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A versatile platform for activity determination of cytokines and growth factors based on the human TSLP (thymic stromal lymphopoietin) receptor

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ABSTRACT

Cytokines and growth factors are signaling proteins involved in communication processes between cells. They are involved in the control of numerous essential physiological processes such as cell proliferation, gene transcription and differentiation; therefore being in the focus of basic and applied research. Many of them are also of relevance for human diseases. When observed as potential targets for pharmacological intervention and objects of structure/function studies, it is important to measure their biological activities, optionally along with potential inhibitors, in a convenient and rational manner. Such tests are frequently laborious to set up and their establishment is complicated by the necessity to employ problematic cell types and sophisticated assays. Here we present a robust and modular activity assay system which can be adapted to virtually all ligands that signal through dimerization of membrane receptors from different families. The technique rests on fusing ligand-binding domains of specific receptors to the transmembrane and intracellular components of the thymic stromal lymphopoietin (TSLP) receptor which translates signals into readily quantifiable luciferase expression in reporter cells. We show that the activation of various hematopoietic cytokine receptors, of receptor tyrosine kinases as well as of receptors bearing serine/threonine kinase domains by their respective ligands was faithfully reflected both upon transient and stable introduction of hybrid receptor and reporter gene constructs into the murine pro-B cell line Ba/F3. Moreover, we demonstrate the suitability of this platform for the functional characterization of cytokine/growth factor receptor inhibitors.

1. Introduction

Cytokines are small proteins involved in intercellular signaling, regulating widespread functions throughout multicellular organisms. In humans and other vertebrates, they are of central importance for small and long-range communication in development and homeostasis, particularly in hematopoiesis and activities of the immune system [1,2]. Importantly, cytokine function and malfunction is crucial in numerous diseases and pathological situations, e.g. in inflammation, immune disorders and cancer. Since the 1980 s, cytokine structure and function as well as cytokine-mediated effects on cells and tissues have been intensively studied, also with regard to the potential employment of cytokines and cytokine derived biologicals in diagnostics, prognostics, and therapy [3,4].

Cytokines exert their function on target cells by interacting with specific surface receptors which, upon activation, trigger intracellular signal transduction, leading to gene regulation and ultimately to the control of cellular programs such as survival, proliferation or apoptosis.

Abbreviations: CD140α, platelet-derived growth factor receptor-α; CSF-1, colony stimulating factor-1; DCs, dendritic cells; EGF, epidermal growth factor; EPO, erythropoietin; ex, extracellular; FCS, fetal calf serum; h, human; HEK cells, human embryonic kidney cells; IFNα2b, interferon α2b; IL, interleukin; in, intracellular; JAK, Janus kinase; LPS, lipopolysaccharides; m, murine; PLB, passive lyse buffer; PBMCs, peripheral blood mononuclear cells; PDGF, platelet-derived growth factor; PBS, phosphate buffered saline; r, rat; R, receptor; RSTK, serine/threonine kinase receptors; RTKs, receptor tyrosine kinases; s, soluble receptor domain; SCF, stem cell factor; STAT, signal transducer and activator of transcription; TGF-β, transforming growth factor-β; ALK-5, TGF-β type I receptor; T, transmembrane; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin; Zeo, zeocine; γc, common γ receptor chain

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The productive contact between the cytokine and its receptor is, hence, the critical event in cytokine-specific function on the target cell and is of particular interest as a target for potential pharmaceutical interference [5,6].

The categorization of cytokines and their receptors is not consistent throughout the literature and depends for instance on whether a structural/biochemical or functional/medical point of view is being taken. Cytokines in a strict biochemical sense are structurally characterized by a four-helix bundle, their dimeric receptors share a common ligand binding domain containing two fibronectin-like subdomains and signal via the Janus kinase/Signal transducer and activator of transcription (JAK/STAT) pathway. The group of JAK associated cytokine receptors can further be subdivided into hematopoietic (class I) and class II cytokine receptors. Examples for ligands of class I cytokine receptors are interleukin-2 (IL-2), interleukin-4 (IL-4) and erythropoietin (EPO), class II receptors are utilized e.g. by interferons [7]. Notably, some signaling proteins not regarded as cytokines but rather as hormones (e.g. Leptin and Somatotropin) and their respective receptors are structurally and mechanistically analogous to four helix bundle cytokine/cytokine receptor systems [8,9]. Another group of cytokines including e.g. interleukin-1 (IL-1), colony stimulating factor-1 (CSF-1) and stem cell factor (SCF) signal via receptor tyrosine kinases (RTKs) with immunoglobulin-like domains in their ligand binding extracellular segment [10]. RTKs are also employed by a wide range of growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), which are occasionally also subsumed under the cytokine category [11]. A further group of receptors showing an entirely different signal transduction mechanism based on intracellular serine/threonine kinase activity is represented by the receptors for transforming growth factor-β (TGF-β) [12]. An extracellular cysteine-rich domain is typical for the tumor necrosis factor (TNF) receptor superfamily [13]. Relatively recently, the group of interleukin-17 (IL-17) receptors has been defined which consists of five members and shows unique structural features that distinguish it from other cytokine receptor subclasses [14].

Among the multitude of cytokines signaling through the different types of cytokine receptors, many are of considerable interest in basic and applied research. Many are also potential targets for the design of agents capable of blocking or enhancing receptor activation. However, numerous cytokines act on specific target cells difficult to isolate from primary sources. Moreover, permanent cell lines responsive to particular cytokines are not always available or cellular responses may be difficult to quantify.

While there is a great variety in receptor types, cell activation and intracellular signaling mechanisms involved in molecular cytokine action, a common denominator is ligand-induced receptor dimerization. We and others have shown in the past that the fusion of receptor ligand binding domains of cytokine receptors to transmembrane and intracellular domains of other members of the receptor family can result in functional hybrid receptors with exchanged ligand specificities [15,16]. In the last years, we studied activation and function of the receptor for human thymic stromal lymphopoietin (hTSLPR), a type I cytokine receptor and identified its cytoplasmic portion as a convenient platform for the construction of signaling competent hybrids with different ligand specificities.

TSLP is an interleukin-7 (IL-7) like cytokine known to play a key role in the pathogenesis of *Asthma bronchiale* by triggering dendritic cells (DCs) to maturate and prime native $CD4^+$ T cells to differentiate into Th2 cells [17]. TSLPR is a heterodimeric receptor complex consisting of the IL-7 receptor α subunit (IL-7Rα, shared with the IL-7 cytokine receptor system) and a TSLP specific receptor unit – TSLPRα, the latter mediating specific gene regulation [18,19]. TSLP-induced heterodimerization of TSLPRα and IL-7Rα leads to activation of the JAK/STAT pathway and ultimately to transcriptional up-regulation of genes controlled by transcription factors STAT3, STAT1, and STAT5 [20].

In this work, we developed an adaptable bioassay for cytokine/growth factor activity resting on hybrid receptors with the intracellular domains of the hTSLPR as a constant intracellular dimerization and signal transducing unit and STAT1-mediated activation of a luciferase gene stably integrated into the genome of a reporter cell line as a readout. We show that this basal set-up can be conveniently modified to characterize biological activities and specific inhibition of a wide range of protein ligands that signal through receptors from diverse families such as cytokine receptors, RTKs and serine/threonine kinase receptors (RSTK).

2. Materials and methods

2.1. Cells and cell culture

Ba/F3 cells were obtained from the German Collection of Microorganisms and Cell Lines (DSMZ, Braunschweig, Germany) and cultured in RPMI1640 medium (PAN Biotech™, Aidenbach, Germany) supplemented with 10% of heat inactivated fetal calf serum (FCS) (PAN Biotech™, Aidenbach, Germany), 1% Gentamycin (Ratiopharm®, Ulm, Germany) and 10 ng/ml murine interleukin-3 (mIL-3) (Invigate GmbH, Jena, Germany). The human embryonic kidney cell line HEK 293 (DSMZ, Braunschweig, Germany) was used for the production of soluble receptor exodomains. Cells were cultured in DMEM medium (PAN Biotech™, Aidenbach, Germany), 10% of heat inactivated FCS and 1% Gentamycin. All cells were maintained at 37 °C with 5% CO_2.

2.2. DNA constructs for cell transfection

All expression constructs were based on vector pcDNA3.1™(+)Zeo (Invitrogen™, Massachusetts, USA). Standard cloning procedures were performed using DreamTaq™ DNA polymerase, T4 DNA Ligase and FastDigest® restriction enzymes (Thermo Scientific™, Massachusetts, USA) employing *Escherichia coli* (*E. coli*) Top10 strain (Invitrogen™, Massachusetts, USA).

Chimeric receptor expression constructs were based on previously described constructs pcDNA-hTSLPRα and pcDNA-hIL-7Rα [20]. Extracellular receptor domains of representative cytokines and growth factors were cloned into those vectors replacing hTSLPRα and hIL-7Rα extracellular domain by using NotI and XhoI restriction sites. Introduced extracellular domains fragments were obtained by PCR from cDNA synthesized from mRNA of lipopolysaccharides (LPS) treated human and murine peripheral blood mononuclear cells (PBMCs) as a template using standard procedures. Expression constructs contain a hIL-4Rα signal peptide for extracellular expression and a P5D4 epitope tag.

The following primers were used to amplify the extracellular domains (ex) to generate chimeric receptor chains of human IL-13 receptor (hIL-13R), murine macrophage colony-stimulating factor receptor (mM-CSFR), rat IL-4R (rIL-4R), hIL-17A/FR, hIFNα2bR, hPDGFαR, mTSLPR and hEGFR:

Primer name	Primer sequence (5′-3′)
hIL-13R	
hIL-4Rα$_{ex}$ forward	5′-GTCCAGCGGCCGCGGGGTGGCTTTGCTCTGGGCT-3′
hIL-4Rα$_{ex}$ reverse	5′-ATAAACTCGAGGCCCTTCGAGCAGCAC-3′
hIL-13Rα$_{ex}$ forward	5′-GTCCAGCGGCCGCGGGCGCCGCGCCTACGGAAAC-3′
hIL-13Rα$_{ex}$ reverse	5′-ATAAACTCGAGTGTGGAATTGCGCTTCTTACC-3′
mM-CSFR	
mM-CSF-R$_{ex}$ forward	5′-TCCAGCGGCCGCGGCCCCTGTCATCGAGC-3′
mM-CSF-R$_{ex}$ reverse	5′-GTAAGATAGGCTCGAGGGACTCATCGGG-3′
rIL-4R	
rat common gamma receptor chain exodomain (rγc$_{ex}$) forward	5′-GTCCAGCGGCCGCGTCCAAGGTCCTCATGTCCAG-3′
rγc$_{ex}$ reverse	5′-AATAAACTCGAGAGCTTCCAGTGCAAACAAGG-3′
hIL-17A/FR	
hIL-17AR$_{ex}$ forward	5′-GTCCAGCGGCCGCGCTGCGACTCCTGGACCACCG-3′
hIL-17AR$_{ex}$ reverse	5′-ATAAACTCGAGCCACAGGGGCATGTAGTCCG-3′
hIL-17CR$_{ex}$ forward	5′-GTCCAGCGGCCGCGCTGGAGAGGCTTGTGGGGCC-3′
hIL-17CR$_{ex}$ reverse	5′-ATAAACTCGAGGCGCTTGTGGATGTATTTGTCC-3′
hIFNα2bR	
hIFNαR1$_{ex}$ forward	5′-GTCCAGCGGCCGCGAAAAATCTAAAATCTCCTC-3′
hIFNαR1$_{ex}$ reverse	5′-ATAGGCTCGAGTTTAGAGGTATTTCCTGG-3′
hIFNαR2$_{ex}$ forward	5′-GTCCAGCGGCCGCGATTTCATATGATTCGCCTG-3′
hIFNαR2$_{ex}$ reverse	5′-ATAGGCTCGAGTTTGGCAGATTCTGCTGATTC-3′
hPDGFαR	
hCD140α$_{ex}$ forward	5′-GTCCAGCGGCCGCGCAGCTTTCATTACCCTCTATC-3′
hCD140α$_{ex}$ reverse	5′-AATAAACTCGAGTTCAGAACGCAGGGTGGGAG-3′
mTSLPR	
mTSLPRα$_{ex}$ forward	5′-GTCCAGCGGCCGCGGCGGCGGCGGTGACG-3′,
mTSLPRα$_{ex}$ reverse	5′- ATAGGCTCGAGGAGGGGCGGGGCCAGGGCC-3′
mIL-7Rα$_{ex}$ forward	5′-GGTCCAGCGGCCGCGGAAAGTGGAAATGCCCAGG-3′
mIL-7Rα$_{ex}$ reverse	5′-ATAGGCTCGAGATCCCATCCTCCTTGATTC-3′
hEGFR	
hEGFR$_{ex}$ forward	5′-GTCCAGCGGCCGCGCTGGAGGAAAAGAAAG-3′
hEGFR$_{ex}$ reverse	5′-ATAGGCTCGAGGGACGGGATCTTAGGC-3′

Constructs for eukaryotic production of soluble receptor domains were based on the vector pcDNA-hTSLPRα$_{ex}$-6His that has been described previously [21]. The coding sequence for soluble mM-CSF-R and soluble hIL-17AR was obtained by PCR from cDNA synthesized from mRNA of LPS-treated human and murine PBMCs as template using standard procedures. The PCR product was ligated into pcDNA-hTSLPRα$_{ex}$-6His after restriction at the XhoI and NotI restriction sites. Expression constructs contain a hIL-4Rα signal peptide for extracellular expression, a P5D4 epitope tag and six His residues at the carboxyl terminus of the receptor domain for purification purposes. The following primers were used to amplify soluble extracellular receptor domains: hIL-17AR$_{ex}$ forward 5′-GTCCACTCGAGCTGCGACTCCTGGAC CACCG-3′, hIL-17AR$_{ex}$ reverse 5′-CAGATGCGGCCGCCCACAGGGGCAT GTAGTCCG-3′; mM-CSF-R$_{ex}$ forward 5′-GTCCACTCGAGGCCCCTGTCA TCGA-3′, mM-CSF-R$_{ex}$ reverse 5′-CAGATGCGGCCGCGGACTCATCGGG GAGC-3′.

Transmembrane rotation constructs were based on the previously described constructs pcDNA-hTSLPRα and pcDNA-hIL-7Rα [20] used as templates for PCR to generate altered variants (introduction of up to four Ala residues) of the hTSLPRα and hIL-7Rα transmembrane and intracellular domains. PCR products were ligated into these constructs using restriction sites for XhoI and XbaI, flanking transmembrane and intracellular receptor domain. The following primers were used to introduce additional Ala residues into the transmembrane domain: hTSLPRα transmembrane domain: +1 Ala forward 5′-AAGCCCTCGA GTTTATTTTAGCTATTTCCAGCCTGGCCATC-3′; +2 Ala forward 5′-AAGCCCTCGAGTTTATTTTAGCTGCCATTTCCAGCCTGGCCATC-3′; +3 Ala forward 5′-AAGCCCTCGAGTTTATTTTAGCTGCCGCAATTTCC AGCCTGGCCATC-3′; +4 Ala forward 5′-AAGCCCTCGAGTTTATTTTA GCTGCCGCAGCTATTTCCAGCCTGGCCATC-3′; hTSLPRα intracellular (hTSLPRα$_{in}$) reverse 5′-GCCCTCTAGACTATCACAACGCCACGT-3′, hIL-7Rα transmembrane domain: +1 Ala forward 5′-AAGCCCTCGAGCCT ATCTTAGCTCTAACCATCAGCATTTTG-3′; +2 Ala forward 5′-AAGCC CTCGAGCCTATCTTAGCTGCCCTAACCATCAGCATTTTG-3′; +3 Ala forward 5′-AAGCCCTCGAGCCTATCTTAGCTGCCGCACTAACCATCAGC ATTTTG-3′; +4 Ala forward 5′-AAGCCCTCGAGCCTATCTTAGCTGCC GCAGCTCTAACCATCAGCATTTTG-3′; hIL-7Rα$_{in}$ reverse 5′- GGCCCTC TAGACTATCACTGGTTTTGGTAG-3′.

Sequences encoding extracellular domains for human TGF-β type I receptor (hALK-5) and human TGF-β type II receptor (hTGF-ßRII) were obtained by PCR from cDNA synthesized from mRNA of LPS-treated human PBMCs and ligated into transmembrane rotation constructs described above using restriction sites NotI and XhoI, flanking extracellular receptor domains. Primers employed to amplify the extracellular domains to generate hybrid receptor chains for hALK-5 and hTGF-ßRII were: hALK-5$_{ex}$ forward 5′-TCCAGCGGCCGCGGCGGCGCT GCTCCCG-3′, hALK-5$_{ex}$ reverse 5′-AGGCTCGAGTTCCACAGGACCAAG GCC-3′; hTGF-ßRII$_{ex}$ forward 5′-TCCAGCGGCCGCGACGATCCCACCGC ACG-3′, hTGF-ßRII$_{ex}$ reverse 5′-AAACTCGAGGTCAGGATTGCTG GTG-3′.

Reporter gene construct pGL3-IRF1-luc was previously described [22]. All constructs were verified by DNA sequencing (GATC Biotech AG, Constance, Germany).

Table 1
List of cytokines/growth factors and corresponding hybrid receptors based on hTSLPR-derived intracellular (in) domains and ligand-specific extracellular (ex) domains with maximal factors of stimulation (ratio of measurements for stimulated and unstimulated reporter cells).

Receptor type	Activating ligand	Receptor combination (extracellular/intracellular)	Maximal stimulation factor
Cytokine receptors	rIL-4	$r\gamma c_{ex}$/hTSLPRα_{in} + rIL-4Rα_{ex}/hIL-7Rα_{in}	150.0
	hIL-7	$h\gamma c_{ex}$/hTSLPRα_{in} + hIL-7Rα_{ex}/hIL-7Rα_{in}	76.6
	hIL-13	hIL-4Rα_{ex}/hTSLPRα_{in} + hIL-13Rα_{ex}/hIL-7Rα_{in}	7.1
	hIL-17A	hIL-17AR$_{ex}$/hTSLPRα_{in} + hIL-17CR$_{ex}$/hIL-7Rα_{in}	119.0
	hIL-17F		42.2
	mTSLP	mTSLPRα_{ex}/hTSLPRα_{in} + mIL-7Rα_{ex}/hIL-7Rα_{in}	16.4
	hIFN-α2b	hIFNαR1$_{ex}$/hTSLPRα_{in} + hIFNαR2$_{ex}$/hIL-7Rα_{in}	48.1
RTKs	hEGF	hEGFR$_{ex}$/hTSLPRα_{in} + hEGFR$_{ex}$/hIL-7Rα_{in}	3.3
	hPDGFα	hCD140α_{ex}/hTSLPRα_{in} + hCD140α_{ex}/hIL-7Rα_{in}	5.7
	hM-CSF	mM-CSF-R$_{ex}$/hTSLPRα_{in} + mM-CSF-R$_{ex}$/hIL-7Rα_{in}	68.3
	mM-CSF		87.9
RSTKs	hTGF-β1	hALK-5$_{ex}$/hTSLPRα_{in} + hTGFßRII$_{ex}$/hIL-7Rα_{in}	1.9
	hTGF-β3		1.6

Table 2
Biological activities of variant hybrid receptors with relative rotational shifts between extra- and intracellular domains. Based on the DNA constructs shown in Fig. 4C, four respective variants of both hALK-5$_{ex}$/hTSLPRα_{in} and hTGFßRII$_{ex}$/hIL-7Rα_{in} hybrid receptors were generated with one to four inserted Ala residues within the transmembrane domain. All 16 possible combinations of obtained hybrid receptors were transfected into Ba/F3 cells, stimulated with 100 ng/ml hTGF-ß3 and quantified for resulting relative luciferase activity induction (maximal stimulation factor). Shown is one representative value out of three experiments.

Receptor chain 1	Receptor chain 2	Stimulation factor (at 100 ng/ml of hTGF-ß3)
hALK-5$_{ex}$/hTSLPRα_{in} + 1Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 1Ala	3.79
hALK-5$_{ex}$/hTSLPRα_{in} + 2Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 1Ala	3.11
hALK-5$_{ex}$/hTSLPRα_{in} + 3Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 1Ala	1.63
hALK-5$_{ex}$/hTSLPRα_{in} + 4Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 1Ala	1.48
hALK-5$_{ex}$/hTSLPRα_{in} + 1Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 2Ala	4.24
hALK-5$_{ex}$/hTSLPRα_{in} + 2Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 2Ala	3.79
hALK-5$_{ex}$/hTSLPRα_{in} + 3Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 2Ala	2.50
hALK-5$_{ex}$/hTSLPRα_{in} + 4Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 2Ala	2.28
hALK-5$_{ex}$/hTSLPRα_{in} + 1Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 3Ala	0.62
hALK-5$_{ex}$/hTSLPRα_{in} + 2Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 3Ala	0.59
hALK-5$_{ex}$/hTSLPRα_{in} + 3Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 3Ala	0.88
hALK-5$_{ex}$/hTSLPRα_{in} + 4Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 3Ala	0.96
hALK-5$_{ex}$/hTSLPRα_{in} + 1Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 4Ala	3.12
hALK-5$_{ex}$/hTSLPRα_{in} + 2Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 4Ala	4.22
hALK-5$_{ex}$/hTSLPRα_{in} + 3Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 4Ala	1.59
hALK-5$_{ex}$/hTSLPRα_{in} + 4Ala	hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 4Ala	1.02

2.3. Cell transfection

Chimeric receptor expression constructs containing intracellular chains of hTSLPR (hIL-7Rα_{in} and hTSLPRα_{in}) fused to different extracellular receptor domains of cytokines of interest (Tables 1 and 2) were used to transiently transfect Ba/F3 cells for receptor expression on the cell surface. 5x10^5 cells were transfected with 5 µg of DNA by electroporation using the Nucleofector® device and Cell Line Nucleofector® Kit V (Lonza, Basel, Switzerland) as described previously [15]. Mock-transfected Ba/F3 cells were used as a control. For obtaining a stable expression of representative hybrid receptors on cell surface, transfected cells were cultured for 24 h without mIL-3, additionally stimulated with the corresponding ligand concentrated in its EC_{50} range and cultured for four weeks. Dose dependent responsiveness to the respective ligand was tested weekly by proliferation assay.

2.4. Cytometric detection of specific hybrid receptor chain surface expression

Aliquots of 1x10^5 cells were washed three times with phosphate buffered saline (PBS), incubated with 1 µg of α-P5D4 antibody for 1 h, washed three times in PBS and stained with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (ImmunoTools GmbH, Friesoythe, Germany) in PBS at 1:100 dilution for 1 h. Mock-transfected cells incubated with primary and secondary antibody were used as a control. Antibody binding to the receptor chains was detected with the device CyFlow® Space (Sysmex Partec GmbH, Görlitz, Germany), the software Cyflogic® 1.2.1 (http://www.cyflogic.com) was used to analyze the results.

2.5. Reporter gene assay

Transiently or stably transfected Ba/F3 cells expressing hybrid receptors were cytokine starved for 6 h to address STAT tyrosine phosphorylation in response to ligand stimulation. For reporter gene assays, cells were additionally transfected with the STAT1 reporter construct pGL3-IRF1-luc containing two STAT1 binding sites of the IRF1 promoter upstream of a thymidine kinase minimal promoter and the sequence encoding the luciferase enzyme [21]. The total amount of all transfected constructs was 5 µg of DNA per transfection. Cytokine starved cells were divided into aliquots of 1 × 10^5 cells and incubated with varying concentrations of cytokine in a volume of 100 µl. As a positive control, 50 ng/ml of mIL-3 was used [23]. Cells were harvested after 16 h incubation and lysed using Passive Lyse buffer (PLB, Promega, Mannheim, Germany) for 20 min. Luciferase activity was measured using the Luciferase Assay System (Promega, Mannheim, Germany) in a GloMax® 96 Microplate Luminometer (Promega, Mannheim, Germany). The stimulation factor was expressed as the ratio of luciferase generated luminescence units determined in ligand-stimulated to untreated cells.

2.6. Cell proliferation assay

Transfected Ba/F3 cells were growth factor-starved for 6 h and 1 × 10^4 of cells were distributed in 100 µl of culture medium and incubated with increasing ligand concentrations for proliferation screening. After 48 h of incubation, cell proliferation was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Mannheim, Germany) in a GloMax® 96 Microplate Luminometer according to manufacturer's protocol. To obtain the "-fold induction" value of proliferation, proliferation measurements of ligand-stimulated cells were normalized against measurements from unstimulated cells.

2.7. Production of soluble mM-CSF-R and IL-17R proteins

For production of soluble mM-CSF-R and hIL-17AR, 2x10^7 HEK 293 cells were transfected by electroporation using the Nucleofector® device or Cell Line Nucleofector® Kit V (Lonza, Basel, Switzerland),

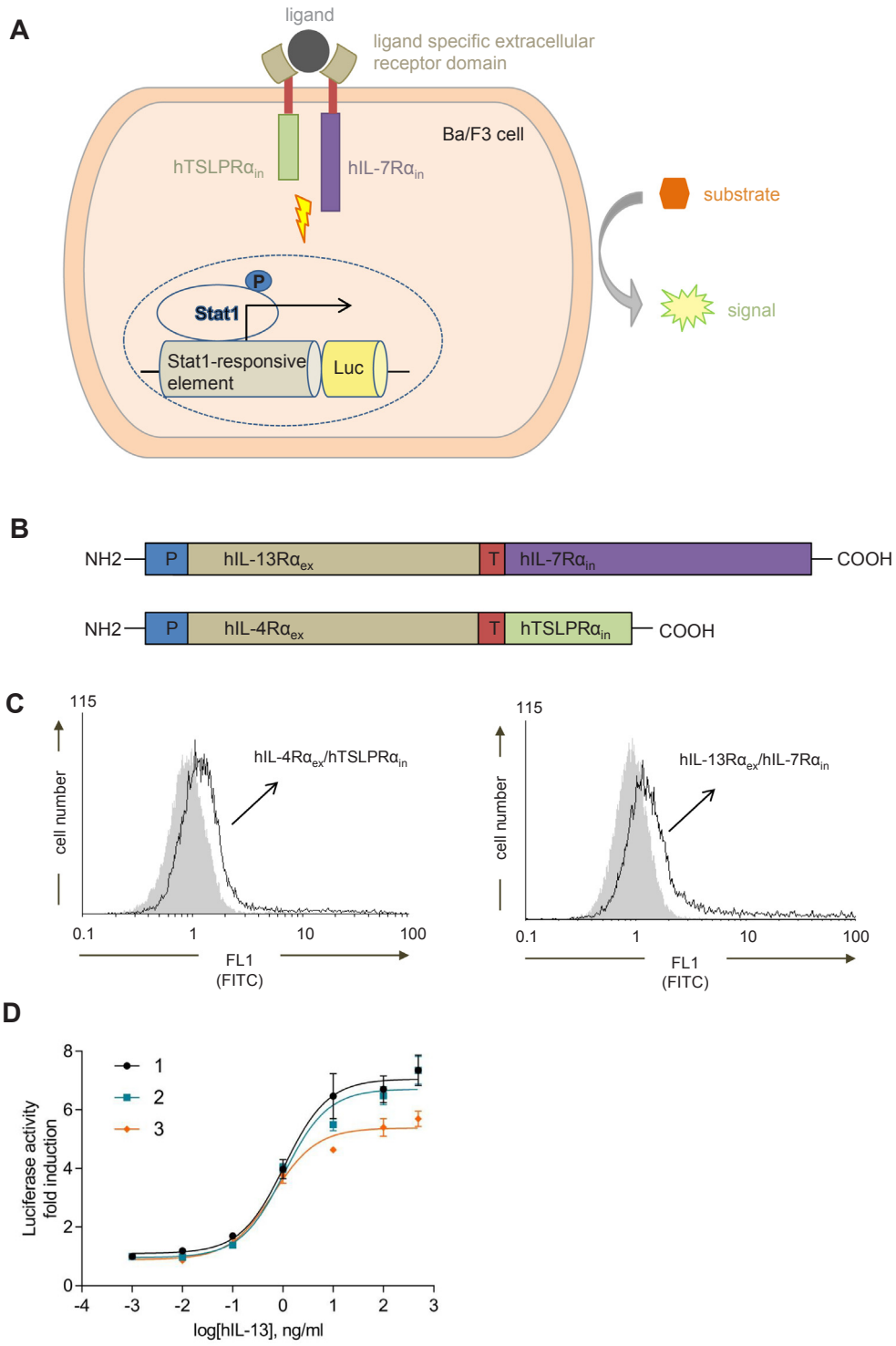

Fig. 1. Construction, expression and functional analysis of a hybrid receptor assembled from the extracellular portion of the hIL-13R and the intracellular segment of the hTSLPR. (A) Concept of cytokine activity determination using hTSLPR based hybrid receptors in Ba/F3 cells and a STAT1-responsive luciferase reporter gene construct. (B) Schematic depiction of hybrid receptor pairs hIL-13Rα$_{ex}$/hTSLPRα$_{in}$ and hIL-4Rα$_{ex}$/hIL-7Rα$_{in}$, P (blue boxes) - P5D4 epitope tag, extracellular domains of hIL-13R (beige boxes), T (dark red boxes) – transmembrane domain, intracellular domains of hTSLPR - hTSLPRα$_{in}$ (violet box) and hIL-7Rα$_{in}$ (light green box). (C) Cytometric analysis of hIL-13Rα$_{ex}$/hTSLPRα$_{in}$ and hIL-4Rα$_{ex}$/hIL-7Rα$_{in}$ surface expression. Ba/F3 cells were transfected with pcDNA-hIL-13Rα$_{ex}$/hTSLPRα$_{in}$ (left) and pcDNA-hIL-4Rα$_{ex}$/hIL-7Rα$_{in}$ (right) expression constructs. 12 h post-transfection, samples containing 1x10^5 cells each were incubated with anti-P5D4 antibody for 1 h, followed by incubation with FITC-labeled goat anti-mouse secondary antibody and analysis by flow cytometry. A control sample (grey) was incubated with FITC-labeled goat anti-mouse only. Fluorescence intensity is plotted against the cell number. A typical out of three experiments is shown. (D) Dose-dependent stimulation of STAT1-mediated luciferase activity by hIL-13 in Ba/F3 cells transiently transfected with a combination of hIL-13R hybrid receptor chains (pcDNA-hIL-13Rα$_{ex}$/hTSLPRα$_{in}$ and pcDNA-hIL-4Rα$_{ex}$/hIL-7Rα$_{in}$) along with luciferase reporter gene construct pGL3-IRF1-luc. 18 h post-transfection, samples of 1 × 10^5 transfected Ba/F3 cells were cytokine-starved for 6 h, stimulated with the indicated concentrations of three different preparations of recombinant hIL-13 and incubated for 16 h. Cells were subsequently lysed and subjected to luciferase activity quantification as described in Materials and Methods. Data from a typical out of three independent determinations is shown, stimulation factors are determined as the ratios of luciferase activities obtained with stimulated vs. unstimulated cells.

respectively, with 5 µg expression construct pcDNA3.1-mMCSF-R$_{ex}$-6His and pcDNA3.1-hIL-17RA$_{ex}$-6His, respectively. To generate stably expressing cells, HEK 293 cells were incubated with 200 µg/ml Zeocin^{TM}(InvivoGen, San Diego, USA) for one to two weeks. Soluble receptors were produced and purified as previously described for soluble hTSLPR$_{ex}$ protein [21]. Purified proteins were dialyzed against PBS and receptor purity was estimated to be > 90% based by silver staining analysis of SDS-polyacrylamide gel (data not shown).

2.8. Reproducibility and statistics

Detection of receptor expression on cell surface was conducted by flow cytometry after each cell transfection. For activity assays, each of the experiments was performed at least three times in duplicates or triplicates. The error bars were calculated as standard deviation using the GraphPad Prism® software (GraphPad Software Inc., La Jolla, CA, USA) in which the data were fitted to a log agonist versus response curve. EC$_{50}$ values in terms of molarity were calculated based on the stoichiometries of cytokines or growth factors as monomers/dimers. For recombinant factors expressed in eukaryotic cells, molecular masses of

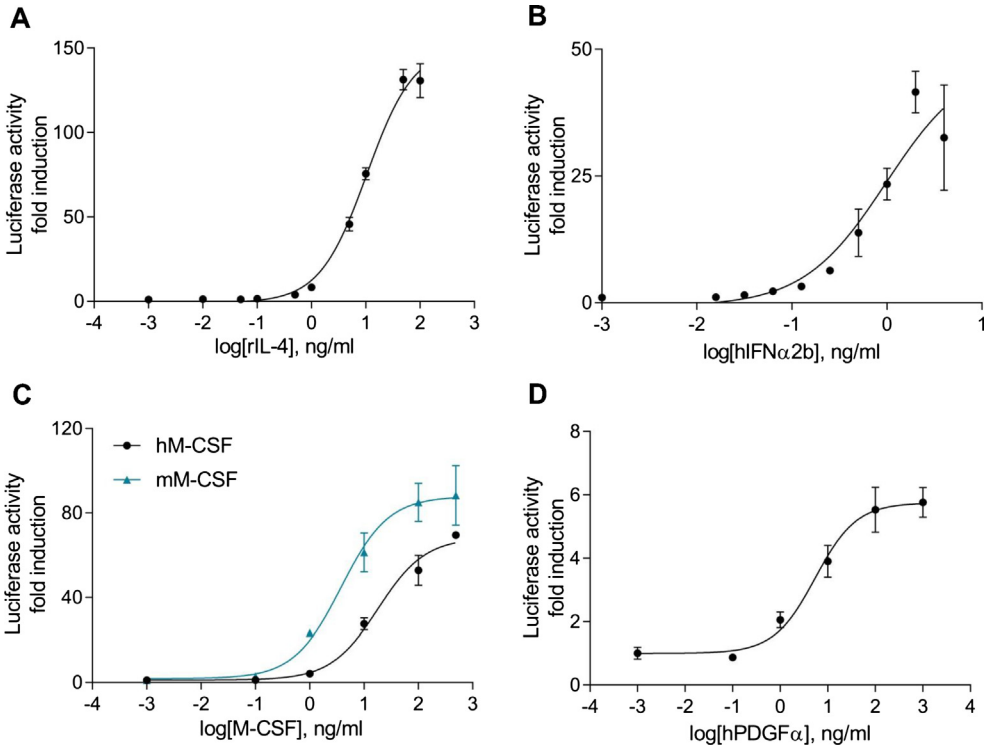

Fig. 2. Dose-dependent induction of STAT1-mediated luciferase activities by hTSLPR-derived hybrid receptors encompassing ligand-binding domains of cytokine/growth factor reporters from different receptor families. Ba/F3 cells were transiently transfected with combinations of expression constructs encoding hTSLPR-based dimerizing hybrid receptors ((A) hybrid receptor pair rIL-2Rγ_{ex}/hTSLPRα_{in} and rIL-4Rα_{ex}/hIL-7Rα_{in}; (B) hybrid receptor pair hIFNαR1$_{ex}$/hTSLPRα_{in} and hIFNαR2$_{ex}$/hIL-7Rα_{in}; (C) hybrid receptor pair mM-CSF-R$_{ex}$/hTSLPRα_{in} and mM-CSF-R$_{ex}$/hIL-7Rα_{in}; (D) hybrid receptor pair hCD140α_{ex}/hTSLPRα_{in} and hCD140α_{ex}/hIL-7Rα_{in}) along with the luciferase reporter gene construct pGL3-IRF1-luc. 18 h post transfection, samples containing 1×10^5 transfected Ba/F3 cells were factor-starved for 6 h and incubated with the indicated concentrations of corresponding factors ((A) rIL-4; (B) hIFNα2b; (C) hM-CSF (black), mM-CSF (blue); (D) hPDGFα). After 16 h of incubation, cells were lysed and luciferase activity was quantified luminometrically as described in Materials and Methods. One experiment out of three independent experiments is shown. 'Fold induction' is the ratio between values obtained for stimulated and unstimulated cells.

glycosylated proteins were judged by the apparent size determined by SDS-polyacrylamide electrophoresis.

3. Results

3.1. Generation and functional analysis of hybrid receptors with the intracellular portion of the hTSLPR as a common signal-transducing platform

The intracellular portions of the heterodimeric hTSLPR were employed as a platform to generate a set of hybrid receptors with ligand binding, extracellular domains from various other receptor systems (Table 1 and Fig. 1A). As a first example for this approach, we fused the extracellular, ligand-binding segments of the heterodimeric hIL-13R (hIL-13Rα_{ex} and hIL-4Rα_{ex} chain) to the transmembrane and intracellular portions of hTSLPR (hTSLPRα_{in} and hIL-7Rα_{in} chain) (Fig. 1B).

Expression constructs encoding two hybrid receptors pcDNA-hIL-4Rα_{ex}/hTSLPRα_{in} and pcDNA-hIL-13Rα_{ex}/hIL-7Rα_{in} were transiently transfected into Ba/F3 cells individually to assess surface expression (Fig. 1C) or along with a STAT1-dependent luciferase reporter gene construct to test for hIL-13-dependent gene activation. Cells were then stimulated with a concentration range of three different hIL-13 preparations and maximum stimulation factors were calculated from the ratio of luminescence signals measured in stimulated and unstimulated cells. Fig. 1D shows distinguishable activation kinetics with an obtained maximum stimulation factors of approximately 5–7. The determined EC_{50} values in the range from 0.64 to 1.05 ng/ml (~50–80 pM) are in good concordance with EC_{50} values reported for natural hIL-13 responsive cells [24].

Next, we explored the potential of the hTSLPR complex as a platform for the generation of similar activity determination setups for a broad range of cytokines and growth factors. A selection of hybrid receptors was generated in which ligand binding domains from representative members of various receptor families (rIL-4R, hIFNαR, mM-CSF-R, hCD140α) are fused to the transmembrane and intracellular signaling domain of the hTSLPR. Respective combinations of hybrid receptors were transfected into Ba/F3 cell along with the STAT1-dependent reporter gene construct as described for the hIL-13-based hybrid receptors before cells were stimulated with specific ligands. Fig. 2 shows by four examples that in all cases, stimulation of cells transfected with hybrid receptor combination and reporter gene with receptor-specific ligands resulted in dose-dependent luciferase expression. The full range of generated and tested hybrid receptors is presented in Table 1. Maximal stimulation indices varied between 7 and roughly 150. We found no clear correlation of maximal signal output with similarity to the native hTSLP receptor complex, some of the studied hybrid receptor systems even yielded higher maximal stimulation indices than the natural hTSLPR.

3.2. Employment of hTSLPR-based hybrid receptors for characterization of cytokine inhibitors

Identification and characterization of specific inhibitors of cytokine and growth factor receptors is an important task in pharmacology. We chose two representatives to demonstrate that the flexible hybrid receptor approach based on hTSLPR-mediated reporter gene is conveniently adaptable to quantitative analysis of cytokine/factor blockade by antagonistic binders.

As a first example, we transfected Ba/F3 cells with the STAT1-driven reporter gene plus a combination of hybrid receptors conferring responsiveness to hM-CSF (mM-CSF-R$_{ex}$/hTSLPRα_{in} and mM-CSF-R$_{ex}$/hIL-7Rα_{in}). Cells were then stimulated with a constant concentration of hM-CSF in the presence of increasing concentrations of the soluble extracellular domain of the mM-CSF-R (smM-CSF-R). As shown in Fig. 3A, smM-CSF-R can completely inhibit hM-CSF-induced receptor activation with an IC_{50} of 35.29 μg/ml (~0.4 μM).

As a second example, we employed a pair of hybrid receptor chains based on the as yet incompletely understood heterodimeric IL-17A/C receptor system for inhibition studies. Ba/F3 cells were transfected with a combination of expression constructs for hybrid receptors hIL-17AR$_{ex}$/hTSLPRα_{in} and hIL-17CR$_{ex}$/hIL-7Rα_{in}, along with a STAT1-

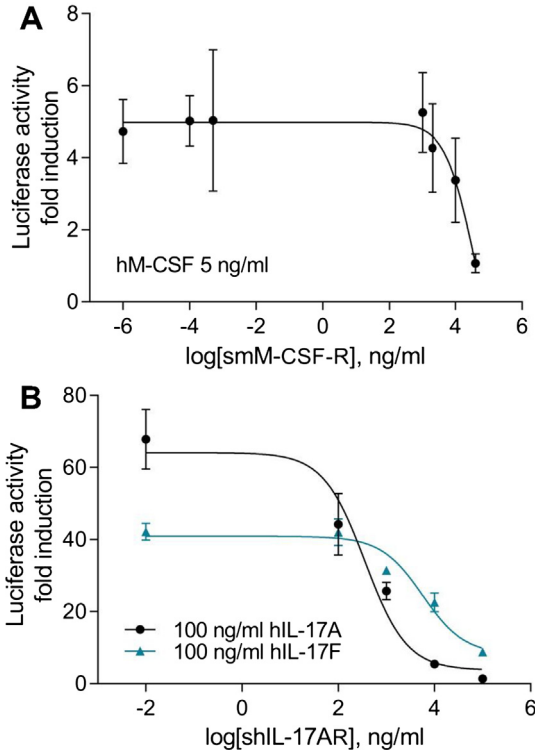

Fig. 3. Employment of hTSLPR-derived hybrid receptors to assess inhibitory properties of antagonistic protein domains on ligand-induced receptor function. Ba/F3 cells were transiently transfected with hybrid receptor combinations and luciferase reporter gene construct pGL3-IRF1-luc and subsequently stimulated with receptor ligands as in Fig. 2. On top of the indicated concentrations of stimulatory factor, increasing amounts of ligand-specific soluble receptor exodomains were added as indicated. Receptor activities were determined by relative luciferase expression as in Fig. 2. (A) Hybrid receptor pair mM-CSF-R_{ex}/hTSLPRα_{in} and mM-CSF-R_{ex}/hIL-7Rα_{in}, stimulus 5 ng/ml hM-CSF, inhibitory receptor domain smM-CSF-R. (B) hIL-17AR_{ex}/hTSLPRα_{in} and hIL-17CR_{ex}/hIL-7Rα_{in}, stimulus 100 ng/ml hIL-17A (black) and 100 ng/ml hIL-17F (blue), respectively, inhibitory receptor domain shIL-17AR.

responsive reporter gene construct. Transfectants were stimulated with 100 ng/ml hIL-17A or hIL-17F, respectively, and subjected to reporter gene activity measurements. As a potential competitor for ligand-induced receptor activity, varying concentrations of the recombinant soluble exodomain of hIL-17AR (shIL-17AR) were added. Receptor-mediated receptor activation as assessed by reporter gene expression was reduced to 2% for hIL-17A stimulation and to roughly 20% when hIL-17F was the ligand (Fig. 3B). IC_{50} values were 0.36 µg/ml for stimulation with hIL-17A (~5 nM) and 5.69 µg/ml (~80 nM) for stimulation with hIL-17F.

3.3. Optimization of hybrid receptor functionality by engineering of transmembrane domains

As documented in Table 1, a hTSLPR-based hybrid receptor for hTGF-β, consisting of constructs hALK-5_{ex}/hTSLPRα_{in} and hTGFßRII$_{ex}$/hIL-7Rα_{in} was functional and allowed for the estimation of biological activities of hTGF-ß1 and hTGF-ß3. However, stimulation factors for STAT1-mediated reporter gene activation were relatively small (1.9 for hTGF-β1 and 1.6 for hTGF-β3 (data not shown)).

In an attempt to enhance signal output, we varied the relative spatial orientation of the intracellular, signal generating domains of the two hybrid receptors. To this aim, a series of hALK-5_{ex}/hTSLPRα_{in} and hTGFßRII$_{ex}$/hIL-7Rα_{in} derivatives was constructed in which one, two, three or four Ala residues, respectively were inserted into both the transmembrane domains originating from the hTSLPRα and hIL-7Rα component. These manipulations were designed to result in relative rotational shifts of the extracellular vs. the intracellular domains of the hybrid receptors by +110, +220, +330 or +440 deg, respectively (Fig. 4A–D). All 16 possible combinations were transfected into Ba/F3 cells along with the STAT1-dependent reporter gene construct and quantified for hTGF-ß3 induced luciferase activity. As shown by stimulation factors in Table 2, the mutual rotational shift of dimerizing hybrid receptor intracellular domains had a profound influence on ligand-dependent signal output. The most efficient combination (hALK-5_{ex}/hTSLPRα_{in} + 1xAla and hTGFßRII$_{ex}$/hIL-7Rα_{in} + 2xAla) showed an almost fivefold stronger reporter gene signal in response to hTGF-ß3 than the pair of original constructs Besides this combination, variants hALK-5ex/hTSLPRαin + 1Ala with hTGF-ßRIIex/hIL-7Rαin + 1Ala, hALK-5ex/hTSLPRαin + 2Ala with hTGF-ßRIIex/hIL-7Rαin + 2Ala and hALK-5ex/hTSLPRαin + 2Ala with hTGF-ßRIIex/hIL-7Rαin + 4Ala also showed high stimulation factors of up to 4. Receptor combinations including hIL-7Rαin + 3Ala were not responsive to the ligand at all. All other combinations led to maximum stimulation factors between 1 and 3.1 (Fig. 4E).

3.4. Generation of stable reporter cell lines responsive to hybrid receptor ligands

Transient transfection of Ba/F3 cells with pairs of hTSLPR-derived hybrid receptors enabled us to study activities of various cytokines and growth factors on a co-transfected reporter gene. To further develop convenience and reproducibility of the assays we set out to generate stable reporter cells for defined ligands.

We had previously shown that the factor dependency of Ba/F3 cells can be exploited to select for transfected cells stably expressing a signaling-competent hTSLPR dimer by replacing mIL-3, the natural survival factor in the medium with hTSLP as a survival and proliferation factor [20]. Here we used this approach to create cell lines that express functional hTSLPR-derived hybrid receptor suitable to characterize signal release by a range of relevant ligands.

Ba/F3 cells were transfected with a representative set of hybrid receptor combinations from the collection successfully tested for functionality in transient transfection experiments (Table 1) and incubated them for four weeks in medium devoid of mIL-3, but instead containing the respective ligands of the extracellular domains concentrated in their EC_{50} range. In all cases, it was possible to establish cell lines that had developed a dependence on the respective factors due to the surface expression of their specific ligand binding domains fused to the hTSLPR signal transducing intracellular segments. While all four cell lines did not survive in the absence of respective ligands (data not shown), they exhibited dose-dependent proliferation in their presence (Fig. 5). EC_{50} values determined in this experiments were in good concordance with those reported for the activity of the individual factors on natural target cells ~ 234.16 pM (2.81 ng/ml) for hIL-13, ~2.56 nM (39.75 ng/ml) for hIL-17A, ~1.9 nM (11.6 ng/ml) for hEGF and ~ 11.8 nM (332 ng/ml) for hPDGFα.

4. Discussion

The intracellular portion of the heterodimeric hTSLPR provides an efficient platform to assess the biological activities of various receptors that transmit signals into the cell upon ligand-induced dimerization. We have devised a modular building block system for functional expression that allows for the convenient fusion of ligand binding receptor exodomains onto the transmembrane/cytoplasmic domains of the two hTSLPR subunits. In combination with a specific luciferase signal output, activity measurement was found possible for a range of receptor systems that far exceed the boundaries of the hematopoietic cytokine receptor family. Kinetic data for various factors determined by means of the presented general hybrid receptor approach reflect findings obtained with natural factor responsive cells, e.g. for hIL-13 (EC_{50}

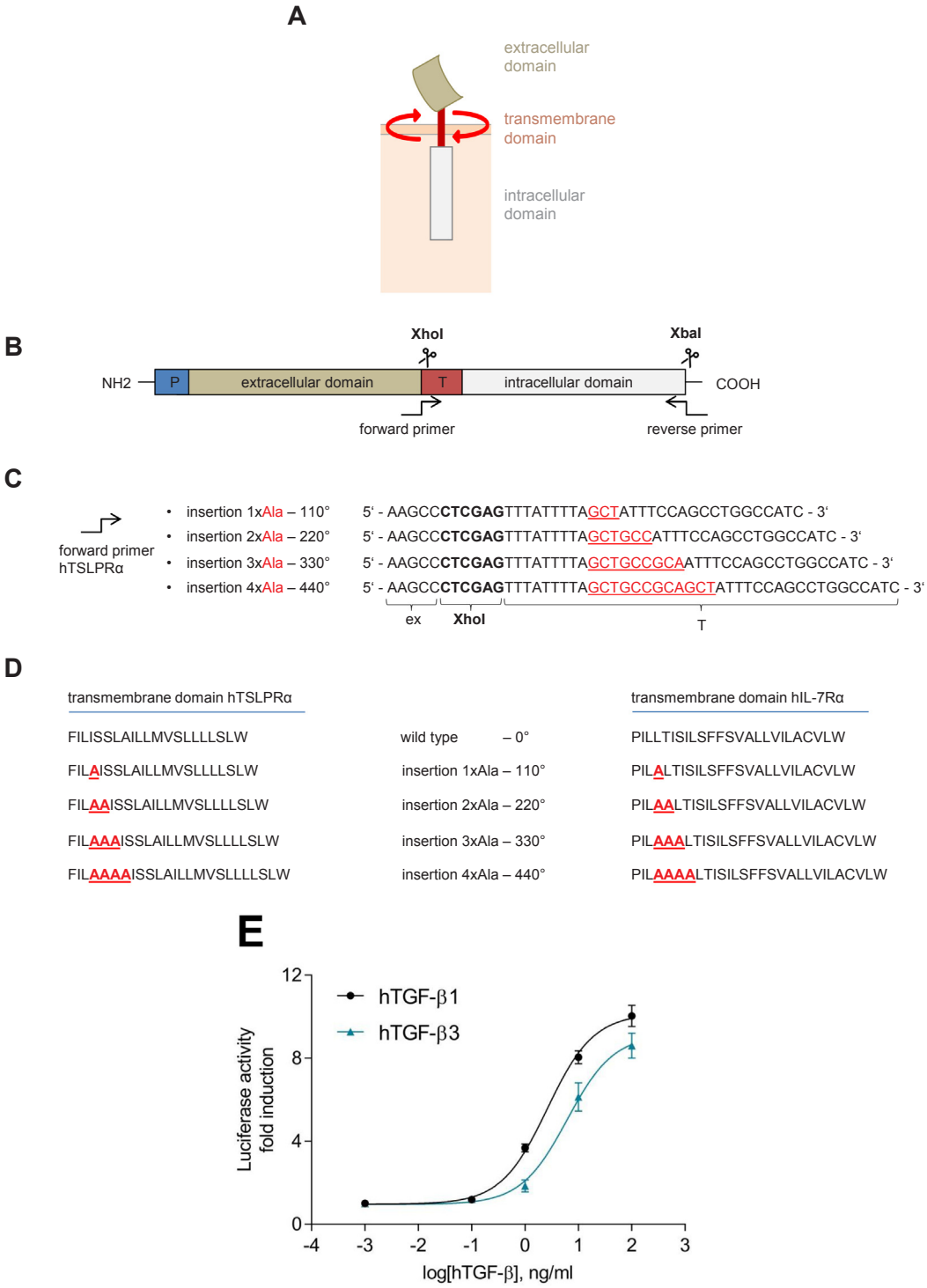

Fig. 4. Design and functional analysis of hTGF-ß/hTSLPR hybrid receptor variants with inter-domain rotational shifts. (A) Alteration of mutual rotational orientation of extracellular and intracellular domains of hybrid receptors by changes within the connecting transmembrane domain. (B) Schematic depiction of hybrid receptor domain structure (P – P5D4-tag (blue box), ex - extracellular domain (beige box), T - transmembrane helical domain (dark red box), intracellular domain (light grey box). (C) Design of mutagenic forward primers with one to four Ala codons (red, underlined) to construct XhoI/XbaI fragments encoding extended transmembrane-intracellular segments by PCR together with the indicted reverse primer. The sequences for the hTSLPRα are shown as an example. (D) Resulting altered primary structures of transmembrane domains of hTSLPRα (left) and hIL-7Rα (right) upon introduction of one, two, three or four additional Ala residues (red, underlined) with the assumed relative rotational shift. (E) Dose-dependent induction STAT1-mediated luciferase activity by hTSLPR-derived hybrid receptor combination hALK-5_{ex}/hTSLPRα_{in} + 1Ala and hTGF-ßRII$_{ex}$/hIL-7Rα_{in} + 2Ala (the most efficient variant of the series, see Table 2). Ba/F3 cells were transfected with DNA constructs encoding the two receptor chains along with luciferase reporter gene construct pGL3-IRF1-luc and stimulated with hTGF-ß1 (black) and hTGF-ß3 (blue). Representation of results is as in Fig. 2. One representative out of three independent experiments is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

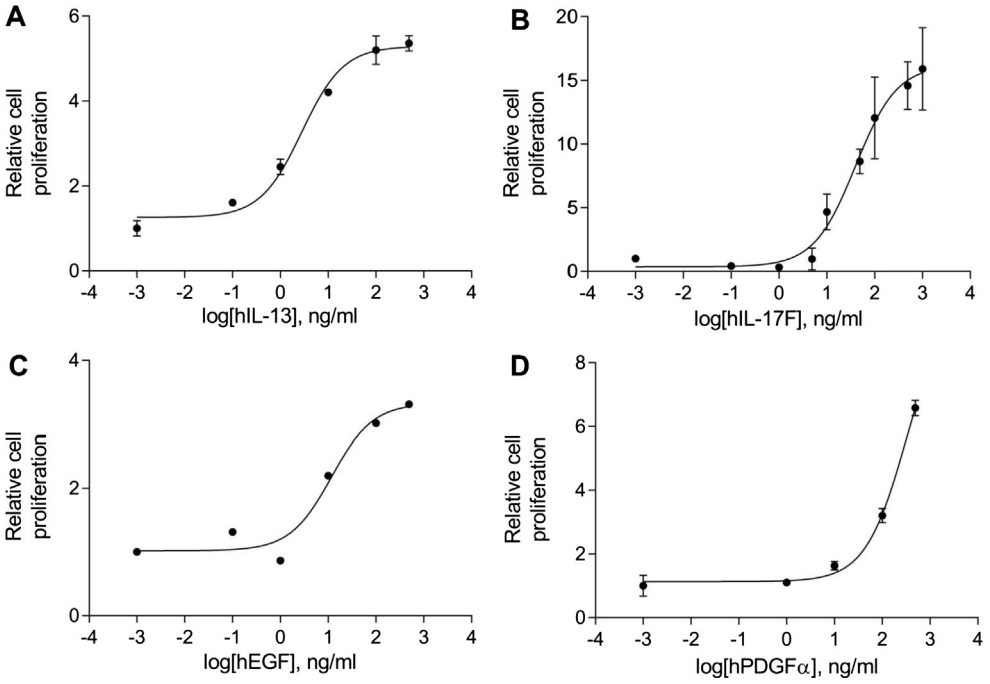

Fig. 5. Ligand-dependent proliferative reponses of Ba/F3 cells stably expressing hybrid receptors. Ba/F3 cells stably transfected with (A) the hIL-13-responsive hybrid receptor combination hIL-13_{ex}/hTSLPRα_{in} and hIL-4Rα_{ex}/hIL-7Rα_{in}; (B) the hIL-17F-responsive hybrid receptor combination hIL-$17AR_{ex}$/hTSLPRα_{in} and hIL-$17CR_{ex}$/hIL-7Rα_{in}; (C) the hEGF-responsive hybrid receptor combination $hEGFR_{ex}$/hTSLPRα_{in} and $hEGFR_{ex}$/hIL-7Rα_{in} and (D) the hPDGFα–responsive hybrid receptor combination $hCD140\alpha_{ex}$/hTSLPRα_{in} and $hCD140\alpha_{ex}$/hIL-7Rα_{in} were factor-starved for 6 h followed by stimulation with increasing concentrations of the indicated, receptor-activating respective stimuli for 48 h. Proliferation was determined using the CellTiter-Glo® Luminescent Cell Viability Assay as described in Materials and Methods. Relative cell proliferation shown is expressed as the ratio of measurements for stimulated and unstimulated cells. One out of three independent experiments is shown.

~8–80 pM) or mM-CSF (~270 pM) [24,25]. For some factors studied, ligand sensitivity was even higher than in more "physiological" assays, e.g. for hPDGFα (EC_{50} ~0.2 nM observed for the hybrid receptor approach employed here compared to previously published data (EC_{50} ~3.5–4.8 nM) [26,27]. In other cases, however, the hybrid receptor approach yielded sensitivities clearly lower than those observed with the respective native receptor. An example for this is the assay for hTGF-β activity, where the most efficient "rotational" hybrid receptor combination showed a roughly hundredfold lower ligand responsiveness than the native receptor (EC_{50} ~100–250 pM compared to 1.4 pM in hybrid receptor assay) [28]. We attribute these differences to structural dissimilarities occurring in the course of assembly of the respective signaling competent ligand-receptor complexes.

The original construct for a heterodimeric hTGF-βR/hTSLPR hybrid receptor yielded only small maximal stimulation indices in response to stimulation by hTGF-β1 and hTGF-β3. One reason for this finding may lie in the fact that the native TGF-β receptor apparently forms a tetrameric complex with TGF-β factors, which are themselves dimers. The active receptor complex consists of two chains of each ALK-5 (TGF-βRI) and TGF-βRII. This situation is probably putting particular constraints on the productive, signal transmitting mutual orientation of the intracellular domains. In addition to this complication, the natural TGF-β-TGF-βR complex recruits membrane protein β-glycan as an accessory stabilizing component [29,30]. It is, thus, notable that despite this convolute setting, a TGF-βR/hTSLPR hybrid receptor complex proved functional in the context of the modular system presented here. The fact that significant enhancement of signal output could be achieved by systematic alterations suggests that further improvement of dimerization-dependent signaling intensity can probably be obtained for any TSLPR based hybrid receptor system. Optimization of the relative intracellular orientation of subunits is likely to result in enhancement of interactions between receptors and signaling mediators. This notion is supported by earlier reports which showed that the efficiency of JAK/STAT signal transduction is greatly influenced by the relative mutual orientation of cytokine receptors dimerized by ligand [22,31,32].

Besides the application of this hybrid receptor system for the estimation of biological activity of cytokines and receptor domains, the approach exploited here is also potentially useful to study the composition of signal releasing ligand-induced assembly of receptor subunits. We have pursued this possibility on the example of the hIL-17 and hIL-17 receptor families, which consist of six hIL-17 variants and five receptor chains, respectively, which can form various signaling competent complexes [33]. Testing a range of hIL-17R/hTSLPR hybrid receptors for their signal output in response to hIL-17 type cytokines, we observed productive dimerization of hIL-17RC and hIL-17RA-derived hybrids by both hIL-17A and hIL-17F as well as hIL-17E-induced signaling through an hIL-17RA/hIL-17RB-derived receptor combination. While the functionality of the corresponding natural ligand-receptor aggregates is established [14], we also observed minor, though significant signaling upon hIL-17-induced formation of as yet uncharted complexes of hIL-17 receptor subunits, e.g. hIL-17D with hIL-17RC/hIL-17RB or hIL-17B with hIL-17RA/hIL-17RB (data not shown).

This hIL-$17AR_{ex}$/hTSLPRα_{in} and hIL-$17CR_{ex}$/hIL-7Rα_{in} hybrid receptor combination also served as an efficient tool for testing already known inhibitory properties of soluble hIL-17AR exodomain when reporter cells were stimulated with hIL-17A and hIL-17F, respectively. In our experiments, we determined the IC_{50} of this soluble receptor domain in competition experiments with hIL-17A as 0.36 μg/ml (~5 nM of glycosylated protein). This value is in relatively good concordance with previous reports (~1.2 nM) [34]. In the case of hIL-17F stimulation, we were able to determine the IC_{50} of soluble hIL-17AR at 5.69 μg/ml (~80 nM).

An interesting perspective of this set-up is the option of identifying unknown or additional ligands for signal transducing receptors from various families. This may be approached by generating cell lines which depend for growth on the dimerization of hybrid receptors. We have previously shown that a crosslinking monoclonal antibody to an epitope tag fused to cytokine receptor amino termini can elicit receptor activity [35]. Long-term incubation of Ba/F3 cells expressing pairs of epitope-tagged hTSLPR-based hybrid receptors with an anti-tag antibody in the absence mIL-3 as growth factor should allow for the selection of cell lines which depend for survival on stimulus-induced dimerization of the respective hybrid receptor complexes.

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