the gene induction level since only IFNβ is able to induce a gene called β-R1 in 2fTGH cells (22) and to activate the promoter of the 6-16 gene in Tyk2-deficient 2fTGH cells (23). Additionally, some recent findings suggest that IFNα and IFNβ utilize different regions of the IFNαR. For instance, co-immunoprecipitation of IFNαR1 and IFNαR2–2 can be achieved upon IFNβ but not IFNα treatment (24, 25), and two monoclonal antibodies or a polyclonal anti-serum, directed against IFNαR1, block the activity of IFNβ but not that of IFNβ (26, 27). In addition, mutagenesis studies of the IFNαR2–2 chain showed differential receptor activation since (i) intracellular deletions abrogated only the IFNβ and not the IFNα-induced antiviral response, while activation of the JAK/Stat pathway was equal in both cases (28), and (ii) extracellular mutations created receptor complexes that showed preferential binding for each of the ligands (27). It is also possible to obtain mutations in IFNβ that mimic IFNα activity (29). These data suggest that other mechanisms may be responsible for generation of signal diversity apart from utilization of cytokine-specific receptor components.

Little is known about the exact stoichiometry of the activated IFNαR complex. High affinity ligand binding and activation of the IFNα/β signaling pathways requires clustering of the IFNαR1 with the IFNαR2–2 chain (4, 5, 7, 30, 31), but it has been postulated that IFNβ may also be capable of clustering the IFNαR2–2 subunit alone (27).

To further explore the aggregation of the IFNαR1 and IFNαR2–2 chains and their effects on IFNαβ signaling, we fused the extracellular domains of the erythropoietin receptor (EpoR) to the intracellular and transmembrane domains of IFNαR1 or IFNαR2–2, thereby generating the chimeric receptors EpoR/IFNαR1 and EpoR/IFNαR2–2. Since erythropoietin homodimerizes the EpoR (32), dimerization of the IFNαR intracellular domains could be obtained, and the resulting effects on several interferon functions were studied.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Human fibrosarcoma 2fTGH cells and 11.1 cells (both gifts from Sandra Pelligrini, Institut Pasteur, Paris, France) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% fetal calf serum (Globepharm, Surrey, United Kingdom) at 37 °C in 10% CO2. Recombinant human Epo was purchased from R&D Systems (Abingdon, UK). IFNα was purchased from PeproTech inc. (Rocky Hill, NJ), and IFNβ was a gift of Dr. P. Hochman (Biogen Inc., Cambridge, MA).

DNA Constructs—The p6-16SEAP vector was constructed by transfecting a HindIII fragment containing the entire 6-16 promoter from the plasmid p6-16lici (gift from Sandra Pelligrini) to the HindIII-opened pSEAP vector (Tropix, Bedford, MA), so that the 6-16 promoter was cloned upstream of the SEAP coding region. EpoR/IFNαR receptor chimeras were constructed as follows. RNA was isolated from 5 × 106 TF-1 cells using the RNeasy kit (Qiagen). According to standard RT-PCR procedures, cDNA was prepared and the PCR performed using Pfu enzyme (five units; Stratagene). Forward (CGGGGTACCATGGCACCCCTGGGGGTGTC) and reverse (CCCTTAATTAAGTCCAGGTCGCTAGGCGTCAG) primers were designed to amplify the extracellular part of the EpoR between a KpnI and PacI site. A band of correct size was visualized and the DNA was digested with KpnI and PacI and inserted into the KpnI-PacI opened pSV-SPORT IL-5Rα/IFNαR2–2 or pSV-SPORT β/IFNαR1 vectors. These vectors contain chimeric receptors that have the extracellular domain of the receptors for IL-5, IL-5Rα, or βc, fused to the transmembrane and intracellular domains of IFNαR1 or IFNαR2–2. By site-specific mutagenesis, a PacI site was added to the fusion point by means of the site-directed mutagenesis kit (Stratagene, La Jolla, CA), which resulted in the insertion of two amino acids (Leu-Ile) before the last extracellular membrane-proximal amino acid (Lys) of IFNαR1 and IFNαR2–2.2 Hence, using the KpnI site that precedes the coding sequence and the PacI site that follows the coding sequence, the extracellular domain of IL-5Rα or βc could be exchanged by the one of EpoR, as described above. The resultant vectors were named pSV-SPORT-EpoR/IFNαR2–2 and pSV-SPORT-EpoR/IFNαR1, respectively.

Transfection Conditions and Development of Stable Cell Lines—All transfections were performed using the calcium phosphate method (33). 2fTGH cells, stably transfected with p6-16SEAP, were obtained by co-transfection of 20 μg of p6-16SEAP with 2 μg of pBSpac/deltap also using the calcium phosphate transfection method. The pBSpac/deltap plasmid contains a gene for puromycin resistance under control of the constitutive SV40 early promoter. Optimal puromycin concentration was determined as 3 μg/ml. Single colonies were isolated by limited dilution in 96-well microtiter plates. The clone 2fTGH6-16SEAP clone 5 was selected based on an optimal IFNα and IFNβ stimulation window of SEAP secretion.

For development of stable cells expressing the EpoR/IFNαR chimeras, either 2fTGH 6-16SEAP clone 5 or 2fTGH cells were transfected with 20 μg of pSV-SPORT-EpoR/IFNαR2–2 and 2 μg of pcDNA1/Neo. Stable transfecants were selected in medium containing G418 (400 μg/ml).

Clones of the Tyk2-deficient 11.1 cells with the stable integration of pSV-SPORT-EpoR/IFNαR2–2 were obtained by cotransfection of the latter plasmid with pBSpac/deltap. Surviving clones on puromycin were tested on their integration of the pSV-SPORT-EpoR/IFNαR2–2 plasmid by transfecting the individual clones with 6-16SEAP. 11.1 EpoR/F2–2 cl. 4 was selected.

SEAP Measurements—The amount of secreted alkaline phosphatase was determined with the Phospha-light kit (Tropix), using disodium 3′-4′-methylenespiro[1,2-dioxetane-3,2′-(5′-chlorotricyclo[3.3.1.1decan]-4′-y]phenyl phosphosphate as the lumogenic substrate. Assays were performed in a 96-well microtiter plate following the manufacturers guidelines, and were counted in a Topcount luminometer (Canberra Packard, Zellik, Belgium).

Antiviral Assays—For antiviral assays, cells were treated with different IFN or Epo concentrations as indicated in the figures. VSV or EMCV was added after 24 h, and the cytopathic effect was scored another 20 h later by removing the medium and staining of living cells for 10 min with 100 μl of a crystal violet solution (0.5% crystal violet (w/v), 3% formaldehyde, 30% ethanol, and 0.17% NaCl (w/v)). After this, the plates were extensively washed and dried and staining was quantified by solubilizing in 100 μl 30% acetic acid and measurement in an enzyme-linked immunosorbent assay reader (Dynatech Laboratories Inc., Chantilly, VA) at 590 nm. As a control on growth inhibition, a similar experiment was set up, but without virus addition. All experiments were performed in triplicate.

Northern and Western blot Analysis—For the Northern blot analysis, total RNA was prepared from 2fTGH 6-16SEAP EpoR/IFNαR2–2 cells using the RNeasy method (Qiagen). RNA (10 μg) was separated on a 1.5% agarose, 6% formaldehyde gel, transferred to a nylon membrane (Zetaprobe; Bio-Rad), and cross-linked using UV radiation. The filters were hybridized for 1 h at 68 °C in ExpressHyb solution (CLONTECH) with [32P]dCTP-labeled DNA probes and washed three times with 0.1× SSC, 0.05% SDS at room temperature and twice in 0.1× SSC, 0.1% SDS at 50 °C. Blots were exposed to film using intensifying screens at –70 °C. β-Actin was used for normalization.

For the Western blot analysis, whole cell extracts of 2fTGH 6-16SEAP EpoR/IFNαR2–2 cells were obtained by lysis in Laemmli SDS-polyacrylamide gel electrophoresis sample buffer, sonication and boiling. Proteins from equal amounts of cell lysate were resolved by 15% polyacrylamide gel electrophoresis sample buffer, sonication and boiling. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). Ponceau S staining (Serva, Heidelberg, Germany) controlled the transfer of proteins to the membrane and allowed normalization of the different samples. Membranes were blocked and incubated with primary antibodies. Specific bands were revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech). Membranes were exposed to Enhanced chemiluminescence renaissance reagents (NEN Life Science Products). Polyclonal rabbit antisera to PKR was obtained from Santa Cruz Bio-technology, and mouse monoclonal anti-PKR was from Transduction Laboratories.

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indicated that homodimerization of the IFNαR2–2 receptor is sufficient for activation of the 6-16 promoter. Induction levels comparable to IFNα were observed. Since both chimeric receptor complexes probably appear at the cell membrane of the double-transfected cells, we were not able to attribute the Epo-induced 6-16 promoter activation to EpoR/IFNαR2–2 homodimerization or to EpoR/IFNαR1 + EpoR/IFNαR2–2 heterodimerization. No mRNA, coding for the wild-type EpoR, is present in the cells as determined by RT-PCR analysis on 2TGH 6-16SEAP clone 5 cells (data not shown).

To eliminate possible cross-talk with the endogenous IFNαR1, we transfected the EpoR/IFNαR2–2 chimeras in 2TGH cells that lack the IFNαR1-associated Tyk2 kinase (11, 1 mutant cells). These cells are deficient in signaling via the IFNαR1 component of which they express barely detectable levels (34, 35). A clear Epo-induced SEAP expression in the 11,1 transfectants was observed (Fig. 3B), underscoring that signaling occurred through homodimerization of the IFNαR2–2 cytosolic domains. Similar 6-16SEAP induction by Epo was observed in 11,1 cells stably expressing the EpoR/IFNαR2–2 chimera (11, 1 EpoR/IFNαR2–2 clone 4) (Fig. 3A). Interestingly, in transiently transfected cells and in this stable clone, but not in the parental 11,1 cells transfected with the 6-16SEAP construct alone, a loss of IFNβ-induced 6-16 promoter activity was observed (Fig. 3, panels A–C). This effect is likely due to recruitment of the available JAK1 pool to the chimeric receptor.

**Induction of Antiviral Activity**—Certain mutations of the IFNαR receptor can uncouple IFN-induced activation of the JAK/Stat pathway from the antiviral activity (28), indicating that additional pathways are involved in the latter activity. Because induction of SEAP production by the 6-16 promoter depends largely on JAK/Stat activation, we wished to compare the function of the chimeric receptors in antiviral assays versus the SEAP induction assay.

2TGH 6-16SEAP and 2TGH cell lines that stably expressed the EpoR/IFNαR2–2–2 chimeric receptor were developed by selection on G418 as described under “Experimental Procedures” (2TGH 6-16SEAP EpoR/IFNαR2–2 clone 4, and 2TGH EpoR/IFNαR2–2 clones 1 and 8, respectively). 2TGH 6-16SEAP EpoR/IFNαR2–2 clone 4 cells were treated for 24 h with serial dilutions of Epo or IFNα, incubated for 20 h with VSV or EMCV and subsequently examined for survival.

Fig. 4A shows a protective effect of Epo against EMCV thereby providing evidence that the IFNαR1 subunit is not dispensable for antiviral activity. However, when compared with IFNα, this effect was at least 10-fold lower with significantly reduced plateau levels. Furthermore, Epo was completely unable to protect cells from infection with VSV (Fig. 4B)
even at high concentrations. Similar results were obtained on the other clones (2fTGH EpoR/R2–2 clones 1 and 8, data not shown). On 11.1 EpoR/R2–2 clone 4 cells, a similar low protection by Epo was observed against EMCV (Fig. 4C), while Epo was completely inactive on parental 11.1 cells (Fig. 4D). This observation lends further support to the fact that no functional signaling via the endogenous IFNaR1 chain is required to explain the observed effects via the EpoR/IFNaR2–2 chimera.

Since the reduction or absence of antiviral activity could also be interpreted as an induction of growth inhibition by the cytokine, we examined the effect of Epo versus IFNa on cell growth in the absence of virus. A small growth-inhibitory effect was seen after treatment with the same Epo concentrations as used for the antiviral assay. This effect was slightly weaker when compared with IFNa treatment (Fig. 4E). This indicates that there is no direct relation between growth inhibition and antiviral activity in our experiments. The results we see in the antiviral assays may, however, be the net result of antiviral and antiproliferative activities. This could explain why we see a reduction in cell number with VSV infection upon Epo treatment since a small, growth-inhibitory effect of Epo may become enlarged upon VSV infection. In the case of IFNa, this effect may be masked by the protective, antiviral effect that this cytokine exerts on the cells. We cannot exclude however, that aberrant Epo/IFNaR2–2 receptor activation resulting from high Epo concentrations could lead to an increased viral infection.

To ascertain that the reduced antiviral activity by EpoR/IFNaR2–2 homodimerization was not the result of instability of Epo, we incubated medium containing Epo, IFNa, or no cytokine in the presence or absence of the 2fTGH 6-16SEAP EpoR/IFNaR2–2 clone 4 cells for 6, 11, 24, 36, and 48 h. After this, the media were tested for activity by transferring them to the 2fTGH 6-16SEAP EpoR/IFNaR2–2 clone 4 cells and measuring SEAP induction. Fig. 5 shows that no clear reduction in Epo or IFNa activity could be measured when ligands were incubated in the absence of cells, indicating that the intrinsic stability of both cytokines was sufficient for longer incubation times. Pre-incubation of Epo and IFNa in the presence of cells caused a reduction in activity, probably due to ligand internalization or to proteases released by the cells, but this reduction was small and equal for both ligands and could therefore not account for the difference in antiviral activity (Fig. 5). Moreover, addition of Epo or IFNa every 8 h during the antiviral assay did not narrow the window in antiviral protection between both cytokines (data not shown).

Induction of p56, PKR, 2'-5'A Synthetase, and MxA mRNA—We further investigated the EpoR/IFNaR2–2-dependent induction of mRNAs of genes that are strongly induced by interferon. PKR, 2'-5'A synthetase and MxA are known to have a central role in the antiviral protection mechanism of the cell (14). p56 was also investigated because it is very strongly induced by interferons (36), and it was recently shown to be involved in translation inhibition (37). RNA levels of these genes were measured 4 h after addition of Epo or IFNa by Northern blot analysis. We used the same molar range of both cytokines as done in the antiviral assays. As shown in Fig. 6, a strong mRNA induction of the four genes was observed. Levels of induction were comparable between Epo and IFNa, again indicating that the IFNaR1 component is not essential for gene induction.

In addition, a time-response Northern blot analysis was performed (Fig. 7). 1. 4, 6, 12, 24, 48, and 72 h after stimulation with IFNa (1.7 ng/ml) and Epo (5 ng/ml), cell lysates were made and RNA was prepared. We could not see a difference in gene induction of the considered genes up to 24 h after stimulation. However, over longer periods (48 and 72 h) Epo-induced mRNA levels clearly declined as compared with IFNa-induced levels.

Expression of PKR and TRBP Proteins—We next tested whether the differences in Epo or IFNa-induced mRNA levels, observed after 24 h, were also seen at the protein level. We took PKR as an example since it is one of the pivotal actors in the interferon induced cellular antiviral response and strongly affects VSV infection (38). Western blot analysis was performed on total cell lysates of cells treated for 6, 12, 24, and 48 h with IFNa (1.7 ng/ml) or Epo (5 ng/ml) as described for the Northern blot analysis in Fig. 7. Blots were revealed by rabbit antibodies raised against the C-terminal end of human PKR (Fig. 8). Similar results were obtained using a mouse monoclonal antibody raised against the RNA binding part of PKR (data not...
shown). Both IFNα and Epo induced the expression of PKR; however, the induction of PKR by IFNα was significantly stronger compared with Epo. Differences could be noticed already following 12 h of treatment (Fig. 8).

Benkirane et al. (60) have shown that treatment of HeLa cells with IFNα up-regulated the steady state levels of PKR and down-regulated those of its cellular inhibitor TRBP. It was suggested that this alteration in the intracellular stoichiometry of PKR and TRBP adversely affects the formation of PKR-TRBP heterocomplexes and thus allows an increase in the antiviral activity of PKR. We therefore revealed the same blots also with antisera directed against TRBP (Fig. 8). Although

Fig. 4. Antiviral activity mediated by different receptor chimeras. A, efficiency of the EpoR/IFNaR2–2 chimera in mediating Epo-induced antiviral activity against EMCV on the 2fTGH 6-16SEAP EpoR/R2–2 clone 4 cells. The cells were treated with the indicated concentrations of Epo or IFNα, and 24 h later virus was added. After another 20 h, surviving cells were stained with crystal violet and quantified by spectroscopy at 590 nm. B, the same conditions were used, except that VSV was used. C, similar to panel A, except that Tyk2-deficient 11.1 EpoR/R2–2 clone 4 cells were used. D, similar to panel C, using parental 11.1 cells. E, control for antiproliferative activity of Epo and IFNα on non-infected 2fTGH 6-16SEAP EpoR/IFNaR2–2 clone 4 cells. Experimental conditions were exactly the same as in the antiviral assays, with the exception that no virus was added. The panel show typical experiments on the 2fTGH 6-16 SEAP EpoR/R2–2 clone 4 cells, but similar results were obtained with other clones (2fTGH EpoR/R2–2 clone 1 and 8) (data not shown).

Fig. 5. Stability of Epo and IFNα. Dulbecco's modified Eagle's medium was supplemented with Epo or IFNα and incubated at 37 °C in the presence or absence of 2fTGH 6-16SEAP EpoR/IFNaR2–2 clone 4 cells for 8, 11, 24, 36, and 48 h. The medium was then transferred to new 2fTGH 6-16SEAP EpoR/IFNaR2–2 clone 4 cells, and activity of the ligands was measured by induction of SEAP expression.
Functional Aspects of IFNαR2–2 Dimerization

The results shown here provide additional evidence for IFNαR activation via differently clustered complexes. Since IFNα and IFNβ both bind to the same IFNαR components, but induce different in vivo or in vitro effects, it is conceivable that these ligands use different ways to activate the receptor. Indeed, a similar uncoupling of JAK/Stat pathway activation and TRBP levels show a slight reduction, 48 h after stimulation, no clear difference in Epo and IFNα-induced levels could be observed.

DISCUSSION

The use of chimeric receptors allows to evaluate the specific contribution of each of the two IFNαR receptor components, IFNαR1 and IFNαR2–2 in type I IFN signaling. In particular, use of receptor chimeras with the EpoR extracellular domain and the IFNαR transmembrane and intracellular domains offers the advantage of stimulating the IFN intracellular pathway without activation of the endogenous IFNαR complex, and without interference of other unknown receptor subunits that may associate with the receptor complex upon IFN activation. This latter phenomenon cannot be excluded in transfection experiments that make use of the species-specific activity of IFN, since an endogenous unknown receptor component may still cross-species interact with the ligand. Furthermore, since EpoR extracellular domains are used, we know precisely that the chimeric receptors aggregate by homodimerization.

Transfection of the EpoR/IFNαR chimeric receptor constructs in 2TGH 6-16SEAP clone 5 cells showed that Epo-induced dimerization of the IFNαR2–2 was sufficient for activation of several interferon-inducible genes, such as the 6-16 gene (as measured by reporter protein synthesis), p56, PKR, 2'-5' A synthetase, and MxA (as shown by Western and/or Northern blot analysis). Unexpectedly, with the same stimulus, only a weak antiviral response was noticed when the cells were incubated with EMCV and no protection was observed against VSV, despite the involvement of the above mentioned gene products in the IFN-induced antiviral state. This decreased antiviral potency of the Epo/IFNαR2–2 chimeras is probably not the result of an inefficiently functioning chimeric receptor, since no difference was seen between Epo- or IFN-induced gene transcription up to 24 h after stimulation. Moreover, IL-5-induced formation of a chimeric IL-5R/IFNαR receptor complex that includes the IFNαR1 and IFNαR2–2 intracellular domains proved to be functional in antiviral activity against EMCV as well as VSV. Since the stability of both ligands, Epo and IFNα, is equal in the antiviral assay, our results imply that it is the absence of the IFNαR1 intracellular domain in the activated receptor complex that is responsible for the decreased antiviral activity.

Kotenko et al. (39) showed that IFNγ-induced dimerization of receptor chimeras, consisting of the IFNγR1 extracellular domains and the IFNαR2–2 intracellular domains, resulted in induction of ISGF3 formation and major histocompatibility complex class I antigen induction. However, this receptor configuration was also able to protect the cells against EMCV although no quantitative measurements were performed preventing comparison with our data. A possible explanation for this discrepancy could be the involvement of the wild-type IFNγR2 receptor subunit in their experiments, a subunit that, although it does not recruit Stat molecules, might recruit other signaling molecules, required for full antiviral activity. The above model therefore only serves for analyzing IFN-induced effects that act solely through Stat activation. Since involvement of other pathways in many IFN-induced functions has been described (14), we favored the use of EpoR/IFNαR receptor chimeras.

The results shown here provide additional evidence for IFNαR activation via differently clustered complexes. Since IFNα and IFNβ both bind to the same IFNαR components, but induce different in vivo or in vitro effects, it is conceivable that these ligands use different ways to activate the receptor. Indeed, a similar uncoupling of JAK/Stat pathway activation and...
antiviral activity as seen here with EpoR/IFNaR2–2 homodimerization has been observed before with a deletion of the C-terminal end of the IFNaR2–2 intracellular domain, resulting in formation of receptor complexes that are capable of inducing antiviral activity if clustered by IFNα, but not by IFNβ, while activation of the JAK/Stat pathway is equal for both ligands (28). Although the involvement of a murine receptor component that cross-reacts with one of the two human ligands cannot be excluded in these experiments, these studies suggest a difference in clustering or receptor conformation between IFNα and IFNβ, resulting in a qualitatively different signal. There are two reasons to believe that activation via IFNaR2–2 dimerization is not a merely artificial situation, but rather mimics (partial) IFNaR activation by IFNβ. First, involvement of IFNaR1 in IFN signaling in 11.1 cells is very unlikely, due to the Tyk2 deficiency and the resulting low levels of IFNaR1 in these cells (34, 35). Since we observed a clear Epo activity on 11.1 cells, transfected with the EpoR/IFNaR2–2 chimeras (Fig. 3), it is conceivable that the partial 6-16 promoter activation by IFNβ, seen on these cells (23), is also transmitted via IFNaR2–2 homodimerization. Interestingly, our results also imply that some residual antiviral protection can occur in cells devoid of Tyk2. A second reason refers to studies done with monoclonal antibodies directed against the IFNaR1 chain. Such antibodies were able to block IFNα but not IFNβ activation (26). In addition, Lewerenz et al. (27) showed that anti-IFNaR1 polyclonal antibodies could only partially neutralize IFNβ activity, leading to a loss of antiviral protection against VSV, consistent with our data where EpoR/IFNaR2–2 dimerization by Epo confers only partial protection to EMCV, but not to VSV.

The mechanism by which IFNaR2–2 homodimerization elicits a signal, leading to gene activation, is at present unclear partly because of the conflicting reports on the requirement of IFNaR1 for Stat activation. Initially, Tyr466 on IFNaR1 was reported to be crucial for Stat activation since peptides containing Tyr466 phosphotyrosines could inhibit IFN signaling in permeabilized cells and interact with Stat1 and Stat2 (40, 41). In contrast, later reports indicated the dispensability of the IFNaR1 chain for gene activation. For instance, mutation of all four Tyr residues in the IFNaR1 chain resulted in a functional receptor complex (42) and Nadeau et al. (43) postulated that Stat2 binding always required the membrane proximal tyrosines of the IFNaR2–2 intracellular domain and at least another Stat2 binding site (Tyr466 or Tyr481 on IFNaR1 or the C-terminal end of IFNaR2–2). Moreover, although they postulated that Stat1 and Stat2 molecules need to be translocated to the IFNaR1 chain, Li and co-workers (41) showed that the recruitment of both Stats initially happens on the IFNaR2–2 subunit in a ligand-independent manner. Finally, dimerization of the IFNaR2–2 chain was performed by Kotenko et al. (39), using IFNγR1/IFNaR2–2 chimeras, and was shown to be fully functional in terms of Stat activation. Hence, activation and heterodimerization of the Stat proteins in the EpoR/IFNaR2–2 homodimer may occur via recruitment to one IFNaR2–2 subunit, followed by binding on a phosphorylated tyrosine residue in the other, juxtaposed IFNaR2–2 intracellular domain. Alternatively, no such juxtaposed domain is needed, as has been shown for Stat1 homodimer formation (44). Since Stat1 activation by the type 1 interferons is supposed to be Stat2-dependent, probably by interaction with IFNaR1 via Stat2 (41, 45, 46), recruitment and activation of Stat1 by the EpoR/IFNaR receptor chimera may follow the same route as Stat2.

Which additional pathways may be activated through recruitment of IFNaR1 in the activated receptor complex? A first possibility is the membrane distal interferon receptor tyrosine activation motif (IRTAM) motif that is perfectly conserved between human, mouse and bovine IFNaR1 and from which the tyrosine residues are the only completely conserved tyrosine residues in the IFNaR1 chains (47). It has been reported that this motif may serve as a docking site for Stat3, which on its turn could act as an adapter for the IFN-dependent interaction of IFNaR1 with the regulatory subunit (p85) of phosphatidylinositol 3′-kinase (48, 49). Apart from the serine phosphorylation of Stat3, phosphatidylinositol 3′-kinase also plays an important role in the regulation of many cellular processes and is an upstream element in a serine kinase phosphorylation cascade (50, 51). It may therefore be involved in the cytosolic activation of a set of proteins, involved in antiviral action. This may explain why a cell line, resistant to the antiviral and antiproliferative actions of IFNα, was found to be defective in Stat3 activation and why expression of Stat3 complemented these effects (52). On the other hand, removal of the IRTAM sequence blocked IFN-dependent IFNaR1 down-regulation, resulting in a prolonged antiviral effect (53). However, as it has been demonstrated that phosphorylation of tyrosine residues in the IFNaR1 intracellular domain is not required for Stat3 activation (43), other Stat3 docking sites, apart from the IRTAM sequence and not necessarily containing tyrosine residues, may exist on the IFNaR1 intracellular domain. In addition, phosphatidylinositol 3′-kinase activation may also be regulated by insulin receptor substrate-1 (IRS-1) for which activation via Tyk2 and JAK1 has been described (54). Although association of MAP kinase (ERK2) with the IFNaR1 subunit has been reported (55), caution must be taken when linking this pathway to antiviral activity since inhibition of mitogen-activated protein/extracellular signal-regulated kinase kinase and mitogen-activated protein kinase activation had no effect on the antiviral activity of IFNα (56). Another uncoupling from the antiviral pathway was seen in Lck−, CD45–, and ZAP 70− Jurkat cells where no growth inhibition could be exerted by IFNα while the induced resistance to measles virus was unaffected. These components were associated with IFNaR1, as judged by co-immunoprecipitation experiments and are essential for signaling via the T-cell receptor (57). Requirement of T-cell receptor signaling components was also reported by other groups, who showed that Stat5, which is constitutively associated with Tyk2, provides upon phosphorylation a docking site for the SH2 domain of CrkL, a protein that is located upstream of the T-cell receptor induced growth-inhibitory C3G/Rap-1 cascade. The Stat5-CrkL complex then migrates to the nucleus to regulate gene transcription via the IFNγ-activated site elements (58, 59). Taken together, and since IFN receptors are found on every cell type, cell-specific pathways, involved in the induction of the antiviral state, must exist. Therefore, with the above mentioned data in mind, we suggest that IFNaR2–2 may serve mainly to activate the JAK/Stat pathway, resulting in the activation of a large set of IFN-inducible genes. In addition, engagement of the IFNaR1 chain in the activated receptor complex may mainly serve to provide additional cell-specific signals required for full antiviral protection.

The observed prolonged high mRNA level of the IFN effector genes and of the PKR protein, when IFNaR1 is involved in the activated receptor complex, is very intriguing and may help explain the difference in antiviral protection, induced by Epo and IFNα. Although the effects of IFNα and Epo on the expression of the PKR inhibitor TRBP were very similar, the differences in the PKR/TRBP ratio may vary and, according to Benkirane et al. (60), may correlate with the observed differences in antiviral activity. Effects both at the transcriptional and at the post-transcriptional level may explain the above-mentioned
findings. In fact, Zhou and co-workers (61) reported that PKR, Rnasel, and MxA do not exclusively carry out antiviral activity against EMCV and VSV. The authors showed a clear residual antiviral activity against both viruses in mice that were triple-deficient in these three effector genes and concluded that additional antiviral pathways must exist (61). Possibly, the involvement of IFNaR1 is required to activate these mechanisms. Analyses are under way to evaluate these alternative possibilities.

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