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## STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

*Cytokines and their receptors are critical for the formation of mature blood cells and for the function of the immune system. We study the structure and function of several cytokine receptors, such as those for erythropoietin (Epo), thrombopoietin (Tpo), Granulocyte Colony Stimulating Factor (G-CSF), and interleukins (IL) 2 and 9. Activation of these receptors is triggered by cytokine-induced changes in receptor dimerization/oligomerization, which lead to the activation of cytosolic Janus tyrosine kinases (JAK). Regulation by JAK kinases of receptor traffic, the assembly of cell-surface receptor complexes, the mechanisms of dimerization of transmembrane (TM) and cytosolic juxtamembrane (JM) domains, and mechanisms of JAK catalytic activation are major points of interest. The laboratory identified constitutively active mutants of JAK2, JAK1 and Tyk2 and of thrombopoietin receptor and is actively investigating the mechanisms by which JAK2 V617F and thrombopoietin receptor W515 mutants induce, in humans, Myeloproliferative Neoplasms, such as Polycythemia Vera, Essential Thrombocythemia or Primary Myelofibrosis*

### THE MECHANISMS BY WHICH THE MUTANT JAK2 V617F INDUCES POLYCYTHEMIA VERA AND OTHER MYELOPROLIFERATIVE NEOPLASMS IN HUMANS

*A. Dusa, C. Pecquet*

The JAK-STAT pathway is emerging as a key player in cancer, with several mutations in genes coding for JAKs being identified in the past three years (1). Janus kinases possess two kinase domains, one active and the other, denoted as the pseudokinase domain, inactive.

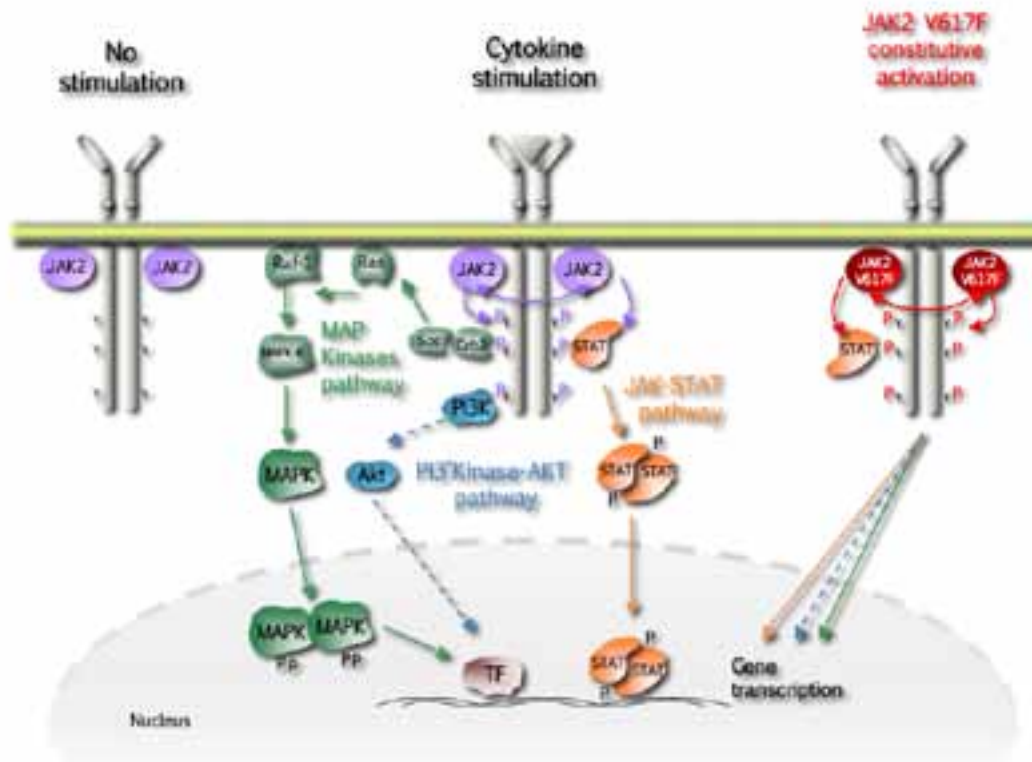
JAK2, one of the four known JAKs (JAK1, JAK2, JAK3 and Tyk2) is crucial for signaling by several cytokine receptors, such as the erythropoietin receptor (EpoR), the thrombopoietin receptor (TpoR), the G-CSF receptor (G-CSFR), the interleukin 3 receptor and the growth hormone receptor. JAKs are appended to the cytoplasmic juxtamembrane domains of receptors and are switched-on upon ligand binding to the receptors' extracellular domains.

Polycythemia Vera (PV), or the Vaquez disease, is characterized by excessive production of mature red cells and sometimes of platelets and granulocytes. Erythroid progenitors in PV are hypersensitive to Epo or independent of

erythropoietin (Epo) for proliferation and differentiation. Strikingly, the traffic of TpoR is defective in myeloid progenitors from PV. A hint that JAK2 or JAK2-binding proteins may be involved in PV came when we showed that the wild type JAK2 strongly promotes the maturation and cell-surface localization of TpoR, the very process that is defective in PV (2).

In collaboration with William Vainchenker at the Institut Gustave Roussy in Paris, we have been involved in the discovery of the JAK2 V617F mutation, that is responsible for >98% of Polycythemia Vera and for >50% of Essen-

tial Thrombocythemia (ET) and Primary Myelofibrosis (PMF) cases (3, 4). The mutation in the pseudokinase domain alters a physiologic inhibition exerted by the pseudokinase domain on the kinase domain and allows the mutated JAK2 to bind and activate EpoR, TpoR and G-CSFR in the absence of cytokines (Figure 1). Saturation mutagenesis at position V617 showed that not only Phe, but also Trp, Leu, Ile and Met can activate JAK2, although Trp is the only mutation that exhibits comparable activity with V617F (5). The homologous V617F mutations in JAK1 and Tyk2 also enable these kinases to be activated without ligand-binding



**Figure 1.** In the absence of cytokine ligands, cytokine receptors (left complex) are preassembled with tyrosine kinases JAK (Janus kinases) in inactive complexes. Cytokine binding to the extracellular domains of receptors (middle complex) induces a conformational change which allows the appended JAKs to cross-phosphorylate and activate each other. In turn, JAKs phosphorylate tyrosine residues (Py) on the cytosolic regions of receptors, which attract SH2- and PTB-containing signaling proteins. These proteins become themselves phosphorylated and either translocate to the nucleus to regulate gene expression (such as STATs, Signal Transducers and Activators of Transcription) or initiate kinase signaling cascades (such as Mitogen Activated Protein-Kinases, MAPK, phosphatidylinositol-3-kinase, PI3K, and Akt). The mutant JAK2 V617F binds to the cytosolic domains of receptors and can trigger signaling in the absence of any cytokine binding to the extracellular receptor domain (right complex). As a consequence, signaling is induced permanently and myeloid progenitors survive, proliferate and differentiate in an uncontrolled manner. (Jean-Michel Heine)

to cytokine receptors (4). Similarly, at least Trp, Leu and Ile also can activate JAK1, besides Phe, at the homologous V658 position. These results suggested that point mutations in JAK proteins might be involved in different forms of cancers (1). An example of such involvement is the identification of mutants in the pseudokinase domain of JAK1 in ~ 20% of adult T-lymphoblastic leukemia.

### **Involvement of TpoR in myeloproliferative diseases**

*C. Pecquet, M. Girardot, J.-P. Defour*

When the protein sequences of TpoR and the closely related EpoR were aligned, we realized that the TpoR contains a unique amphipathic motif (RWQFP) at the junction between the transmembrane and cytosolic domains. Deletion of this motif ( $\Delta 5$ TpoR) results in constitutive activation of the receptor (6), suggesting that these residues maintain the receptor inactive in the absence of TpoR. Mutagenesis of the RWQFP motif showed that W515 (W508 in the murine) is the key residue maintaining human TpoR normally inactive. In vivo, in bone marrow reconstituted mice, the  $\Delta 5$ TpoR and TpoR W515A induce massive expansion of platelets, neutrophils and immature erythroid progenitors and myelofibrosis by day 45 (7) (Figure 2). We predicted that mutations in the amphipathic motif W515 may exist in patients with myelofibrosis (6). Indeed, residue W515 has been found to be mutated to either leucine or lysine by the groups of D. G. Gilliland and A. Tefferi. Why the phenotype induced by TpoR W515 mutants is much more severe than that of JAK2 V617F is under investigation in our group. We recently established that the myelofibrosis phenotype induced by TpoR W515 mutants depends on cytosolic Y112 of TpoR, and appears to involve excessive MAP-kinase signaling (7). Thus, small molecules targeting phosphorylated Y112 might be useful in the treatment of myelofibrosis.

At present, our laboratory is performing under the auspices of an ARC grant (Action de Recherche Concertée of the Université catholique de Louvain) with the St Luc Hospital departments of Hematology (Prof. Cédric Hermans, Prof. Augustin Ferrant), Clinical Biology (Prof. Dominique Latinne) and HORM-PHOS Unit of de Duve Institute (Prof. Mark Rider) a large study on the presence and signaling of JAK2 and TpoR mutations in patients with myeloproliferative neoplasms. Close collaborations with Drs. Laurent Knoops and Jean-Baptiste Demoulin are supported by the ARC project.

### **DETERMINATION OF THE INTERFACE AND ORIENTATION OF THE ACTIVATED EPOR, TPOR AND G-CSFR DIMERS**

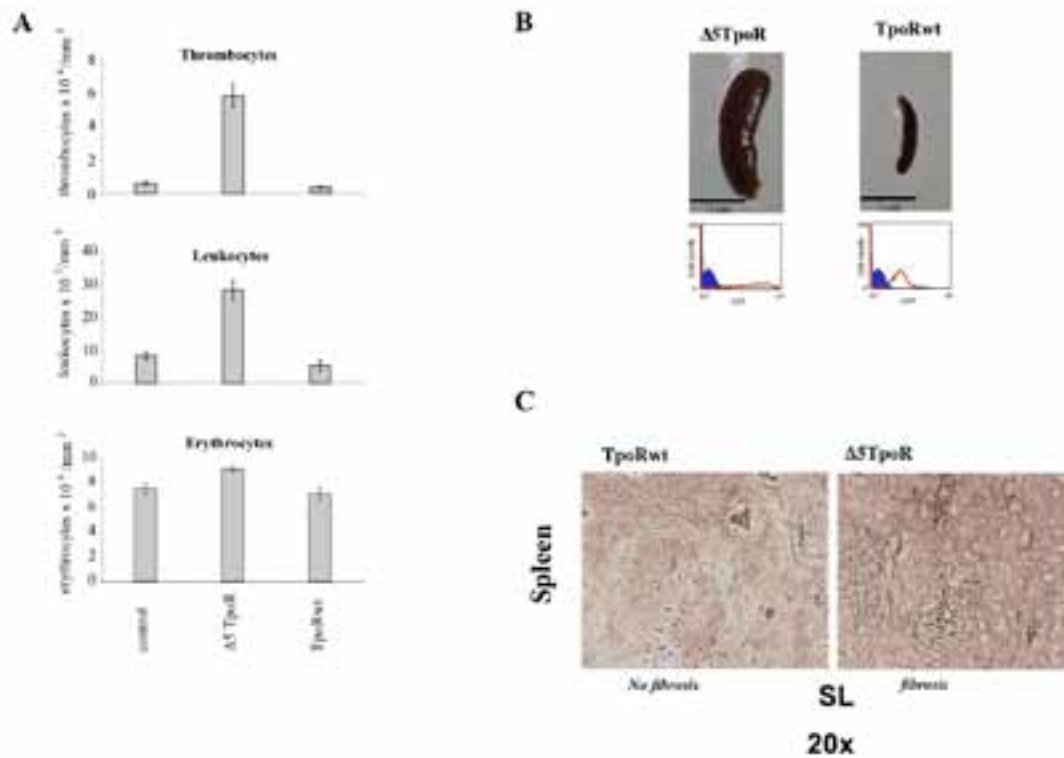
*N. Caceres, A. Dusa, J.-P. Defour*

Epo binding to the erythropoietin receptor (EpoR) results in survival, proliferation and differentiation of erythroid progenitors into mature red blood cells. In the absence of Epo, the cell-surface EpoR is dimerized in an inactive conformation, which is stabilized by interactions between the TM sequences. Epo binding to the extracellular EpoR domain induces a conformational change of the receptor, which results in the activation of cytosolic JAK2 proteins (8).

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  $\alpha$ -helices (9). Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (one), d (four), the register of the coiled-coil  $\alpha$ -helices is imposed on the downstream TM  $\alpha$ -helix and intracellular domain.

When each of the seven possible dimeric orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR, only two fusion proteins stimulated the proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells (9). Since the predicted dimeric interfaces of the two active fusion proteins are very close, a unique dimeric EpoR conformation appears to be required for activation of signaling. In this active conformation TM residues L241 and L244 and JM residue W258 are predicted to be in the interface.

Similar studies are undertaken for the related TpoR and G-CSFR. 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.



**Figure 2.** Bone marrow adoptive transfer in lethally-irradiated mice with hematopoietic stem cells expressing the constitutively active  $\Delta 5TpoR$  induces severe myeloproliferative disorder, splenomegaly and fibrosis of the spleen.  $\Delta 5TpoR$  is a mutant where the amphipathic RW515QFP motif is deleted, which results in constitutive activation of receptor signaling. (A) Peripheral cell counts recorder 45 days after reconstitution indicates leukocytosis and thrombocytosis induced by  $\Delta 5TpoR$ . (B) Splenomegaly was induced by  $\Delta 5TpoR$  at day 45 post reconstitution. The spleen size in TpoRwt mice was equivalent to that in control healthy mice. Green fluorescence protein (GFP) levels were equal after transduction, but enhanced migration to the spleen and proliferation explain the high GFP levels in  $\Delta 5TpoR$  spleens. (C) Histology of spleen sections of mice reconstituted with the indicated constructs. Silver staining (SL) for reticulin indicates fibrosis of the spleen in the  $\Delta 5TpoR$  mice (C. Pecquet and J. Staerk).

## **STRUCTURE AND FUNCTION OF JUXTAMEMBRANE AND TRANSMEMBRANE SEQUENCES OF CYTOKINE RECEPTORS**

*R.-I. Albu, A. Dusa, J. Van Hees, C. Mouton*

We have previously shown that the EpoR as well as a fraction of IL2/IL9 receptors exist on the cell surface as a preformed ligand-independent inactive dimers (homomeric and heteromeric in the case of IL2/IL9 receptor complexes). For the EpoR, transmembrane domain interactions stabilize the inactive dimer at the surface and the EpoR TM sequence is an example of TM dimer based on purely hydrophobic sequences (Proc. Natl Acad. Sci USA 2001, 98, 4379-84; EMBO J., 1999, 18, 3334-47). We study potential transmembrane interactions in the context of other transmembrane proteins, such as TpoR, G-CSFR. We use cell surface immunofluorescence co-patching of differentially epitope tagged receptors in order to determine the ligand-independent state of cell surface complexes. Preformed cytokine receptor oligomers might be important for supporting signaling by mutated JAKs in the absence of ligand. In addition to cytokine receptors, we study the role of transmembrane dimerization in the amyloidogenic processing of Amyloid Precursor Protein (APP) in collaboration with the group of Prof. Jean-Noel Octave. We identified three Gly-X-X-X-Gly motifs in the juxtamembrane and transmembrane domain of APP and showed that these motifs promote amyloidogenic processing of APP (J. Biol. Chem. 2008 283, 7733).

## **TRAFFIC OF CYTOKINE RECEPTORS TO THE CELL-SURFACE**

*C. Pecquet, R.-I. Albu*

We have observed that, in hematopoietic cells, over-expression of JAK proteins leads to enhanced cell-surface localization of cytokine

receptors (i.e. EpoR TpoR, IL9R, IL2R, gc). For some receptors, the effect of the cognate JAK is to promote traffic from the endoplasmic reticulum (ER) to the Golgi apparatus, while for others, such as the TpoR, JAK2 and Tyk2 also protect the mature form of the receptor from degradation by the proteasome, and thus JAKs enhance the total amount of cellular receptor (2). In collaboration with Pierre Courtoy, we are employing confocal microscopy of epitope tagged receptors in order to define the precise intracellular compartments where receptors and JAKs interact. 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. Furthermore, the extracellular fibronectin type III modules of TpoR (D1, D2, D3 or D4) appear to be critical for efficient cell surface localization of the receptor. The W515K activating mutation was introduced in TpoR mutants that lack segments of the extracellular domain; these mutants are normally impaired in their traffic. Selection in the absence of Tpo leads to enhanced cell surface localization of N-terminally truncated TpoR mutants that also possess the activating W515K mutation. Microarray experiments are determining which chaperones or signaling proteins are overexpressed in selected cells, that might stimulate TpoR traffic.

## **CONSTITUTIVE ACTIVATION OF JAK-STAT SIGNALING PATHWAYS AND GENES TARGETED BY STAT5 IN TRANSFORMED HEMATOPOIETIC AND PATIENT-DERIVED LEUKEMIA CELLS**

*M. Girardot*

Cytokine stimulation of cytokine receptors, induces transient activation of the JAK-STAT pathway. In contrast, oncogenic forms of re-

ceptors or of JAKs (JAK2 V617F) transmit a continuous signal which results in constitutive activation of STAT proteins. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors or JAKs in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously (1, 9). A similar picture has been noted in patient-derived leukemia cells. The critical question is which genes are specifically regulated by constitutively active STAT proteins in leukemic cells. Using chromatin immunoprecipitation and sequencing of native promoters bound by STAT5 we noted that, in transformed cells, STAT5, and mainly STAT5B, can also bind to low affinity N4 (TTCNNNN-GAA) DNA sites, not only to the high affinity N3 sites, which are characteristic of ligand-activated STAT5. We are attempting to identify the promoters actually bound by STAT proteins in living cells in physiologic and pathologic situations. We identified one specific target gene of constitutive active STAT5B signaling in megakaryocytes of MPN patients, namely Lipoma Preferred Partner (LPP) (10), a gene found to be translocated in rare leukemias. LPP is the host gene for miR-28, which we found to down-modulate TpoR translation, impair megakaryocyte differentiation. miR-28 is pathologically overexpressed in 30% of MPNs (10). Targets of miR-28, such as E2F6, are critical cell cycle regulators that might influence the phenotype of myeloproliferative disorders (10), thus linking specific gene induction by constitutive STAT signaling to phenotype of disease.

## SELECTED PUBLICATIONS

- Constantinescu SN, Girardot M, Pecquet C. *Mining for JAK-STAT mutations in cancer.* **Trends Biochem Sci** 2008;33:122-31.
- Royer Y, Staerk J, Costuleanu M, Courtoy PJ, Constantinescu SN. *Janus kinases affect thrombopoietin receptor cell surface localization and stability.* **J Biol Chem** 2005;280:27251-61.
- James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, Garcon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval JL, Constantinescu SN, Casadevall N, Vainchenker W. *A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera.* **Nature** 2005;434:1144-8.
- Staerk J, Kallin A, Demoulin J-B, Vainchenker W, Constantinescu SN. *JAK1 and Tyk2 activation by the homologous Polycythemia Vera JAK2 V617F mutation: cross-talk with IGF1 receptor.* **J Biol Chem** 2005;280:41893-9.
- Dusa A, Staerk J, Elliott J, Pecquet C, Poi-rel HA, Johnston JA, Constantinescu SN. *Substitution of JAK2 V617 by large non-polar amino acid residues causes activation of JAK2.* **J Biol Chem** 2008;283:12941-8.
- Staerk J, Lacout C, Smith SO, Vainchenker W, Constantinescu SN. *An amphipathic motif at the transmembrane-cytoplasmic junction prevents autonomous activation of the thrombopoietin receptor.* **Blood** 2006;107:1864-71.
- Pecquet C, Staerk J, Chaligné R, Goss V, Lee KA, Zhang X, Rush J, Van Hees J, Poi-rel HA, Scheiff JM, Vainchenker W, Giraudier S, Polakiewicz RD, Constantinescu SN. *Induction of myeloproliferative disorder and myelofibrosis by thrombopoietin receptor W515 mutants is mediated by cytosolic tyrosine 112 of the receptor.* **Blood** 2010;115:1037-48.
- Constantinescu SN, Huang LJ, Nam H, Lodish HF. *The erythropoietin receptor cytosolic juxtamembrane domain contains an essential, precisely oriented, hydrophobic motif.* **Mol Cell** 2001;7:377-85.
- Seubert N, Royer Y, Staerk J, Kubatzky KF, Moucadel V, Krishnakumar S, Smith SO, Constantinescu SN. *Active and inactive orientations of the transmembrane and cytosolic domains of the erythropoietin receptor dimer.*

**Mol Cell** 2003;12:1239-50.

10. Girardot M, Pecquet C, Boukour S, Knoops L, Ferrant A, Vainchenker W, Giraudier S, Constantinescu SN. *miR-28 is a thrombopoietin receptor targeting microRNA detected in a fraction of myeloproliferative neoplasm patient platelets.* **Blood** 2010;DOI 10.1182/blood-2008-06-165985.

#### Links

Group: Ludwig Institute for Cancer Research Ltd. NewsLink Sept 2005 of our group (<http://www.licr.org/12124501528/newslink/0509/>)  
European Commission Marie Curie Research Training Network ReceptEUR ([www.recepteur.org](http://www.recepteur.org))

#### Research:

Whitehead Institute for Biomedical Research, MIT, Lodish Lab (<http://www.wi.mit.edu/lodish/>)

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<http://sos.bio.sunysb.edu> lab homepage

<http://csb.sunysb.edu> structural biology homepage

<http://csb.sunysb.edu/bsb> graduate program in biochemistry and structural biology

Hematology (American Society of Hematology Education Book)

<http://www.asheducationbook.org/>

#### Learning:

Biologie moléculaire de la cellule (Molecular Cell Biology) French Edition

[http://universite.deboeck.com/livre/GCOI=28011100737460&fa=author&person\\_id=108&publishercode=28011](http://universite.deboeck.com/livre/GCOI=28011100737460&fa=author&person_id=108&publishercode=28011)

#### Bioinformatics:

Institute of Bioinformatics Bangalore, India  
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