

STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

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Cytokines and their receptors are critical for formation of mature blood cells and for the function of the immune system. Signaling by cytokine receptors is triggered by ligand-induced changes in receptor dimerization/oligomerization, which induces the activation of cytosolic Janus tyrosine kinases (JAK). Activated JAK proteins phosphorylate downstream proteins, like receptors themselves, signal transducers and activators of transcription (STAT) proteins and a variety of other signaling proteins. Phosphorylated STAT proteins dimerize in the cytosol and are translocated to the nucleus where they bind to specific promoter sequences and regulate transcription. We study the signal transduction mechanisms and biologic functions of cytokine receptors such as the receptors for erythropoietin (Epo), thrombopoietin (Tpo), interleukin 2 (IL2) and interleukin 9 (IL9). The assembly of cell-surface receptor complexes, the structure and orientation of the transmembrane (TM) and cytosolic juxtamembrane (JM) domains, and the regulation by JAK kinases of receptor traffic are major focuses. We also study the mechanisms by which STAT proteins become constitutively activated and how they function in transformed hematopoietic or patient-derived leukemia cells.

Determination of the interface and orientation of the activated erythropoietin receptor dimer

Nadine Seubert, Yohan Royer, Katharina Kubatzky, Nicole El-Najjar

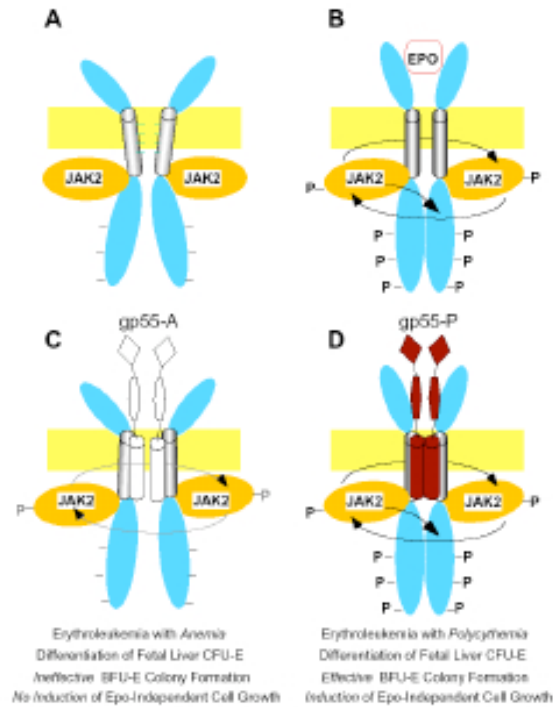
Epo binding to the erythropoietin receptor (EpoR) results in survival, proliferation and differentiation of erythroid progenitors into mature red blood cells (1). We have shown that, in the absence of Epo, the cell-surface EpoR is dimerized in an inactive conformation which is stabilized by interactions between the TM sequences (2). Epo binding to the extracellular EpoR domain induces a conformational change of the receptor which results in the activation of cytosolic JAK2 proteins. The α -helical orientation of the (TM) and cytosolic JM domains is crucial for receptor activation (3). Specifically, in collaboration with Lily Huang and

Harvey Lodish, Whitehead Institute, Cambridge, MA, USA, we have identified a number of key residues in the EpoR cytosolic JM domain which are required for switching on the activity of JAK2 and initiate signaling (4).

To identify the residues that form the interface between the receptor monomers in the activated EpoR dimer we have replaced the EpoR extracellular domain with a coiled-coil dimer of α -helices. Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (1), d (4), and a (7), the register of the coiled-coil α -helices is imposed on the downstream TM α -helix and intracellular domain. This allows the prediction of the position (on an α -helix) of the residues that will be in the interface of the activated EpoR dimer. We have generated seven different constructs where all seven possible orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR. All seven fusion proteins are expressed when transduced in

cytokine-dependent cell lines and reach the cell-surface. However, only two of the seven fusion proteins showed activity represented by stimulation of proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells. The predicted dimeric interfaces of the two active fusion proteins are very close, emphasizing the notion that a unique dimeric EpoR conformation is

required for activation of signaling. At present we are characterizing the signaling events induced by the two active coiled-coil-EpoR fusion proteins and we are performing Cys scanning of the EpoR TM domain and cross-linking assays in order to prove that the dimeric active interface we have identified can be demonstrated in the wild type receptor dimer



(A) The erythropoietin receptor (EpoR) is an inactive dimer on the cell-surface in the absence of ligand due to interactions between the transmembrane (TM) domains (interrupted line). Cytosolic Janus kinase 2 (JAK2) is bound to the receptor juxtamembrane domain and stimulates receptor folding and traffic to the cell-surface.

(B) Erythropoietin (Epo) binding induces a conformational change in the extracellular domain which is transmitted via the α -helical TM domain to receptor residues in the juxtamembrane domain that contact JAK2 and switch its activity on. Activated JAK2 phosphorylates (-P) itself, the receptor cytosolic domain and many other signaling proteins leading to survival, proliferation and differentiation of erythroid progenitors into mature red blood cells.

(C) Co-expression of the EpoR with the gp55-A envelope protein of the Spleen Focus Forming Virus results in cell-surface complex formation due to specific interactions between the TM domains. The EpoR transmembrane dimer, which maintains the EpoR inactive, is disrupted by the interaction with gp55-A. The receptor acquires a conformation, which allows weak constitutive activation resulting in erythroblastemia with low numbers of red blood cells (*anemia*).

(D) Co-expression of the EpoR with the viral gp55-P envelope protein results in cell-surface complex formation due to specific interactions between the TM domains. In this complex the EpoR acquires a dimeric conformation very similar to that induced by Epo, which results in strong constitutive activation of EpoR signaling leading to erythroblastemia and massive production of mature red blood cells (*polycythemia*).

Structural studies on the transmembrane and juxtamembrane cytosolic sequences of the EpoR

Katharina Kubatzky, Nicole El-Najjar

The structure of the transmembrane and cytosolic domains of cytokine receptors remains a mystery. Due to our previous work showing that the junction between the EpoR TM and intracellular domain is rigid (3) we hypothesized that the important structured segments of the cytosolic domain must contain the cytosolic JM segment. We have cloned the cDNAs coding for the seven coiled-coil-EpoR fusion proteins in the pET31b vector in order to produce recombinant fusion proteins in quantities amenable for biophysical and structural studies. In collaboration with the group of Steven Smith, SUNY, Stony Brook, NY, USA, we will determine the NMR structure of the EpoR TM and cytosolic JM domains in the active and inactive coiled-coil-EpoR fusion proteins. The structural studies will be facilitated by the already known structure of the coiled-coil part of the fusion proteins. Comparing the structure and biophysical properties of the active and inactive coiled-coil-EpoR fusion proteins we aim to determine the conformational requirements of the activated state of the receptor. The information obtained by studying the model coiled-coil-EpoR fusion proteins will be then tested on the wild type EpoR. In addition, the recombinant form of the two active coiled-coil-EpoR fusion proteins will be used as baits in order to identify receptor binding proteins in cellular lysates.

Traffic of cytokine receptors to the cell surface

Yohan Royer

Traffic and cell-surface expression of the EpoR critically depend on the ability of EpoR to bind JAK2 intracellularly (4). In the absence of JAK2 the receptor does not get transported from the endoplasmic reticulum (ER) to the Golgi apparatus and does not acquire EndoH resistance. Strikingly, chimeric proteins that contain the EpoR intracellular domain and the extracellular domains of IL9R α , IL2R α or of the common β chain, are expressed on the cell-surface as a function of coexpressed JAK2. Moreover, we have observed that in hematopoietic cells overexpressing JAK proteins several cytokine receptors are expressed at significant higher levels on the cell-surface. Particularly, the IL9R α which requires JAK1 for signaling is expressed at higher levels on the cell-surface when JAK1 but not JAK2 or JAK3 is

overexpressed. Our working hypothesis is that the N-terminus FERM domain of JAK proteins exerts a generic pro-folding effect on cytosolic domains of cytokine receptors. We are testing this hypothesis on several different cytokine receptors and are investigating the link between proper folding in the ER and transport to the cell-surface.

Structure and function of the extracellular domain of the EpoR (Katharina Kubatzky, Yohan Royer). Recombinant Epo is the leading drug of modern biotechnology and is widely used in the clinic for treatment of anemias of different causes, such as chronic renal failure, anemia induced by cancer chemotherapeutic agents, anemia of AIDS patients receiving AZT, or anemias of prematurity, rheumatoid arthritis and myelodysplasia. Apart from being expressed in erythroid progenitors, megakaryocytes and endothelial cells, the EpoR is expressed in neurons where it can protect from apoptosis induced by hypoxia. The present challenge is to design a small molecule mimetic or partial mimetic of Epo which should be non-toxic and non-immunogenic. The mechanism by which Epo binding to the extracellular domain changes the conformation of the EpoR is not known but is relevant for the efforts to isolate small molecule activators of EpoR. Epo binds to three loops on each monomer of the EpoR extracellular domain (EpoR-ECD). The same loops are involved in binding of a mimetic peptide and in contacts between un-liganded extracellular domains. Using insect cell produced EpoR-ECD we have shown that Cys181 of the ECD can be used to cross-link two ECD monomers in the presence of ligand. Using mutagenesis and recombinant production of the EpoR-ECD in insect cells we will investigate the conformation of the EpoR-ECD in the presence and absence of ligand as well as the conformation of a mutant constitutively active form of the EpoR-ECD (EpoR R129C).

Signaling by the thrombopoietin receptor

Judith Staerk

The thrombopoietin receptor (TpoR) is essential for formation of platelets, for renewing hematopoietic stem cells and for expanding myeloid progenitors (5). Like the EpoR, the TpoR is thought to signal by activation of JAK2, of several STATs (STAT1, 3 and 5) as well as of MAP-kinase, PI-3-kinase and AktB. However, TpoR and EpoR signal quite differently since only TpoR can induce hematopoietic differentiation of embryonic stem cells or stimulate the earliest stages of hematopoiesis in immature hematopoietic cells. In contrast, only EpoR can support efficient formation of mature red cells. Since both EpoR and TpoR are members of the homodimeric class of cytokine

receptors we have started to compare signaling and gene expression induced by these two receptors as well as the orientation of their intracellular domains in the activated state. We have already identified a major difference between TpoR and the EpoR, namely that several orientations of the TpoR are compatible with inducing cell proliferation but only a restricted dimeric conformation is compatible with the induction of intercellular adhesion. By constructing chimeric receptors we attempt to identify the relevant sequences which confer to the TpoR and EpoR their specific biologic activities in immature hematopoietic progenitors and committed erythroid progenitors, respectively

Signaling by the receptors for IL2 and IL9 via the common γ chain (γ -c)

Yohan Royer

γ -c is a cytokine receptor that is shared by the receptor complexes of several cytokines, such as IL2, IL4, IL7, IL9 and IL15. γ -c binds and activates JAK3. Humans that lack the γ -c or have mutations in JAK3 develop severe combined immunodeficiency. We are investigating the assembly of IL9R γ and IL2R γ with the common γ chain. We have employed PCR-directed mutagenesis to test the involvement of a conserved cytokine receptor JM hydrophobic motif (3) in IL9R γ and IL2R γ signaling. It appears that IL9R γ has quite different sequence requirements than IL2R γ for signaling, although both utilize γ -c. While IL9R γ is rather similar to the EpoR, IL2R γ does not require hydrophobic residues at positions -1, -2 and -6 from Box 1. We are also investigating the sequence requirements of γ -c JM domain for activating JAK3 and interacting with JAK1. In collaboration with Jean-Christophe Renault we attempt to isolate novel IL9R γ and γ -c mutants that would reveal the precise domains of IL9R γ and γ -c that trigger activation of JAK1 and JAK3.

Sequence-specific interactions between transmembrane domains

Nicole El-Najjar

Two transmembrane viral envelope proteins (gp55-P and gp55-A) belonging to the polycythemic (P) and anemic (A) Spleen Focus Forming Viruses (SFFV) strains, can activate the EpoR when co-expressed in the same cell (6). In collaboration with Yoav Henis, Tel-Aviv University, Israel, we have shown that both the gp55-A and gp55-P TM domains specifically interact with the TM domain of the EpoR. gp55-A weakly activates the receptor leading to

erythroleukemia with low number of red blood cells (*anemia*). However, gp55-P fully activates the EpoR to stimulate proliferation and differentiation of erythroid progenitors leading to both erythroleukemia and massive red blood cell production (*polycythemia*). The activation of EpoR by gp55-P results from a highly specific interaction between the membrane spanning sequences of the two proteins: Ser238 of the murine EpoR TM and Met390 of the gp55-P TM are critical determinants of this interaction (7). Taking advantage of this specific interaction we are constructing a genetic system where the TM sequence of gp55-P is randomized or replaced with short sequences derived from a lymphoid cDNA library in order to select for novel sequences capable of functioning as TM domains (transmembrane domain trap), of binding EpoR and of activating the EpoR. Several genetic approaches exist to probe the ability of a particular TM sequence to homodimerize/homooligomerize but no assay has been reported for selection of hetero-interactions between TM sequences. In our system activation of EpoR signaling will result in cell survival and proliferation which represent a powerful selection.

Constitutive activation of JAK-STAT signaling pathways and genes targeted by STAT5 in transformed hematopoietic and patient-derived leukemia cells

Virginie Moucadel, Yohan Royer, Judith Staerk

Cytokine stimulation of cytokine receptors, induces transient activation of the JAK-STAT pathway. In contrast several mutant forms of cytokine receptors (i.e. EpoR R129C or TpoR S498N) have been isolated that signal constitutively (reviewed in (1, 8, 9)). Such receptors are permanently dimerized in an activated state and induce the biologic effects of the wild type receptors as well as leukemic cell transformation. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously, in the absence of any cytokine. In the transformed cells many of the transient signaling events induced by cytokines are detectable permanently, i.e. ligand-independent phosphorylation of JAK and STAT proteins or high levels of nuclear activated STATs especially STAT5 and STAT3. A similar picture has been noted in patient derived leukemia cells, where constitutively active STAT proteins have been reported in a majority of myeloid and lymphoid leukemia patients. The critical questions we would like to answer concern the mechanisms by which the JAK-STAT remain permanently activated in transformed cells and which genes are regulated by constitutively active STAT proteins in leukemic cells.