Profilig of Early Gene Expression Induced by Erythropoietin Receptor Structural Variants*

Tom Büchse, Holger Prietzsch, Tilo Sasse, Sandra Körbel, Gunnar Stigge, Simon Bogdanow, Josef Brock, and Thomas Bittorf

From the Institute of Medical Biochemistry and Molecular Biology, Medical Faculty, University of Rostock, 18057 Rostock, Germany

The development of erythroid progenitor cells is triggered via the expression of the erythropoietin receptor (EPOR) and its activation by erythropoietin. The function of the resulting receptor complex depends critically on the presence of activated JAK2, and the complex contains a large number of signaling molecules recruited to eight phosphorylated tyrosine residues. Studies using mutant receptor forms have demonstrated that truncated receptors lacking all tyrosines are able to support red blood cell development with low efficiency, whereas add-back mutants containing either Tyr343 or Tyr479 reconstitute EPOR signaling and erythropoiesis in vivo. To study the contribution of tyrosines to receptor function, we analyzed the activation of essential signaling pathways and early gene induction promoted by different receptor structural variants using human epidermal growth factor receptor/murine EPOR hybrids. In our experiments, receptors lacking all tyrosine residues or the JAK2-binding site did not induce mitogenic and anti-apoptotic signaling, whereas add-back mutants containing single tyrosine residues (Tyr343 and Tyr479) supported the activation of these functions efficiently. Profiling of early gene expression using cDNA array hybridization revealed that (i) the high redundancy in the activation of signaling pathways is continued at the level of transcription; (ii) the expression of many genes targeted by the wild-type receptor is not supported by add-back mutants; and (iii) a small set of genes are exclusively induced by add-back receptors. We report the identification of several early genes that have not been implicated in the EPOR-dependent response so far.

Erythropoietin (EPO),2 the prime growth factor regulating the erythroid lineage, induces the proliferation of immature red blood cells and their differentiation into mature erythrocytes (1). The corresponding EPO receptor (EPOR), a member of the type 1 cytokine receptor family, is expressed on the surface of erythroid burst- and colony-forming units (2). In contrast to other receptor types, these receptors lack any intrinsic activity and are activated by the induction of a special conformation of pre-existing homodimers after ligand binding (3). The recruitment and activation of the cytosolic tyrosine kinase JAK2 have been shown to be the first and crucial steps in the formation of a multiprotein receptor complex that is responsible for the initiation of diverse signaling pathways (4). Activated JAK2 mediates the phosphorylation of tyrosine residues within the intracellular receptor domain, providing docking sites for Src homology 2 (SH2) domain-encoding effector molecules, including the JAK2 substrate STAT5, a transcription factor that is rapidly translocated to the nucleus (5). Among additional proteins that have been shown to be physically associated with the EPOR following ligand binding are phospholipase Cγ1 (6) and phosphatidylinositol 3-kinase (7). Furthermore, negative regulators are also engaged and terminate receptor-dependent signaling by affecting JAK2, such as the tyrosine phosphatase SHP1 (8) and the suppressor of cytokine signaling SOCS3 (9), or compete for receptor-binding sites, as shown for CIS (10). The signaling events initiated by the receptor complex result in post-translational, transcriptional, and translational changes and mediate different responses of erythroid cells, induction of mitogenesis, and differentiation but also anti-apoptotic pathways. Some of the signals and biological responses reported have been related to distinct receptor substructures by studies using mutant receptors expressed in cell lines. For example, STAT5 activation has been assigned to phosphotyrosines 343 and 401, whereas the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) was shown to interact with phosphotyrosine 479 (11). Although these and other interactions have been confirmed in different cell lines, other studies revealed the ability of receptor forms that are heavily truncated or that lack all tyrosine residues to support the maturation of erythroid cells (12–14). Despite the existence of extensive data on the signaling abilities of EPOR structural variants, it is still not clear how these molecules transform their potential into a cellular response. The first step in the initiation of a certain cellular program is the induction of a specific set of immediate-early genes. Many of these genes that are rapidly transcribed after cytokine challenge encode transcription factors, which in turn regulate additional genes involved in the cellular response.

The aim of this study was to compare the ability of different receptor structural variants to transmit the induction of early gene transcription. We employed a set of hybrid receptors consisting of the ligand-binding domain of the human epidermal growth factor receptor (EGFR) (EGFR) and the transmembrane and intracellular domains of the murine EPOR to bypass endogenous EPO responsivity of the Ba/F3 cells studied. EGFR-stimulated hybrid receptors were used to analyze the role of distinct receptor substructures in transcriptional regulation by cDNA array hybridization.

To define the relative importance of positively acting tyrosine residues in receptor function, we studied receptors lacking all tyrosine residues or the JAK2-binding site as well as receptors containing single tyrosine residues (Tyr343 and Tyr479) that are believed to reconstitute the entire functionality of the receptor. By analysis of gene expression induced following activation of the EGFR/EPOR hybrids, we identified

1 The abbreviations used are: EPO, erythropoietin; EPOR, erythropoietin receptor; JAK2, Janus kinase 2; SH2, Src homology 2; STATS, signal transducer and activator of transcription; PI3K, phosphatidylinositol 3-kinase; EGFR, epidermal growth factor receptor; FCS, fetal calf serum; IL-3, interleukin-3; dapsyl, 5-dimethylaminophenylhexyl-1-sulfonyl; MAPK, mitogen-activated protein kinase; WT, wild-type; MEK3, mitogen-activated protein kinase/extracellular signal-regulated kinase; MEK1, mitogen-activated protein kinase/extracellular signal-regulated kinase; TTP, tristetraprolin.

Received for publication, August 2, 2005, and in revised form, December 23, 2005. Published, JBC Papers in Press, December 27, 2005.

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several new early genes that have not been implicated in the context of an EPOR-dependent response so far.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Cytokine Stimulation, Inhibitors, and Expression of EGFR/EPOR Hybrids**—Ba/F3 cells were maintained in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS) and conditioned medium from WEHI-3 cells (15) as a source of murine interleukin-3 (IL-3). Unless stated otherwise, cells were depleted of growth factor before stimulation with 200 ng/ml recombinant human EGF (PeproTech EC Ltd., London, UK) or 100 pm murine IL-3 (R&D Systems, Minneapolis, MN) for the times indicated. When applying inhibitors, cells were preincubated in the presence of drugs for 1 h prior to cytokine challenge (PD98059 (Promega Corp., Mannheim, Germany) or wortmannin (New England Biolabs Inc., Frankfurt am Main, Germany)). The chimeric receptor constructs were generated as described previously (16). Briefly, constructs were established by fusing wild-type and mutant forms of the murine EPOR intracellular and transmembrane domains to the human EGFR extracellular domain, followed by stable transfection in murine Ba/F3 cells. All constructs used in this study were verified by dyeoxy sequencing of the complete coding region. Clones expressing matched levels of EGFR/EPOR were initially selected as described (16), and their surface expression of transfected hybrid receptors was periodically verified by flow cytometric analysis (see Fig. 2).

**Analysis of EGFR/EPOR Expression**—To confirm EGFR/EPOR expression, cells (1 × 10⁶) were incubated with an R-phycocerythrin-labeled antibody directed against the EGFR extracellular domain (clone EGFR.1, BD Biosciences, Heidelberg, Germany) and diluted in phosphate-buffered saline, 0.5% bovine serum albumin, and 2 mM EDTA (pH 7.4) for 45 min on ice. The cells were then washed and analyzed by flow cytometry (FACSCalibur, BD Biosciences). Finally, propidium iodide was added to discriminate between viable and nonviable cells. As negative staining controls, cells were incubated with an R-phycocerythrin-conjugated immunoglobulin subclass isotype control antibody directed against dansyl (clone 27-35, BD Biosciences). Negative (parental Ba/F3) and positive (A431; ATCC CRL-1555) cell controls were included also.

**Proliferation Assay**—Cell viability was measured using metabolic conversion of WST-1 reagent (Roche Diagnostics GmbH, Mannheim) (17) following the manufacturer’s instructions. In short, cells were deprived of IL-3 and immediately restimulated by different concentrations of EGF or IL-3 for 45 h in 96-well microtiter plates (3000 cells/100 μl). For background determination, a control well without cells was treated in parallel for every microtiter plate. Subsequently, 10 μl of WST-1 reagent was added to each well, and metabolic cellular activity was assessed after 2–4 h of incubation by measuring absorbance at 450 nm (with 620 nm as a reference). To compare values obtained from different microtiter plates, data were normalized by inclusion of a set of standard samples on every plate.

**Induction of Apoptosis and Detection of Apoptotic Cells**—Cells were cultured in the presence of 40 ng/ml EGF or 20 pm IL-3 or in medium without supplementation. The optimal time of incubation was determined in preliminary experiments and varied from 9 to 22 h. EGF and IL-3 concentrations were chosen to be in the range of a submaximal cellular response as specified by dose-response experiments. Phosphatidylinerine exposed on the outer side of the plasma membrane as an early apoptotic marker was detected by binding to fluorescein isothiocyanate-labeled annexin V and measured by flow cytometry according to the manufacturer’s instructions using an annexin V-fluorescein isothiocyanate apoptosis detection kit (Merck Biosciences, Bad Soden, Germany). Apoptotic cells were distinguished from necrotic cells by dye exclusion (propidium iodide). For data analysis, the ratio of the percentage of apoptotic (fluorescein isothiocyanate-positive, propidium iodide-negative) cells incubated in the presence of EGF or IL-3, respectively, to the percentage of apoptotic cells in the unstimulated control was calculated.

**Immunoblotting**—Cells were deprived by boiling 5 × 10⁶ cells from each sample in 100 μl of SDS sample buffer (10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.05% (w/v) bromphenol blue). Total protein was separated on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences, Rodgau-Jügesheim, Germany) as described previously (18). Membranes were blocked with 5% bovine serum albumin and incubated with primary antibodies. Excess antibodies were washed out three times with phosphate-buffered saline containing 0.1% (v/v) Tween 20. After a final incubation with a horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG antibody (Amersham Biosciences, Freiburg) and three more washing steps, blots were developed using the ECL Plus chemiluminescent detection system (Amersham Biosciences). Antibodies directed against phospho-Thr²⁰²/Tyr²⁰⁴ p44/p42 MAPK, phospho-Erk²¹⁷/²²¹ (Santa Cruz Biotechnology, Inc., Heidelberg). Membranes were stripped by agitation in 62.5 mM Tris, 2% (w/v) SDS, and 100 mM β-mercaptoethanol (pH 6.8) for 30 min at 50 °C before reprobing.

**Northern Blotting**—Cells were deprived of FCS and growth factors for 6 h and restimulated with EGF or IL-3 or left untreated for the times indicated. Total RNA was purified with TRIzol reagent (Invitrogen), and 10 μg of each RNA sample was loaded onto a 1.2% agarose gel containing 1.2 M formaldehyde. After transfer onto nylon membrane (Hybond-N, Amersham Biosciences), samples were hybridized with [α-³²P]dCTP-labeled DNA probes specific for the mRNAs of the genes investigated. Hybridization was performed with QuikHyb solution (Stratagene, La Jolla, CA) following the manufacturer’s instructions. Data were obtained by exposure to x-ray films or using a Fuji BAS-1800 II bio-imaging analyzer (Raytest, Straubenhardt, Germany) and quantified by densitometry. Membranes were stripped by agitation in 0.1× SSC and 0.1% (w/v) SDS for 10 min at 95 °C. Successful stripping was monitored by autoradiography.

**Real-time Quantitative Reverse Transcription-PCR**—Total RNA was isolated as described above and reverse-transcribed using oligo(dT) priming and Moloney murine leukemia virus reverse transcriptase (Promega Corp.). Analysis of target cDNA levels was performed by real-time PCR using the TaqMan™ Universal PCR Master Mix and an ABI Prism 7000 sequence detection system (Applied Biosystems, Darmstadt, Germany). For the detection of zfp36 cDNA, we used the Assays-on-Demand™ mouse gene-specific fluorescently labeled TaqMan™ MGB™ probe (assay Mm00457144_m1). The lypoxanthine-guanine phosphoribosyltransferase housekeeping gene hprt was quantified using the TaqMan™ MGB™ probe (assay Mm00469684_m1) as a reference. Both probes were purchased from Applied Biosystems and designed to exclude the detection of genomic DNA.

PCR conditions were set up as follows: 95 °C for 10 min and 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. All experiments were performed at least in triplicate. Expression of zfp36 mRNAs was normalized to hprt according to the following equation: ΔCt = C(zfp36) - C(hprt). ΔΔCt values were calculated by subtracting ΔCt of the samples from ΔCt of the
corresponding controls and used to express the factor of zfp36 induction depicted (2ΔΔCt).

**mRNA Expression Analysis Using cDNA Arrays: Isolation of mRNA and Preparation of Radiolabeled cDNA Probes**—RNA was purified using the Atlas pure total RNA labeling system (BD Biosciences). Briefly, a minimum of 4 × 10⁶ cells were collected by centrifugation and lysed. Total RNA was separated from cell debris by several phenol and chloroform washing steps, followed by precipitation with isopropanol alcohol and ethanol. Prior to quality and purity checks of RNA, genomic DNA was digested with DNase I. RNA recovery was performed after treatment with phenol and chloroform by precipitation using sodium acetate and ethanol. Poly(A)+ RNA was isolated using streptavidin-coated magnetic beads after incubation of up to 50 μg of total RNA with biotinylated oligo(dT). Radiolabeled cDNA probes were synthesized and isolated as described in the Atlas cDNA expression array user manual (BD Biosciences). In short, poly(A)+ RNA (see above) was subjected to reverse transcription in the presence of [α-32P]dATP (3000 Ci/mmol; Amersham Biosciences) and a cDNA synthesis primer mixture (specific to immobilized cDNA probes). The labeled cDNA probes were separated from unincorporated [α-32P]dATP by column chromatography following the manufacturer’s instructions.

**Array Hybridization and Data Analysis**—Atlas 1.2 mouse membranes were treated as recommended by the manufacturer. Briefly, radioactive cDNA samples were heated at 100 °C for 2 min, quickly chilled on ice, and subsequently added to the prehybridization mixture. Hybridization was carried out overnight at 68 °C, followed by several washing steps. Sealed arrays were exposed to a bio-imaging screen. Hybridization intensities of spotted genes were detected using the Fuji Bas-1800 II bio-imaging analyzer and AIDA software (Raytest). Data normalization and determination of local background thresholds and expression changes were performed by specially developed algorithms described in detail under “Results.”

**RESULTS**

Structural Variants of an EGFR/EPOR Chimera Differ in Their Ability to Trigger Mitogenesis and Survival of Ba/F3 Cells—To study the role of distinct receptor structures in the induction of EPO-dependent intracellular signaling pathways, we engineered the pro-B cell line Ba/F3 to express hybrid receptors consisting of the extracellular domain of the human EGFR fused to the transmembrane and intracellular domains of the murine EPOR. We have shown previously that these hybrid receptors induce EPOR-specific signaling after EGF challenge and provide a model to investigate the function of stably expressed mutant receptors bypassing endogenous wild-type receptors (16, 19).

In this study, we attempted to analyze a set of mutant receptors for modifications in their ability to induce early gene expression compared with wild-type (WT) EGFR/EPOR (Fig. 1). The YF EGFR/EPOR mutant lacks all eight tyrosine residues (converted to phenylalanine) known to serve as SH2 domain-docking sites and has been designed to study the role of receptor phosphorylation. A second mutant designated W282R EGFR/EPOR contains a single amino acid substitution (tryptophan to arginine) in a region proximal to the membrane, causing an inability to associate with the cytosolic tyrosine kinase JAK2, which has been shown to be an essential part for most but not all of the known receptor functions (20). Early gene induction triggered by this mutant receptor...
should therefore reflect the activity of JAK2-independent signaling events. Two additional receptors (Y343 and Y479 EGFR/EPOR) were designed as add-back mutants to study the role of single tyrosine residues within the EPOR intracellular domain. Single cell clones were established and analyzed for their surface expression of transfected hybrid receptors by flow cytometric analysis. We employed an antibody directed against the EGFR extracellular domain (clone EGFR.1). The results of a representative experiment including all cell clones used in this study are depicted in Fig. 2. Compared with control cells (parental Ba/F3) and isotype controls, transfected cells showed a substantial and comparable expression of receptor isotypes.

Cell clones stably expressing the receptor types described were initially characterized for their EGF-dependent mitogenic potential and their ability to prevent apoptotic death. To prove the responsivity of the cell clones derived by stable transfection, we always included analysis of IL-3-dependent responses. All cell clones used were established from parental IL-3-dependent Ba/F3 cells and showed a comparable proliferative response as depicted in Fig. 3. Although we observed a dose-dependent mitogenic response in each case, slight differences may be due to clonal variation. In contrast, the EGF-dependent proliferation showed dramatic differences. The receptor forms lacking tyrosine residues (YF EGFR/EPOR) and the JAK2-binding site (W282R EGFR/EPOR) failed to induce mitogenesis completely. Compared with the WT protein, add-back mutant receptors (Y343 and Y479 EGFR/EPOR) induced a significant but diminished proliferative capacity.

The ability to transmit anti-apoptotic activities is shared by many cytokine receptors, including the EPOR. Ba/F3 cells rapidly undergo apoptosis after withdrawal of growth factors. IL-3 was a potent inhibitor of apoptosis for all Ba/F3 clones used in this study (Fig. 4). As shown by flow cytometric analysis, the WT EGFR/EPOR hybrid was also able to suppress the induction of apoptosis effectively. As already shown for the mitogenic activity, the existence of an intact JAK2-binding site and tyrosine residues in the intracellular domain is essential for anti-apoptotic
It is important to note that the receptor containing Tyr479 confers the highest potential to suppress apoptosis. Taken together, the existence of Tyr343 or Tyr479 restored an activity comparable with the WT receptor, whereas the Y343 EGFR/EPOR mutant lost a substantial part of its potency. EPOR mutant lost a substantial part of its potency.

Subsequently, we analyzed the signaling capacity of the receptors by investigating pathways known to be critically involved in the induction of EPOR-dependent cellular activities. Again, we included data on the IL-3-dependent response to make sure that the cellular signaling capacity of all cell clones was functionally intact. As shown in Fig. 5A by the phosphorylation of p42 and p44 MAPKs as well as the phosphorylation-induced mobility shift of Raf-1, the Ras/Raf/MAPK signaling pathway was targeted by both the IL-3 receptor and the EGFR/EPOR hybrid. The ability of mutant receptors to trigger mitogenesis was closely correlated with their potency to activate the MAPK pathway. In contrast to the YF and W282R EGFR/EPOR mutants, which obviously lack essential structural changes to couple to the cascade, the Y479 EGFR/EPOR mutant induced an activity comparable with the WT receptor, whereas the Y343 EGFR/EPOR mutant lost a substantial part of its potency.

### TABLE 1

Early gene expression mediated by EGFR/EPOR hybrids

Ba/F3 cells stably expressing the wild-type EGFR/EPOR hybrid and treated with EGF for different times were subjected to cDNA array analysis as described under “Experimental Procedures.” Based on the data of the entire time course, genes were sorted for their transcriptional changes (peak values) regardless if induced or suppressed.

| Peak | Label | Gene name | GenBank accession no. |
|------|-------|-----------|----------------------|
| 28.8 | E06k  | socs1     | U88325*              |
| 17.5 | C04i  | junB      | J03236*              |
| 12.4 | A11f  | Pancreatic and duodenal homeobox gene 1 | X74342 |
| 11.3 | E08e  | frizzled homolog 3 (fzd3, fzd3) | U33205 |
| 11.3 | E08i  | Cornichon-like | A800619 |
| 10.3 | F06i  | Neurofilament, light polypeptide | X02165 |
| 10.1 | A08n  | T-box 4 | U75329 |
| 9.4  | B01d  | SA2 nuclear protein | A8009453 |
| 9.1  | E01d  | Oncostatin M | A0002666 |
| 8.8  | E04i  | Interferon-β, fibroblast | D31942* |
| 8.5  | E03i  | Inhibit β-subunit C | K00020 |
| 8.4  | E09i  | frizzled homolog 7 | U95962 |
| 8.3  | C14i  | Fas antigen | M83649 |
| 8.0  | D09a  | 5-Hydroxytryptamine (serotonin) receptor 3A | X72395 |
| 7.9  | D01m  | Neuronal death protein DP5 (hrk) | D83698 |
| 7.8  | C07i  | Provil integration site 1 (pim-1) | M13945* |
| 7.6  | A12g  | Forkhead box L1 | X92498 |
| 7.1  | D13i  | Inhibit β-subunit A | X69619 |
| 7.0  | F07i  | Thrombospondin-2 | L07803 |
| 6.9  | C05g  | rak23, ras oncogene family member | Z23821 |
| 6.9  | A05a  | Mesoderm posterior 1 | D83674 |
| 0.1  | B12F  | Prothymosin-α | X56135 |
| 6.8  | A03a  | Tristetraproline (zfp36) | M57422 |
| 6.7  | E04l  | IL-11 | U03421 |
| 6.7  | F13n  | B cell translocation gene 2 (btg2/tis21) | M64292 |
| 0.2  | B09d  | Vimentin | X51438 |
| 6.4  | F14i  | Fragile X mental retardation 2 homolog | A001549 |
| 0.2  | B07i  | Y-box protein 1 | A08514 |
| 6.2  | A12d  | Forkhead box D4 | X83638 |
| 6.2  | F13i  | klotho | A8005141 |
| 6.0  | A07i  | Distal-less homeobox 3 | S81932 |
| 6.0  | E12k  | mekk3 | U43187 |
| 0.2  | E08c  | Chemokine (CXXC) receptor 4 | D87747 |
| 5.9  | C05c  | c-myc (myelocytomatosis oncogene) | X1023* |
| 0.2  | B08d  | Embryonic ectoderm development | U78103 |
| 0.2  | A05g  | NK6 transcription factor-related, locus 2 | L08074 |
| 0.2  | B11n  | p27kip1 | U10440 |
| 5.7  | D08k  | Glutamate receptor-β1 | D10171 |
| 0.2  | B13m  | Catenin-α1 | X59990 |
| 5.5  | C11e  | Norepinephrine transporter | U76306 |
| 0.2  | B10f  | Cyclin B1 | X66032 |
| 0.2  | F01i  | p58 inhibitor of PKR | U28423 |
| 5.4  | D09j  | Cholinergic receptor-α, polypeptide 7 | L37663 |
| 5.3  | C04d  | c-jun | J04115* |
| 5.3  | E10d  | Receptor-associated protein of the synapse | J04115* |
| 5.2  | A05b  | atonal homolog 3 | D85845 |
| 5.2  | D07l  | Vasoactive intestinal peptide receptor 2 | D28132 |
| 5.2  | C01h  | pecam-1 | L06039 |
| 5.1  | C08e  | p53 tumor suppressor | K01700 |
| 5.1  | F08f  | Laminin-γ1 | X05211 |
| 0.2  | C05j  | mpl | Z22649 |
| 0.2  | B11j  | Weel-like protein kinase | D30743 |
| 4.9  | C02n  | Transformation termination factor 1 (tff1) | X633794 |
| 4.9  | D05h  | Transforming growth factor-β receptor I | D25540 |
| 4.9  | F12b  | rad21 homolog | D49429 |
| 0.2  | C14b  | gadH | U44088 |
| 4.7  | A01g  | Gap junction membrane channel β5 | M91236 |
| 0.2  | B09a  | stat1 | U06924* |

* Among the 60 genes shown, these genes were already known to be induced by EPOR-mediated pathways.

### Pathways

Our data show that the existence of Tyr543 or Tyr579 restored these activities to levels slightly lower or higher than the WT receptor, respectively. It is important to note that the receptor containing Tyr579 conferred the highest potential to suppress apoptosis. Taken together, the established system of structural receptor variants reflects essential EPOR functions and allows the dissection of the role of receptor substructures in the induction of signaling pathways and transcriptional regulation.

**Distinct Signaling Pathways Are Induced by EGFR/EPOR Variants**—In the next step, we analyzed the signaling capacity of the receptors by investigating pathways known to be critically involved in the induction of EPOR-dependent cellular activities. Again, we included data on the IL-3-dependent response to make sure that the cellular signaling capacity of all cell clones was functionally intact. As shown in Fig. 5A by the phosphorylation of p42 and p44 MAPKs as well as the phosphorylation-induced mobility shift of Raf-1, the Ras/Raf/MAPK signaling pathway was targeted by both the IL-3 receptor and the EGFR/EPOR hybrid. The ability of mutant receptors to trigger mitogenesis was closely correlated with their potency to activate the MAPK pathway. In contrast to the YF and W282R EGFR/EPOR mutants, which obviously lack essential structural changes to couple to the cascade, the Y479 EGFR/EPOR mutant induced an activity comparable with the WT receptor, whereas the Y343 EGFR/EPOR mutant lost a substantial part of its potency.
EPOR-dependent Early Gene Expression

![Graphs showing time course of EGF-induced gene activation in WT EGFR/EPOR cells.](image)

FIGURE 6. Time course of EGF-induced gene activation in WT EGFR/EPOR cells. Atlas 1.2 mouse cDNA arrays were hybridized using $^{[32P]}$dATP-radioabeled cDNAs prepared from poly(A)$^+$ RNA of cells stimulated by EGF for the times indicated. After background subtraction and normalization of hybridization signals for each spot, the results are expressed as the ratios of data from stimulated cells versus unstimulated controls. The time course of four representative genes with different kinetics ($junB$, prothymosin alpha, pim-1, and frizzled homolog 3) is shown.

The second pathway investigated was the JAK/STAT signaling cascade, which has been shown to be involved in diverse cytokine functions. The EPOR specifically activates JAK2 and the latent transcription factor STAT5. The phosphorylation of STAT5 by receptor-dependent activities was studied using a phosphospecific antibody (anti-phospho-Tyr$^{694}$) against STAT5. The results, as well as those observed after reprobing with an antibody directed against STAT5, are shown in Fig. 5B. Besides the WT receptor, only the add-back mutant receptors containing a single tyrosine residue were able to activate STAT5, underlining the necessity of docking sites in the intracellular domain as well as the presence of receptor-associated JAK2. The reprobed blot shows both unphosphorylated and phosphorylated STAT5, which underwent a mobility shift (upper band).

The promotion of cell survival has been shown to be supported by the Akt pathway. We analyzed the activity of two components, the phosphorylation of p85 PI3K and Akt (also known as protein kinase B). The corresponding results are shown in Fig. 5C for both phosphospecific antibodies and antibodies directed against protein-specific epitopes (loading control). Interestingly, only the Y479 EGFR/EPOR mutant triggered this pathway at levels comparable with the WT receptor, whereas all other mutant receptors were no longer able to enhance the activity of Akt. The Y343 EGFR/EPOR mutant partially retained the ability to induce PI3K activity; but at the level of Akt, no response was detectable.

In summary, the data demonstrate the dependence of distinct signaling properties on certain receptor substructures, which should give rise to a different capacity to induce gene expression.

Changes in mRNA Expression Caused by Stimulation of EGFR/EPOR Chimeras: Time Course and Methodology—A primary goal of our studies exploring the intracellular signaling triggered by EGF stimulation of Ba/F3 cells stably expressing exogenous EGFR/EPOR hybrids was to investigate changes in mRNA expression of numerous genes simultaneously. Because current genome-wide DNA array systems show a restricted sensitivity, we employed an Atlas 1.2 mouse array (BD Biosciences), which is a nylon membrane-based cDNA array, in combination with $^{[32P]}$dATP-radioabeled probes. Although exhibiting a high sensitivity, this system focuses on 1185 selected genes, most of them involved in basic cellular pathways.

Ba/F3 cells expressing EGFR/EPOR hybrids were depleted of FCS and growth factors for 6 h and subsequently stimulated with 200 ng/ml EGF in the absence of FCS. Total RNA was extracted at different time points up to 8 h. Cellular responsiveness and quality of RNA were verified by Northern blot analysis using a probe specific for murine bcl-x (data not shown). bcl-x was found to be transiently expressed, with the highest level at 2.5 h after EGF addition and a weak or no signal for the controls in the absence of EGF. Similar loading was verified by reprobing the blot with a probe for hprt. Next, poly(A)$^+$ RNA was isolated and used to generate radiolabeled cDNAs, which were hybridized to Atlas 1.2 mouse cDNA arrays. Differences in mRNA expression profiles were determined by image analysis. To improve the accuracy and reproducibility of the method, both the numerical background subtraction and the normalization between different membranes were optimized. A grid of circles was aligned to the signal spots, and every circle was surrounded by a ring-shaped window, allowing a very accurate determination of the local background and leading to a significantly enhanced reproducibility. The correct alignment and quality of each spot were confirmed visually. Based on the raw local spot intensity and on the overall variance of background intensities, an individual noise threshold was calculated for every spot and used to flag a spot as present or absent. Before determining the signal ratios of corresponding spots of two membranes, it was crucial to define the intensity of absent spots as being equal to or less than the individual noise threshold. Thus, over- or underestimation of spots detected on only one of two compared membranes was prevented, and the accuracy of data analysis was further improved. Normalization of the data from individual arrays can be achieved by comparison of the signal intensities obtained from nine different housekeeping genes spotted on the Atlas 1.2 mouse cDNA arrays or from a subset of them. However, expression of at least some of these housekeeping genes might be affected by stimulation with EGF or withdrawal of FCS and IL-3. Therefore, a different approach for normalization was employed based on the assumption that the expression of the majority of the investigated genes is not specifically affected by the engagement of the EGFR/EPOR chimera by EGF. Both methods of normalization were systematically compared and showed a high correlation (correlation coefficient of 0.96), validating both ways for this special experimental setup.

Of 1185 spotted cDNAs, 681 showed a reproducible hybridization signal (marked as present in at least 75% of the hybridizations). Comparing the signal ratios of stimulated versus unstimulated samples over the full time course, a total of 121 of the detected transcripts showed a change in expression of at least 4-fold, 14 even 8-fold or more. The data of 60 regulated candidate genes showing the highest variation were confirmed by systematic visual assessment of the arrays for every time point and are shown in Table 1. Among these 60 genes, 46 mRNAs were up-regulated following EGF treatment, and 14 were down-regulated under the same conditions. In contrast, considering all differentially expressed genes (threshold of 2-fold or more), the number of up- and down-regulated genes is nearly balanced. The genes whose mRNA expression was altered by EGF fall into several categories, including genes encoding transcription factors, regulators of proliferation and apoptosis, proteins involved in DNA repair, and cytoskeleton and extracellular matrix proteins. Interestingly, the strongest qualitative and quantitative changes in mRNA expression were obtained as early as 30 min following the addition of EGF (239 of all spotted cDNAs and 29 of the 60 most regulated genes, respectively). At this early time point, transcriptional changes are caused directly by receptor-dependent signaling events. It is apparent that a significant number of strongly regulated genes would have not been detected if samples were taken at only one time point of stimulation. The time course of the 60 most affected genes was analyzed in detail, and representative examples are displayed in Fig. 6. The majority of them exhibited a transient fast induction (e.g.
**EPOR-dependent Early Gene Expression**

**TABLE 2**

Changes in mRNA expression induced via structurally different EGFRs/EPORs

| GenBank™ accession no. | WT | W282R | YF | Y479 | Y343 |
|------------------------|----|-------|----|------|------|
| U88325                 | 28.8 | Invalid | >9.4 | >48.0 |
| M13945                 | 2.8 | Invalid | >6.4 | 18.6 |
| J03156                 | 17.5 | >10.4 | >2.8 |
| M21065                 | 0.8 | >15.5 | 4.2 |
| X74342                 | 12.4 | Invalid | |
| X02165                 | 10.3 |      |      |
| U57329                 | 10.1 |      |      |
| X56135                 | 0.1 | 2.0 | 1.7 | 0.4 | 0.9 |
| D31942                 | >9.1 | >3.0 | >3.1 |
| AJ1002636              | 8.8 |      |      |
| X01023                 | 5.9 | 1.3 | 0.5 | >5.7 | 8.6 |
| M83649                 | 8.3 |      |      |
| D83698                 | 7.9 |      |      |
| X92498                 | 7.6 |      |      |
| X69619                 | 7.2 |      |      |
| M331042                | 1.8 | >6.9 | 3.7 |
| M57422                 | 6.8 | >5.1 | >2.0 |
| L07803                 | 6.5 |      |      |
| A001549                | 6.4 |      |      |
| U88328                 | >2.4 | >6.3 | >5.0 |
| X86368                 | 6.2 |      |      |
| A005141                | 6.2 |      |      |
| Z22821                 | 6.2 | Invalid |      |
| S81932                 | 6.0 |      |      |
| U40888                 | 2.5 | 1.5 | 1.2 | >4.9 | 6.0 |
| L08074                 | >5.8 |      |      |
| K00020                 | 5.7 | Invalid |      |
| D10171                 | 5.7 |      |      |
| U03421                 | 5.6 |      |      |
| U76306                 | 5.5 |      |      |
| L37663                 | >5.4 |      |      |
| J04115                 | 5.3 |      |      |
| X15788                 | 5.1 | >1.4 |      | 0.8 |
| D25540                 | 4.9 |      | 1.0 |
| D87747                 | 0.3 | 0.7 | 1.2 | <0.2 | 0.3 |
| L00039                 | 0.2 |      | 0.6 | 1.2 |
| X51438                 | 0.2 | >1.2 | 1.0 | 0.8 |
| X57621                 | 0.2 | 1.5 | 1.1 | 0.6 | 0.8 |
| X83974                 | 4.9 | 1.2 | 0.6 | 1.1 | 0.9 |
| X05211                 | 4.9 |      |      |
| U95962                 | 4.8 |      |      |
| M91236                 | 4.7 |      |      |
| Z21524                 | 2.1 | 2.8 | 1.8 |      |
| U43187                 | 4.5 | >1.3 |      |
| X06746                 | 1.6 | >1.7 | >3.4 |
| D49429                 | 3.3 | 1.7 | 0.8 | 1.1 | 0.8 |
| X64713                 | 0.3 |      | 0.7 |
| X52364                 | 0.3 | 0.7 | 1.1 | >1.7 | 1.1 |
| M05653                 | 0.3 | 0.9 |      |      | 1.0 |

**Gene name**

- soc1
- Proviral integration site 1 (*pim-1*)
- junB
- Interferon regulatory factor 1 (*irf1*)
- Oncostatin M
- Neurofilament, light polypeptide
- Y-box 4
- Prothymosin-α
- SA2 nuclear protein
- c-myc (myelocytomatosis oncogene)
- Neuronal death protein DSP (brk)
- Forkhead box L1
- Inhibin β-subunit A
- Immediate-early response 2 (*ier2*)
- Tristetraprolin (*zfp36*)
- Thrombospondin-2
- Fragile X mental retardation 2 homolog
- soc3
- Forkhead box D4
- klotho
- ras oncogene family member
- Distal-less homebox 3
- T cell death-associated gene (*tdag51*)
- NK6 transcription factor-related, locus 2
- Interferon-β, fibroblast
- Glutamate receptor-β1
- IL-11
- Norepinephrine transporter
- Cholinergic receptor-α, polypeptide 7
- Receptor-associated protein of the synapse
- Transforming growth factor-β receptor 1
- Chemokine (CXC) receptor 4
- pecam-1
- Vimentin
- Y-box protein 1
- Transcription termination factor 1 (*ttfl*)
- Laminin-γ1
- Inhibin β-subunit C
- Gap junction membrane channel β5
- Hematopoietically expressed homeobox
- mek3
- Early growth response 2
- rad21 homolog
- Cyclin B1, related sequence 1
- Intercellular adhesion molecule 1 (*icam-1*)
- Integrin-β7
EPOR-dependent Early Gene Expression

Analysis of Changes in mRNA Expression Induced by Structurally Different EGFR/EPOR Hybrids—To analyze the role of discrete EPOR structures in the induction of gene expression, we applied the methodology described above to a comparative approach including the YF, W282R, Y343, and Y479 EGFR/EPOR mutants and focused on short-term cytokine stimulation (30 min). We detected >150 significant alterations (difference of 2-fold or more) compared with the WT receptor; 49 of them showed an induction-suppression factor of at least 3 (Table 2). Again, the identified genes mostly encode transcription factors, regulators of mitogenesis and apoptosis, and proteins of the cytoskeleton.

In general, the ability of all mutant receptors to induce transcriptional regulation was significantly reduced compared with the WT receptor. Whereas the YF and W282R EGFR/EPOR mutants seemed to be defective in most pathways, both add-back mutant receptor forms (Y343 and Y479 EGFR/EPOR) retained part of their regulatory potential. Interestingly, the induction of several genes by these receptors was found to be stronger than in cells expressing the WT receptor. Examples of such genes are socs1 for Y343 EGFR/EPOR, ier2 for Y479 EGFR/EPOR, and socs3, pim-1, irf1, and tdag51 for both receptor types.

For many of the regulated genes, both receptors containing a single tyrosine residue showed a similar response compared with the WT receptor, pointing to a redundant function of these tyrosine residues in the transmission of signals to the nucleus. However, the regulation of a few genes seems to be preferably transmitted through distinct phosphorylated tyrosine residues serving as docking sites for intracellular signaling proteins. For example, the induction of socs1 was strongly induced by the receptor containing Tyr343 (factor of >48 versus 28.8 for the WT receptor), whereas the induction by the receptor containing Tyr479 was significantly lower (factor of 9.4). However, the same receptor form showed a strong induction of irf1, ier2, and junB compared with the receptor containing Tyr479.

Our first attempts to verify transcriptional changes shown in cDNA array experiments reproduced the effects observed. As shown in Fig. 7, we were able to validate the results for a number of genes by independent methods (Northern blotting and quantitative reverse transcription-PCR) and to prove the reliability of the gene expression data obtained. The ability of receptor subtypes to direct expression changes in the mRNA levels of the well-established EPO-dependent genes junB, socs1, and socs3 analyzed by Northern hybridization corresponds exactly with the data obtained by array analysis (Fig. 7A).

Zfp36, a New Immediate-early Gene Involved in the EPOR-dependent Cellular Response—Initial investigations toward the characterization of newly identified EPOR targets were focused on zfp36, which turns out to be a typical immediate-early gene. Quantitative reverse transcription-PCR studies (mRNA expression levels were related to IL-3-stimulated controls) revealed a rapid EGF-dependent induction of the gene by the WT receptor and receptors containing tyrosine residues (Fig. 7B). Additional experiments were performed to study the EPOR-dependent zfp36 induction in detail. The time course depicted in Fig. 8A shows a rapid up-regulation of the mRNA level, peaking after 30 min and declining to basal levels 1 h following cytokine challenge. Dose-response analysis (Fig. 8B) revealed that the gene was induced at cytokine concentrations at or below levels sufficient for mitogenic activity (see Fig. 3). The addition of inhibitors specific for PI3K (wortmannin) and MEK1 (PD98059) prior to cytokine stimulation revealed that inhibition of the MAPK signaling pathway substantially reduced the induction of zfp36 (Fig. 9). In contrast, the activity of PI3K seemed to be dispensable. Interestingly, however, the simultaneous addition of both inhibitors resulted in a more pronounced inhibition, indicating an interaction of both pathways toward zfp36 gene induction.

DISCUSSION

The cellular response induced by activated cytokine receptors is based on a network of signal transfer cascades that trigger both transcriptional and translational events. The induction of immediate-early genes starting within minutes after ligand binding is the key step in the activation of the genetic program controlling the biological fate of the cells. Regarding the EPOR, intracellular signaling pathways and essen-
primary receptor structures have been characterized intensively. The primary function of the associated cytosolic tyrosine kinase JAK2 and eight tyrosine residues in the intracellular receptor domain have been highlighted by studies employing mutant receptors and knockout mice (13, 16, 20–22). Although the EPOR complex is composed of a wide variety of signaling molecules that have at least partially been shown to interact with distinct receptor substructures, studies using mutant receptor forms have also established that heavily truncated receptors retain the ability to support erythroid development (12, 23, 24). These minimal receptors essentially contain the membrane-proximal JAK2-binding region and at least one of eight intracellular tyrosine docking sites for the recruitment of signaling molecules. Depending on the cellular system used, it has been observed that even receptors without tyrosine residues retain detectable signaling activities and support erythroid differentiation in mouse models (10, 13, 22). Although numerous studies have been directed toward defining the role of single tyrosine residues in the recognition of essential signaling molecules, their relative importance remains a controversial issue.

Our study was conducted to analyze the transcriptional downstream events following activation of receptor subtypes differing in the critical substructures described above. Apart from the WT EGFR/EPOR hybrid, we employed receptors that were unable to bind JAK2 (W282R EGFR/EPOR) or to recruit SH2 domain-containing proteins via tyrosine residues (YF EGFR/EPOR) as well as receptors containing single tyrosine residues (Y343 and Y479 EGFR/EPOR). An initial comparative analysis of the biological effects triggered by these receptors revealed a complete inability of receptors lacking tyrosine residues or an intact JAK2-binding site to transmit mitogenic or anti-apoptotic activities. However, receptors that were modified to contain one single tyrosine residue and a functional JAK2-binding site activated mitogenesis at a lower level compared with the WT receptor and were nearly as effective in the induction of anti-apoptotic signaling. In particular, the receptor containing the most distal tyrosine (residue 479) displayed a high potential to suppress apoptotic death. The data on the regulation of distinct EPOR-dependent signaling pathways generally match the biological effects observed. The tyrosine docking sites at positions 343 and 479 were originally thought to be exclusively responsible for STAT5 and p85 PI3K binding, respectively (7, 25, 26). In contrast to this concept, our results clearly show that these residues redundantly couple to both proteins as well as the Ras/Raf/MAPK signaling cascade. Although able to recruit PI3K, however, the Y343 EGFR/EPOR mutant was not able to activate Akt as a downstream substrate of the PI3K pathway. The PI3K/Akt pathway, which is essential for the prevention of apoptosis and proliferation, has been shown recently to be activated by different EPOR-dependent mechanisms (27). In accordance with our results, both Tyr479 and Tyr343 mediate the activation of PI3K. It was demonstrated that this enzyme directly associates with Tyr479 and is also triggered through Tyr343 by the recruitment of Gab (growth factor-bound protein-associated binder) proteins to the EPOR. In addition, a heavily truncated receptor devoid of tyrosine residues seems to be able to activate PI3K by a pathway including phosphorylated insulin receptor sub- strate 2 in Ba/F3 cells (27). Although we also employed Ba/F3 cells, we did not find PI3K/Akt activation and anti-apoptotic signaling using a receptor without tyrosine residues. In contrast to the study discussed above, this receptor form was created by the replacement of tyrosine residues with phenylalanine and not by truncation. Additionally, we used a EGFR/EPOR hybrid because of the low but significant endogenous EPOR expression in Ba/F3 cells studied in our laboratory.

The ability of receptors lacking tyrosine residues to activate STAT5 is also a controversial issue. Although some studies have revealed an essential role for the recruitment of STAT5 to Tyr343 for growth, Bcl-x expression, and cell survival (23, 28, 29), Tyr343 seems to be dispensable in other model systems (10, 30). However, STAT5 activation through an alternative mechanism conducted by non-tyrosine-containing sequences was shown to be critical for EPO-dependent cell survival in 32D cells (30). However, our findings show that receptors that are negative for STAT5 activation are defective in anti-apoptotic signaling and point to an essential role for STAT5 in the suppression of apoptosis. It is remarkable that Tyr479 alone is able to mediate, at least partially, all functions, some of them to an even higher extent than Tyr343.

Taken together, our attempts to understand the function of structurally different receptor variants reveal a close correlation between their mostly redundant signaling capabilities and their potency to induce cellular activities. Therefore, it is tempting to ask if redundancy observed at the level of the signaling pathways is continued at the level of transcriptional regulation.

Although a number of target genes have been previously linked to EPO signaling, little information is available about the initial molecular events at the transcriptional level. To study transcriptional changes, we employed Atlas 1.2 mouse arrays, which focus on a set of 1185 genes known to be involved in the regulation of basic cellular activities. We first aimed to analyze the time course of gene regulation induced by WT EGFR/EPOR during the first 8 h following EGF challenge. Transcriptional activation of ~120 genes was shown to occur at different time points after receptor activation, but the most prominent transcriptional activities were recorded shortly after ligand binding (30 min). Interestingly, the number of EPOR-dependent genes that are induced or suppressed is nearly balanced, but dramatic changes were found among induced genes preferably. Careful analysis of the expression changes revealed most notably two types of responses, rapid transient or biphasic regulation, respectively. The data clearly underline that kinetic analysis of transcriptional changes is vital to avoid misinterpretation of results. Nevertheless, many studies published to date are restricted to a single time point, a procedure that is not adequate to reflect the course of the complex processes involved.

The set of cDNAs found to be regulated by the WT receptor includes most of the already established EPO-dependent genes, e.g. socs1, junB, c-jun, oncostatin M, and pim-1. The expression changes observed are in accordance with data presented by other groups (31–34). In addition, we were able to identify a substantial number of genes that have not been previously related to EPO-induced gene expression. The gene products that may modulate erythroid cell development fall into differ-
ent functional groups, proteins involved in gene transcription or translation (e.g. ZFP36 and TTF1), regulators of signal transduction (e.g. Rab23 and MEKK3) and cell cycle regulation/mitogenesis/apoptosis (e.g. neuronal death protein DP5 (HRK) and TADG51), and extracellular ligands (e.g. Fas, inhibin-β-subunits A and C, and interferon-β). One of the interesting candidate genes that has not been implicated in the action of EPO encodes the DNA-binding protein tristetraproline (TTP). Our data on the regulation of the corresponding gene clearly show that its activation is an integral part of the EPO-dependent cellular response. TTP was originally identified as ZFP36 in a differential hybridization screen of a cDNA library derived from insulin-stimulated cells and subsequently linked to the action of other mitogens also (35). Recent data show that TTP is targeted by MAPK/p38 kinase-dependent pathways (36, 37), which are already known to be part of the EPO signaling machinery (18, 38). Following activation, TTP is rapidly translocated into the cytosol and exerts its effects on the regulation of target genes by destabilizing mRNAs coding for inflammatory cytokines like tumor necrosis factor-α (39). Among others, TTP destabilizes mRNAs coding for inflammatory cytokines like tumor necrosis factor-α. Notably, EPO was shown to exert inhibitory effects on the burst of inflammatory cytokines in a model of stroke with reperfusion (40). In addition, EPO induces anti-inflammatory effects in models of autoimmune encephalitis and peripheral nerve injuries (41, 42). In light of these findings, our data suggest a possible function of TTP in EPO-dependent cytoprotective signaling.

The strong induction of several genes encoding extracellular ligands that are predestined to act on other cells or, alternatively, by an autocrine mechanism is an interesting new property in the network of EPOR-dependent functions. Inhibin-β-subunits A and C, identified as EPO target genes, form inhibins by dimerization with α-subunits or produce activins by homodimerization. Inhibins and activins, which are members of the transforming growth factor-β superfamily with opposing actions, are known to be involved in the regulation of diverse functions, including erythroid differentiation (43).

Knowing the potency of WT EGFR/EPOR to regulate the expression of a defined set of genes, we extended our study to cells expressing mutant receptors. To obtain insight into the early gene regulation triggered by the pre-existing signaling capacity of the individual receptor complexes, we employed short-term (30 min) stimulated cells. As shown above, this time point is characterized by dramatic changes in gene expression of both a quantitative and qualitative nature. Evaluation of the expression profiles obtained revealed a massive influence of structural receptor modifications on their ability to transmit signals causing gene regulation. It is interesting to note that a substantial number of genes essentially rely on the expression of the complete WT receptor structure to be targeted. This seems to be in contrast to data showing the dispensability of tyrosine residues for the induction of survival signals (22, 30), erythroid colony-forming unit development (13, 22), and c-myc gene expression (10). Experiments in primary erythroid progenitor cells have demonstrated, however, that a JAK2-coupled but phosphotyrosine-null receptor is clearly diminished in its activity in vivo assays, but is assisted in vivo (knock-in mice) by compensatory mechanisms (14). In our experimental system, the W282R and YF EGFR/EPOR mutants, which are not able to induce any signaling and cellular activities, are defective in the induction of most of the genes targeted by the WT receptor. The same applies to genes actively suppressed by the EPOR, e.g. prothrombin-α, which is down-regulated by the WT receptor as well as the Y479 EGFR/EPOR add-back mutant, but is slightly induced in cells expressing the W282R and YF EGFR/EPOR variants. Although the physiological role of this small acidic ubiquitous protein is only partially understood, its function seems to be related to developmental processes, mainly the proliferative response (44). Recent studies have established participation in chromatin remodeling (45) and formation of mitotic spindles (46). In addition, prothrombin-α, which has a strong nuclear localization signal, was shown to target cytokine-activated STAT transcription factors to the nucleus (47). Therefore, down-regulation of prothrombin-α by the WT receptor could be involved in EPO signaling termination.

The gene expression profiles obtained from receptors containing a single tyrosine residue (Tyr343 and Tyr479) clearly demonstrate that the functionality of these molecules can at least partially be restored. Compared with the WT receptor-expressing cells, however, a substantial number of genes are not targeted efficiently. Consequently, these receptors, although activating central EPOR-dependent signaling pathways as shown above and supporting complete erythropoiesis in vivo (48), generate only part of the signals necessary for a complete transcriptional response. The set of genes showing restored expression caused by both add-back mutant receptors are very similar, pointing to redundant mechanisms of induction. The differences observed in the individual potency of these receptors are in almost all cases of a quantitative nature. For example, socs1 and pim1 are predominantly induced in cells expressing the Y434 EGFR/EPOR mutant, whereas junB is preferably regulated via the Y479 EGFR/EPOR mutant. Interestingly, in some cases, the potency of these receptors to induce gene expression exceeds that of the WT receptor. For example, the genes coding for IRF1 and TADG51 are induced exclusively or at a significant higher level by add-back receptors, respectively. irf1 encodes a transcription factor that was originally identified as a nuclear factor involved in the transcription of interferon-β (49) and is currently thought to be a negative regulator of cell proliferation (50). The granulocyte colony-stimulating factor receptor, structurally and functionally closely related to the EPOR, triggers irf1 by a STAT5-dependent pathway (51). The fact that “minimal” receptor forms induce genes that are not responsive through WT receptor signaling points to the existence and importance of negative regulating mechanisms in the EPOR complex and may explain at a transcriptional level the hyper-responsiveness of such cells observed in earlier studies (22, 52).

Taken together, our findings indicate that early gene induction via positively acting tyrosine residues is characterized by a pronounced functional redundancy that reflects the ability of both receptor substructures to support the activation of coincident signaling pathways. The individual Tyr343 and Tyr479 residues trigger the activation of almost identical sets of genes; but, in contrast to the idea of a complete restoration of all receptor functions, they are clearly diminished in their gene regulatory potential compared with the WT receptor. In future experiments, the newly discovered EPOR-dependent immediate-early genes should receive particular attention to further explain the role of receptor substructures and to relate signaling capabilities of the EPOR complex to specific cellular responses.

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