

CHAPTER IV

Discussion

The first evidence for a possible implication of dysregulated JAK kinases in leukomogenesis came from studies in *Drosophila melanogaster* describing that activating point mutations in the *Drosophila*'s Janus kinase, Hopscotch, lead to hematopoietic neoplasia. The first mutation described, designated *hop*^{*Tum-1*}, resulted in an amino acid change of glycine to glutamic acid at residue 341 in the JH4 domain (part of the FERM domain), a residue that is not very well conserved among JAK kinases (174). This dominant mutant is temperature sensitive and leads to a hyperproliferation of phagocytic larval plasmocytes that prematurely differentiate into lamellocytes, aggregate and form melanotic masses at higher temperatures. A further chemical mutagenesis screen led to the identification of a new mutation of the same gene, designated *hop*^{*T42*}, which resulted in a similar phenotype (86). *Hop*^{*T42*} contains a glutamic acid to lysine substitution at amino acid 695 (E695K). This residue is located in the JH2 domain and is conserved among all JAK kinases. Most importantly, Luo and colleagues showed that the corresponding mutation in murine JAK2 activated STAT5 in COS cells, although they also reported that this mutant failed to transform Ba/F3 cells to factor independence (86). These studies suggested that aberrant activation of JAKs might be particularly relevant in pathogenesis of hematological malignancies in humans. Indeed, soon after, activation of JAK2 has been reported as a result of the translocation leading to TEL-JAK2 fusion protein in few cases of acute lymphoblastic leukemia (ALL) (82) and more recently, as a result of the JAK2 V617F mutation in the majority of polycythemia vera patients and other MPNs (88-91). However, numerous hematological malignancies remained where the origin of constitutive STAT activation has not yet been explained. The major aim of this thesis was to identify other, new mechanisms implicated in constitutive activation of JAK-STAT pathway resulting in tumoral transformation.

Our main observations are (i) the identification and characterization of numerous activating mutations in JAK1 occurring *in vitro* as a second genetic hit in BaF3 cells that overcame impaired IL-9 responsiveness by spontaneously overexpressing JAK1 during the first step of selection, (ii) the identification and characterization of constitutively active JAK1 mutants participating in development of B- and T-ALL in humans. Furthermore, our studies of the mechanisms by which JAK1 mutants constitutively activate the JAK-STAT pathway show that: (iii) the ALL-associated JAK1 mutants need association to JAK1-binding receptor, such as IL-9R or IL-2R to allow for constitutive signaling; (iv) in the absence of γ chain, IL-9R α is a preformed homodimer, that provides sufficient scaffold for constitutive activation of mutated JAK1 and STAT factors; (v) the ALL-associated JAK1 mutations confer

hypersensitivity to the anti-proliferative effect of type I IFNs, providing evidence that type I IFNs should be considered as a potential therapy for JAK1 mutation-positive ALL and possibly for other JAK1 mutation-positive malignancies.

The manuscripts that compose the results section of this thesis already contain a discussion of each of these findings. At this stage, I would like to discuss further about the following question:

Why are JAK1 mutations so diverse as compared to JAK2 mutations, and what are the clinical implications of such diversity?

The identification of the JAK2 V617F mutation as a STAT5-activating and disease-causing factor in MPNs has emphasized the oncogenic role of the JAK family and started an era of screening for presence of mutations in JAKs in hematological malignancies. It has been shown that the homologous V617F mutation in JAK1 (V658F) and TYK2 (V678F) are also gain-of-function mutations (101), however at the initiation of this thesis no JAK1 or TYK2 mutations had been reported in human patients. In collaboration with the laboratory of prof. Marco Tartaglia, we had the chance to collaborate to the first description of mutations in JAK1 gene in B- and T-ALL pediatric and adult patients. Beside 3 polymorphisms, in total 8 distinct mutations in JAK1 were identified. 3 of these mutations, namely A634D, R724H and R879C, were shown to be gain-of-function mutations constitutively activating the JAK-STAT pathway and other 5 mutations, K204M, S512L, L653F, R879H and R879S, are still to be tested for their functional and biological activities (166). Subsequently, other groups reported JAK1 mutations either in ALL (167,168) or in AML (167,175). Beside leukemia, JAK1 mutations were reported in two patients with breast cancer (167) and one patient with hepatocellular carcinoma (HCC) (176) (**Table 4.1**). Moreover, in autonomous clones selected in our *in vitro* model of tumorigenesis we identified 14 new, beside 4 previously described in human cancer, activating JAK1 mutations.

Altogether, these observations show a high diversity of JAK1 mutations, which is in strong contrast with the dominant role of JAK2 V617F mutation as almost a unique disease-causing mutation in JAK2 in PV and other MPNs (93).

Amino acid substitution	Proof of activity	JH domain	Reference	Cancer	Activating JAK1 mutations in our <i>in vitro</i> model
K204M	Not done	FERM	Flex et al.	B-ALL	Not present
S512L	Not done	SH2	Flex et al.	T-ALL	Not present
L624_R629>W	Not done	Pseudokinase	Mullighan et al.	T-ALL	Not present
A634D	active	Pseudokinase	Flex et al.	B and T-ALL	Not present, but F635V
S646F	active	Pseudokinase	Mullighan et al.	T-ALL	active
Y652H	Not done	Pseudokinase	Asnafi et al.	T-ALL	active
V658F	active	Pseudokinase	Jeong et al.	T-ALL	active
R724H	active	Pseudokinase	Mullighan et al.	B and T-ALL	Not present, but A723D
R724Q	Not done	Pseudokinase	Flex et al.	T-ALL	Not present
L783F	Not done	Pseudokinase	Asnafi et al.	T-ALL	active
R879S	Not done	Kinase	Jeong et al.	T-ALL	Not present
R879C	active	Kinase	Flex et al.	T-ALL	Not present
R879H	Not done	Kinase	Flex et al.	T-ALL	Not present
T478S	active	SH2	Flex et al.	AML	Not present
V623A	active	Pseudokinase	Xiang et al.	AML	Not present
Q644H, V645F	Not done	Pseudokinase	Xie et al.	HCC	Not present
H647Y	Not done	Pseudokinase	Jeong et al.	Breast cancer	Not present
V658F	active	Pseudokinase	Jeong et al.	AML	active
V715I	Not done	Pseudokinase	Jeong et al.	Breast Cancer	Not present
T782M	Not done	Pseudokinase	Jeong et al.	NSCLC	Not present

Table 4.1. Currently known JAK1 point mutations participating in development of ALL and other malignancies. Some of the JAK1 mutations were reported to be constitutively active (in red), the functional and biological activities of others is still to be tested (in black). Our *in vitro* model of tumorigenesis selects autonomous clones only with activating JAK1 mutations. Some of these mutations were already described in human malignancies and some are localized in residues adjacent to known activating JAK1 mutations (in green).

Beyond the uniqueness of the JAK2 V617F mutation, a major unresolved question is how the very same mutation can lead to different diseases such as polycythemia vera or essential thrombocythemia. One of the hypotheses that have been proposed is that this choice is dictated by the preferential expression of the Epo or Tpo receptors. Indeed, JAK2 V617F needs to associate to homodimeric class I cytokine receptors, such as the EpoR or TpoR, to mediate the cytokine receptor-JAK2-STAT5 constitutive activation responsible for the increased myeloproliferation (97,98).

Our data show that JAK1 mutants also need to associate to JAK1-binding receptors, such as IL-2R, IL-9R or IFN receptors, to promote constitutive activation of the receptor complex and signal transduction via STAT factors (169). We showed that in HEK293 cells the V658F and A634D ALL-associated JAK1 mutants constitutively activate STAT3 or STAT5 when

associated with IL-9R or IL-2R, respectively. However, our observations suggest that different JAK1 mutants might show specificity for some cytokine receptor chains. In IL-3-dependent BaF3 cells, the A634D mutant and the R724H JAK1 mutant induced autonomous growth, whereas the R879C mutant did not. In contrast, this JAK1 R879C protected the BW5147 cell line from dexamethasone-induced apoptosis (166). Moreover our data show, that among the three functionally characterized ALL-associated JAK1 mutants, JAK A634D was the strongest potentiator of the IFN response, while JAK1 R724H weakly potentiated cell response to IFN and JAK1 R879C had no effect.

These findings are best explained by the idea that the capability of each JAK1 mutant to promote intracellular signaling is strictly dependent upon the receptor-dependent context, and by the differential expression of a set of cytokine receptors. Altogether these data document a level of specificity based on the type of mutation in JAK1 and strongly suggest that the different ALL-associated JAK1 mutants do not have a homogeneous dysregulating effect on intracellular signaling. This specificity could be explained by either a mutation-specific conformational change of the kinase resulting in a different binding affinity for the diverse cytokine receptors, a different capacity to cross-activate other JAK bound to the second chain of the receptor complex, or a difference in substrate specificity. Based on these results, we believe that different JAK1 mutations are functionally distinct and should confer specific properties to cells harboring these mutations.

ALL comprises a biologically diverse group of clonal disorders characterized by extremely variable clinical outcome. One can therefore speculate, that the diverse spectrum of JAK1 mutations, where each JAK1 mutation is associated with distinct dysregulating effect on intracellular signaling, might also reflect diversity in types of ALL, with some more and some less aggressive. This hypothesis is very difficult to test in humans, since JAK1 mutation-positive ALL patients were reported to represent 3-20% of ALL patients (166,177) and adult ALL itself is a relative rare malignancy as compared to MPNs. In the cohort of patients studied by the group of Marco Tartaglia, JAK1 mutations were associated with advanced age at diagnosis as well as poor response to therapy and overall prognosis as compared to JAK1-negative patients (166), but the very limited number of patients with a particular mutation does not allow to establish any sensible correlation. We believe that mouse bone marrow reconstitution experiments represent a suitable *in vivo* model to test whether reconstitution by different JAK1 mutants (either ALL-associated or “*in vitro*” selected) will result in similar or diverse leukemia-like phenotypes.

However, even if we assume that ALL is a heterogeneous entity and that each JAK1 mutant can lead to a putative unique sub-entity, these mutations clearly lead to similar type of leukemias. This might simply reflect the redundancy in the signaling pathways activated by receptors for IL-2, IL-4, IL-7 and IL-9. The issue of cytokine redundancy is nicely illustrated by the phenotype of mice that overexpress these factors. Although these mice show distinct immunological phenotypes, very similar ALL-like tumors develop in mice overexpressing either IL-7 or IL-9 in T lymphocytes (160,178). Thus, in contrast with myeloid progenitors in which the Epo and Tpo receptors instruct both the proliferation and differentiation of the cells towards the erythroid or megakaryocytic lineage respectively, the JAK1-binding receptors in immature lymphoid progenitors might be more redundant and promote mainly proliferation in the lymphoid lineages (eventually letting other factors determine cell differentiation).

Of note, this hypothesis might also explain why the V617F mutation in JAK2 is so frequent in myeloproliferative neoplasms (MPNs). Based on the homology of JAK1 and JAK2, we would predict that mutations in JAK2 that are homologous to the mutations in JAK1 would also activate JAK2. However, the V617F mutation is nearly the only one observed in patients with MPNs. This mutation might specifically potentiate signaling pathways that would not be activated by other mutants, thereby representing the “best hit” to mediate myeloproliferation. This could reflect a unique ability to activate the Epo and Tpo receptors, or the fact that this JAK2 V617F mutant manages to activate these receptors in a way that preserves a subtle equilibrium between their proliferation- and differentiation-promoting activities.

Recently, activating mutations in JAK2 have also been identified in 20-30% of Down syndrome-associated ALL (DS-ALL) patients (179) and in pediatric B-ALL patients (180,181). Interestingly, these JAK2 mutations (R683G/S, I682G), also called “lymphoid JAK2 mutations”, are almost exclusively localized in an IREED motif within pseudokinase domain and up to date no V617F mutation has been detected in these patients. Examination of DS-ALL gene expression signature revealed that, in 30-50% of these JAK2 mutation-positive patients the TSLP receptor (or CRLF2) is overexpressed. Importantly, these two genetic events showed co-operation in constitutive activation of JAK-STAT pathway (180,181), similar to what was previously shown for MPNs-associated JAK2 V617F mutant and Epo or Tpo receptor (97,98). Altogether, these data strongly suggest the existence of distinct mutation hotspots in JAK2 that represent the “best hit” to mediate specific proliferative signals via distinct cytokine receptors in each particular cell type and thus would lead to

development of different malignancies, like the V617F JAK2/EpoR in MPNs and “lymphoid JAK2 mutations”/CRLF2 in DS-ALL. This model also implies the possibility that the active conformation or/and mechanisms of constitutive activation of JAK2 by mutations from two different hotspots would be different in order to provide the best affinity of the JAK2 mutants to specific cytokine receptors.

CHAPTER V

Conclusions

and perspectives

The ultimate goal of understanding molecular processes that underpin oncogenesis is to select key cellular targets to design workable drugs (51). The central role of the JAK2 V617F in the physiopathology of MPNs points to this mutant as an excellent therapeutic target. Therefore, several JAK- and JAK2-specific inhibitors are now under development or/and in clinical trials for MPNs. When tested, some of these “JAK2-specific” inhibitors showed remarkable activity on JAK1 as well.

However, there are several potential problems associated with targeting mutated JAK1 in ALL. In general, deregulated tyrosine kinases (such as BCR-ABL1 translocation) appears relatively late during the leukemic process, thus it is associated with the systematic presence of additional genetic lesions like mutations in the RAS, PTEN, NOTCH genes and many others. Among the JAK1 mutation-positive cases, no defect was observed in the RAS and PTEN genes. In contrast, heterozygous mutations in NOTCH1 were observed in all JAK1 mutation-positive individuals (166). A second potential issue is that JAK1 mutations show highly diverse, heterogeneous dysregulating effects on intracellular signaling. Moreover, even through localization of the affected residues in the protein structure suggests that mutations are not completely random and seem to be clustered in specific “hotspot” affecting interfaces important for activation/inhibition of the kinase, there is still high diversity of JAK1 mutations identified in ALL patients as compared with “uniqueness” of the JAK2 V617F in MPNs. All these will complicate development and use of the mutation-specific JAK1 inhibitor in the future, implying the wild-type JAK1 as a constant “off-target” of the JAK inhibitor-based therapy for JAK1 mutation-positive patients. In addition, our *in vitro* data show, that some of the JAK1 mutations might be intrinsically resistant to ATP-competitive inhibitors.

The future of cancer therapy probably resides in combining drugs that target the different hits in a tumor-specific way. Currently, most “specific” therapies, available or in development, that are potentially useful for treatment of ALL are targeting mutated proteins in pathways regulating growth or apoptosis (140). Our observation that JAK1 mutation-positive ALL cells are hypersensitive to the anti-proliferative effect of type I IFN *in vitro* and *in vivo*, is therefore of great importance. In addition, our *in vitro* model for selection of autonomous clones spontaneously expressing constitutively active JAK1 mutants allowed us to monitor the sensitivity of *de novo* JAK1 mutation-positive autonomous clones to the anti-proliferative effect of type I IFNs. Our data show, that 78% of these autonomous cells spontaneously expressing different constitutively activated JAK1 mutants are hypersensitive to type I IFNs.

Anti-proliferative activity of IFN- α is used in the clinic to treat patients with MPNs (173), melanoma or renal cell carcinoma (172), but is not part of the current protocol for ALL patients (140). In contrast to other drugs that need to pass through a long process of testing toxicity, specificity and other pharmacological qualities *in vitro*, *in vivo* and in clinical trials, the potential benefit of IFN- α for JAK1 mutation-positive ALL patient can skip most of these and be rapidly tested in clinic. Therefore, the next step would be to determine whether IFN- α therapy could be of benefit for JAK1 mutation-positive ALL patients and how to combine IFN- α with other target-specific drugs and chemotherapy in a way that takes into account the different hits involved in the pathogenesis. This might be particularly challenging, since T-ALL is a rare disease in adults, and in children the remission rate with current chemotherapeutic-based protocol is already high. The randomized evaluation of the benefits of a new therapeutic option specific for T-ALL subgroups is difficult by itself, irrespectively of the low incidence of JAK1 mutations, affecting maximum 20% of T-ALL and 3% of B-ALL patients (166). Obviously, this ultimate goal goes far beyond of what I could achieve during my PhD thesis.

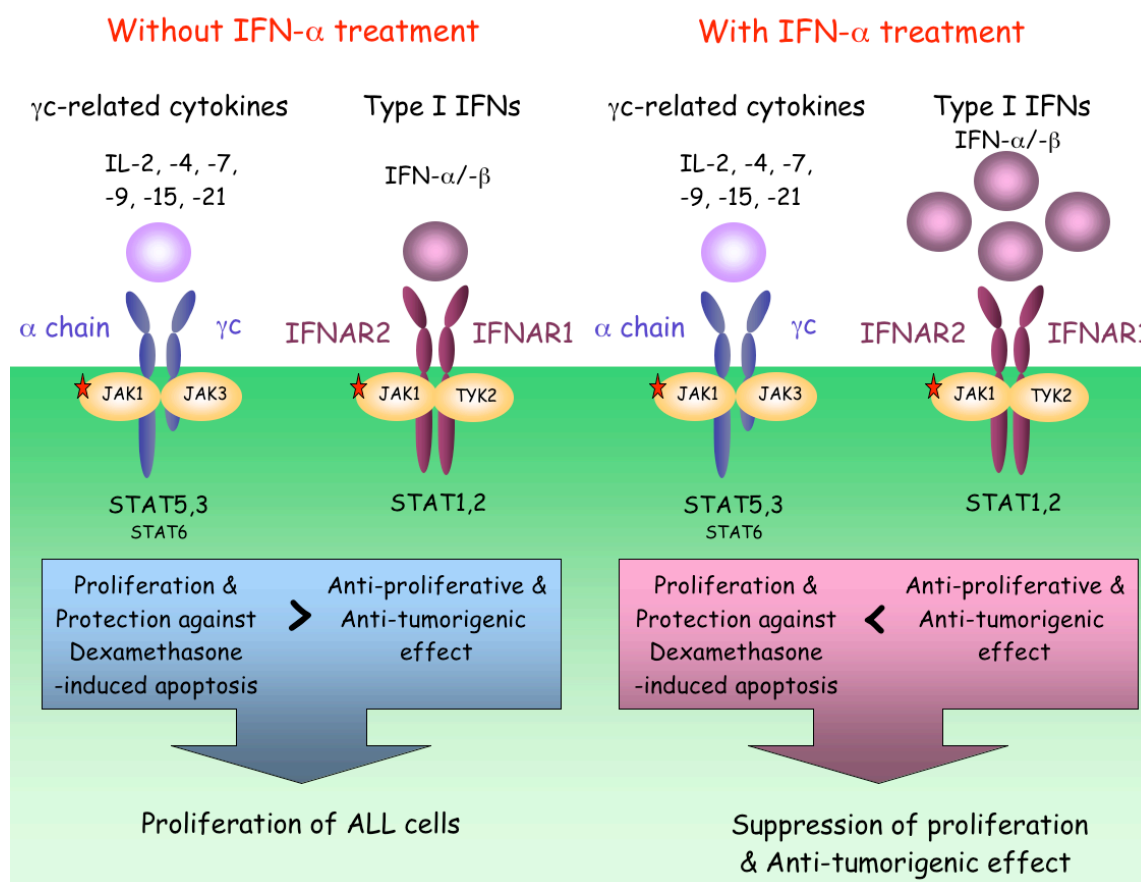


Figure 5.1 Schematic representation of JAK1 mutation-positive ALL evolution with and without type I IFN treatment.

CHAPTER VI

References

1. Thomson, A. W. (2003) *The Cytokine Handbook* (Angus W. Thomson, M. T. L., Ed.), 2. 2 vols.
2. Rozwarski, D. A., Diederichs, K., Hecht, R., Boone, T., and Karplus, P. A. (1996) *Proteins* **26**, 304-313
3. Walter, M. R., Cook, W. J., Zhao, B. G., Cameron, R. P., Jr., Ealick, S. E., Walter, R. L., Jr., Reichert, P., Nagabhushan, T. L., Trotta, P. P., and Bugg, C. E. (1992) *J Biol Chem* **267**, 20371-20376
4. Tanner, J. W., Chen, W., Young, R. L., Longmore, G. D., and Shaw, A. S. (1995) *J Biol Chem* **270**, 6523-6530
5. Leonard, W. J., and O'Shea, J. J. (1998) *Annu Rev Immunol* **16**, 293-322
6. Constantinescu, S. N., Keren, T., Socolovsky, M., Nam, H., Henis, Y. I., and Lodish, H. F. (2001) *Proc Natl Acad Sci U S A* **98**, 4379-4384
7. Brown, R. J., Adams, J. J., Pelekanos, R. A., Wan, Y., McKinstry, W. J., Palethorpe, K., Seeber, R. M., Monks, T. A., Eidne, K. A., Parker, M. W., and Waters, M. J. (2005) *Nat Struct Mol Biol* **12**, 814-821
8. Yang, N., Wang, X., Jiang, J., and Frank, S. J. (2007) *Mol Endocrinol* **21**, 1642-1655
9. Livnah, O., Stura, E. A., Johnson, D. L., Middleton, S. A., Mulcahy, L. S., Wrighton, N. C., Dower, W. J., Jolliffe, L. K., and Wilson, I. A. (1996) *Science* **273**, 464-471
10. Livnah, O., Stura, E. A., Middleton, S. A., Johnson, D. L., Jolliffe, L. K., and Wilson, I. A. (1999) *Science* **283**, 987-990
11. Syed, R. S., Reid, S. W., Li, C., Cheetham, J. C., Aoki, K. H., Liu, B., Zhan, H., Osslund, T. D., Chirino, A. J., Zhang, J., Finer-Moore, J., Elliott, S., Sitney, K., Katz, B. A., Matthews, D. J., Wendoloski, J. J., Egrie, J., and Stroud, R. M. (1998) *Nature* **395**, 511-516
12. Wang, X., Lupardus, P., Laporte, S. L., and Garcia, K. C. (2009) *Annu Rev Immunol* **27**, 29-60
13. Heinrich, P. C., Behrmann, I., Muller-Newen, G., Schaper, F., and Graeve, L. (1998) *Biochem J* **334** (Pt 2), 297-314
14. Boulanger, M. J., Chow, D. C., Brevnova, E. E., and Garcia, K. C. (2003) *Science* **300**, 2101-2104
15. Skiniotis, G., Boulanger, M. J., Garcia, K. C., and Walz, T. (2005) *Nat Struct Mol Biol* **12**, 545-551
16. Chow, D., Ho, J., Nguyen Pham, T. L., Rose-John, S., and Garcia, K. C. (2001) *Biochemistry* **40**, 7593-7603
17. Huyton, T., Zhang, J. G., Luo, C. S., Lou, M. Z., Hilton, D. J., Nicola, N. A., and Garrett, T. P. (2007) *Proc Natl Acad Sci U S A* **104**, 12737-12742
18. Skiniotis, G., Lupardus, P. J., Martick, M., Walz, T., and Garcia, K. C. (2008) *Mol Cell* **31**, 737-748
19. Matadeen, R., Hon, W. C., Heath, J. K., Jones, E. Y., and Fuller, S. (2007) *Structure* **15**, 441-448
20. Tavernier, J., Devos, R., Cornelis, S., Tuypens, T., Van der Heyden, J., Fiers, W., and Plaetinck, G. (1991) *Cell* **66**, 1175-1184
21. Bagley, C. J., Woodcock, J. M., Hercus, T. R., Shannon, M. F., and Lopez, A. F. (1995) *J Leukoc Biol* **57**, 739-746
22. Hansen, G., Hercus, T. R., McClure, B. J., Stomski, F. C., Dottore, M., Powell, J., Ramshaw, H., Woodcock, J. M., Xu, Y., Guthridge, M., McKinstry, W. J., Lopez, A. F., and Parker, M. W. (2008) *Cell* **134**, 496-507
23. Carr, P. D., Gustin, S. E., Church, A. P., Murphy, J. M., Ford, S. C., Mann, D. A., Woltring, D. M., Walker, I., Ollis, D. L., and Young, I. G. (2001) *Cell* **104**, 291-300

24. Quelle, F. W., Thierfelder, W., Witthuhn, B. A., Tang, B., Cohen, S., and Ihle, J. N. (1995) *J Biol Chem* **270**, 20775-20780
25. Kovanen, P. E., and Leonard, W. J. (2004) *Immunol Rev* **202**, 67-83
26. Ghoreschi, K., Laurence, A., and O'Shea, J. J. (2009) *Immunol Rev* **228**, 273-287
27. Waldmann, T. A. (2006) *Nat Rev Immunol* **6**, 595-601
28. Nelms, K., Keegan, A. D., Zamorano, J., Ryan, J. J., and Paul, W. E. (1999) *Annu Rev Immunol* **17**, 701-738
29. Andrews, A. L., Holloway, J. W., Holgate, S. T., and Davies, D. E. (2006) *J Immunol* **176**, 7456-7461
30. Park, L. S., Martin, U., Garka, K., Gliniak, B., Di Santo, J. P., Muller, W., Largaespada, D. A., Copeland, N. G., Jenkins, N. A., Farr, A. G., Ziegler, S. F., Morrissey, P. J., Paxton, R., and Sims, J. E. (2000) *J Exp Med* **192**, 659-670
31. Wang, X., Rickert, M., and Garcia, K. C. (2005) *Science* **310**, 1159-1163
32. LaPorte, S. L., Juo, Z. S., Vaclavikova, J., Colf, L. A., Qi, X., Heller, N. M., Keegan, A. D., and Garcia, K. C. (2008) *Cell* **132**, 259-272
33. Hage, T., Sebald, W., and Reinemer, P. (1999) *Cell* **97**, 271-281
34. Hubbard, S. R., and Till, J. H. (2000) *Annu Rev Biochem* **69**, 373-398
35. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science* **241**, 42-52
36. Wilks, A. F. (1989) *Proc Natl Acad Sci U S A* **86**, 1603-1607
37. Wilks, A. F., Harpur, A. G., Kurban, R. R., Ralph, S. J., Zurcher, G., and Ziemiecki, A. (1991) *Mol Cell Biol* **11**, 2057-2065
38. Krolewski, J. J., Lee, R., Eddy, R., Shows, T. B., and Dalla-Favera, R. (1990) *Oncogene* **5**, 277-282
39. Cance, W. G., Craven, R. J., Weiner, T. M., and Liu, E. T. (1993) *Int J Cancer* **54**, 571-577
40. Velazquez, L., Fellous, M., Stark, G. R., and Pellegrini, S. (1992) *Cell* **70**, 313-322
41. Schindler, C., Shuai, K., Prezioso, V. R., and Darnell, J. E., Jr. (1992) *Science* **257**, 809-813
42. Yan, R., Luo, H., Darnell, J. E., Jr., and Dearolf, C. R. (1996) *Proc Natl Acad Sci U S A* **93**, 5842-5847
43. Sadowski, I., Stone, J. C., and Pawson, T. (1986) *Mol Cell Biol* **6**, 4396-4408
44. Pawson, T. (1988) *Oncogene* **3**, 491-495
45. Lucet, I. S., Fantino, E., Styles, M., Bamert, R., Patel, O., Broughton, S. E., Walter, M., Burns, C. J., Treutlein, H., Wilks, A. F., and Rossjohn, J. (2006) *Blood* **107**, 176-183
46. Boggon, T. J., Li, Y., Manley, P. W., and Eck, M. J. (2005) *Blood* **106**, 996-1002
47. Williams, N. K., Bamert, R. S., Patel, O., Wang, C., Walden, P. M., Wilks, A. F., Fantino, E., Rossjohn, J., and Lucet, I. S. (2009) *J Mol Biol* **387**, 219-232
48. Lindauer, K., Loerting, T., Liedl, K. R., and Kroemer, R. T. (2001) *Protein Eng* **14**, 27-37
49. Saharinen, P., Takaluoma, K., and Silvennoinen, O. (2000) *Mol Cell Biol* **20**, 3387-3395
50. Saharinen, P., and Silvennoinen, O. (2002) *J Biol Chem* **277**, 47954-47963
51. Wilks, A. F. (2008) *Semin Cell Dev Biol* **19**, 319-328
52. Huang, L. J., Constantinescu, S. N., and Lodish, H. F. (2001) *Mol Cell* **8**, 1327-1338
53. Ragimbeau, J., Dondi, E., Alcover, A., Eid, P., Uze, G., and Pellegrini, S. (2003) *Embo J* **22**, 537-547
54. Radtke, S., Hermanns, H. M., Haan, C., Schmitz-Van De Leur, H., Gascan, H., Heinrich, P. C., and Behrmann, I. (2002) *J Biol Chem* **277**, 11297-11305

55. Girault, J. A., Labesse, G., Mornon, J. P., and Callebaut, I. (1998) *Mol Med* **4**, 751-769
56. Miyazaki, T., Takaoka, A., Nogueira, L., Dikic, I., Fujii, H., Tsujino, S., Mitani, Y., Maeda, M., Schlessinger, J., and Taniguchi, T. (1998) *Genes Dev* **12**, 770-775
57. Velazquez, L., Mogensen, K. E., Barbieri, G., Fellous, M., Uze, G., and Pellegrini, S. (1995) *J Biol Chem* **270**, 3327-3334
58. Haan, C., Is'harc, H., Hermanns, H. M., Schmitz-Van De Leur, H., Kerr, I. M., Heinrich, P. C., Grotzinger, J., and Behrmann, I. (2001) *J Biol Chem* **276**, 37451-37458
59. Kohlhuber, F., Rogers, N. C., Watling, D., Feng, J., Guschin, D., Briscoe, J., Witthuhn, B. A., Kotenko, S. V., Pestka, S., Stark, G. R., Ihle, J. N., and Kerr, I. M. (1997) *Mol Cell Biol* **17**, 695-706
60. Chen, M., Cheng, A., Chen, Y. Q., Hymel, A., Hanson, E. P., Kimmel, L., Minami, Y., Taniguchi, T., Changelian, P. S., and O'Shea, J. J. (1997) *Proc Natl Acad Sci U S A* **94**, 6910-6915
61. Yan, H., Piazza, F., Krishnan, K., Pine, R., and Krolewski, J. J. (1998) *J Biol Chem* **273**, 4046-4051
62. Hilkens, C. M., Is'harc, H., Lillemeier, B. F., Strobl, B., Bates, P. A., Behrmann, I., and Kerr, I. M. (2001) *FEBS Lett* **505**, 87-91
63. Royer, Y., Staerk, J., Costuleanu, M., Courtoy, P. J., and Constantinescu, S. N. (2005) *J Biol Chem* **280**, 27251-27261
64. Levy, D. E., and Darnell, J. E., Jr. (2002) *Nat Rev Mol Cell Biol* **3**, 651-662
65. Renauld, J. C. (2003) *Nat Rev Immunol* **3**, 667-676
66. Rodig, S. J., Meraz, M. A., White, J. M., Lampe, P. A., Riley, J. K., Arthur, C. D., King, K. L., Sheehan, K. C., Yin, L., Pennica, D., Johnson, E. M., Jr., and Schreiber, R. D. (1998) *Cell* **93**, 373-383
67. Parganas, E., Wang, D., Stravopodis, D., Topham, D. J., Marine, J. C., Teglund, S., Vanin, E. F., Bodner, S., Colamonici, O. R., van Deursen, J. M., Grosveld, G., and Ihle, J. N. (1998) *Cell* **93**, 385-395
68. Neubauer, H., Cumano, A., Muller, M., Wu, H., Huffstadt, U., and Pfeffer, K. (1998) *Cell* **93**, 397-409
69. Wu, H., Liu, X., Jaenisch, R., and Lodish, H. F. (1995) *Cell* **83**, 59-67
70. Kisseleva, T., Bhattacharya, S., Braunstein, J., and Schindler, C. W. (2002) *Gene* **285**, 1-24
71. Rane, S. G., and Reddy, E. P. (1994) *Oncogene* **9**, 2415-2423
72. Cao, X., Shores, E. W., Hu-Li, J., Anver, M. R., Kelsall, B. L., Russell, S. M., Drago, J., Noguchi, M., Grinberg, A., Bloom, E. T., and et al. (1995) *Immunity* **2**, 223-238
73. Thomis, D. C., Gurniak, C. B., Tivol, E., Sharpe, A. H., and Berg, L. J. (1995) *Science* **270**, 794-797
74. Macchi, P., Villa, A., Giliani, S., Sacco, M. G., Frattini, A., Porta, F., Ugazio, A. G., Johnston, J. A., Candotti, F., O'Shea, J. J., and et al. (1995) *Nature* **377**, 65-68
75. Pepper, A. E., Buckley, R. H., Small, T. N., and Puck, J. M. (1995) *Am J Hum Genet* **57**, 564-571
76. Puel, A., Ziegler, S. F., Buckley, R. H., and Leonard, W. J. (1998) *Nat Genet* **20**, 394-397
77. Shaw, M. H., Boyartchuk, V., Wong, S., Karaghiosoff, M., Ragimbeau, J., Pellegrini, S., Muller, M., Dietrich, W. F., and Yap, G. S. (2003) *Proc Natl Acad Sci U S A* **100**, 11594-11599
78. Shimoda, K., Kato, K., Aoki, K., Matsuda, T., Miyamoto, A., Shibamori, M., Yamashita, M., Numata, A., Takase, K., Kobayashi, S., Shibata, S., Asano, Y., Gondo,

- H., Sekiguchi, K., Nakayama, K., Nakayama, T., Okamura, T., Okamura, S., and Niho, Y. (2000) *Immunity* **13**, 561-571
79. Huse, M., and Kuriyan, J. (2002) *Cell* **109**, 275-282
 80. Noguchi, M., Yi, H., Rosenblatt, H. M., Filipovich, A. H., Adelstein, S., Modi, W. S., McBride, O. W., and Leonard, W. J. (1993) *Cell* **73**, 147-157
 81. Hanratty, W. P., and Dearolf, C. R. (1993) *Mol Gen Genet* **238**, 33-37
 82. Lacronique, V., Boureux, A., Valle, V. D., Poirel, H., Quang, C. T., Mauchauffe, M., Berthou, C., Lessard, M., Berger, R., Ghysdael, J., and Bernard, O. A. (1997) *Science* **278**, 1309-1312
 83. Peeters, P., Raynaud, S. D., Cools, J., Wlodarska, I., Grosgeorge, J., Philip, P., Monpoux, F., Van Rompaey, L., Baens, M., Van den Berghe, H., and Marynen, P. (1997) *Blood* **90**, 2535-2540
 84. Bousquet, M., Quelen, C., De Mas, V., Duchayne, E., Roquefeuil, B., Delsol, G., Laurent, G., Dastugue, N., and Brousset, P. (2005) *Oncogene* **24**, 7248-7252
 85. Griesinger, F., Hennig, H., Hillmer, F., Podleschny, M., Steffens, R., Pies, A., Wormann, B., Haase, D., and Bohlander, S. K. (2005) *Genes Chromosomes Cancer* **44**, 329-333
 86. Luo, H., Rose, P., Barber, D., Hanratty, W. P., Lee, S., Roberts, T. M., D'Andrea, A. D., and Dearolf, C. R. (1997) *Mol Cell Biol* **17**, 1562-1571
 87. Harrison, D. A., Binari, R., Nahreini, T. S., Gilman, M., and Perrimon, N. (1995) *Embo J* **14**, 2857-2865
 88. James, C., Ugo, V., Le Couedic, J. P., Staerk, J., Delhommeau, F., Lacout, C., Garcon, L., Raslova, H., Berger, R., Bennaceur-Griscelli, A., Villeval, J. L., Constantinescu, S. N., Casadevall, N., and Vainchenker, W. (2005) *Nature* **434**, 1144-1148
 89. Baxter, E. J., Scott, L. M., Campbell, P. J., East, C., Fourouclas, N., Swanton, S., Vassiliou, G. S., Bench, A. J., Boyd, E. M., Curtin, N., Scott, M. A., Erber, W. N., and Green, A. R. (2005) *Lancet* **365**, 1054-1061
 90. Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., Wernig, G., Huntly, B. J., Boggon, T. J., Wlodarska, I., Clark, J. J., Moore, S., Adelsperger, J., Koo, S., Lee, J. C., Gabriel, S., Mercher, T., D'Andrea, A., Frohling, S., Dohner, K., Marynen, P., Vandenberghe, P., Mesa, R. A., Tefferi, A., Griffin, J. D., Eck, M. J., Sellers, W. R., Meyerson, M., Golub, T. R., Lee, S. J., and Gilliland, D. G. (2005) *Cancer Cell* **7**, 387-397
 91. Kralovics, R., Passamonti, F., Buser, A. S., Teo, S. S., Tiedt, R., Passweg, J. R., Tichelli, A., Cazzola, M., and Skoda, R. C. (2005) *N Engl J Med* **352**, 1779-1790
 92. Lacout, C., Pisani, D. F., Tulliez, M., Gachelin, F. M., Vainchenker, W., and Villeval, J. L. (2006) *Blood* **108**, 1652-1660
 93. Vainchenker, W., Dusa, A., and Constantinescu, S. N. (2008) *Semin Cell Dev Biol* **19**, 385-393
 94. Zhao, R., Xing, S., Li, Z., Fu, X., Li, Q., Krantz, S. B., and Zhao, Z. J. (2005) *J Biol Chem* **280**, 22788-22792
 95. Vainchenker, W., and Constantinescu, S. N. (2005) *Hematology Am Soc Hematol Educ Program*, 195-200
 96. Wernig, G., Gonneville, J. R., Crowley, B. J., Rodrigues, M. S., Reddy, M. M., Hudon, H. E., Walz, C., Reiter, A., Podar, K., Royer, Y., Constantinescu, S. N., Tomasson, M. H., Griffin, J. D., Gilliland, D. G., and Sattler, M. (2008) *Blood* **111**, 3751-3759
 97. Lu, X., Huang, L. J., and Lodish, H. F. (2008) *J Biol Chem* **283**, 5258-5266
 98. Lu, X., Levine, R., Tong, W., Wernig, G., Pikman, Y., Zarnegar, S., Gilliland, D. G., and Lodish, H. (2005) *Proc Natl Acad Sci U S A* **102**, 18962-18967

99. Hookham, M. B., Elliott, J., Suessmuth, Y., Staerk, J., Ward, A. C., Vainchenker, W., Percy, M. J., McMullin, M. F., Constantinescu, S. N., and Johnston, J. A. (2007) *Blood* **109**, 4924-4929
100. Haan, S., Wuller, S., Kaczor, J., Rolvering, C., Nocker, T., Behrmann, I., and Haan, C. (2009) *Oncogene* **28**, 3069-3080
101. Staerk, J., Kallin, A., Demoulin, J. B., Vainchenker, W., and Constantinescu, S. N. (2005) *J Biol Chem* **280**, 41893-41899
102. Walters, D. K., Mercher, T., Gu, T. L., O'Hare, T., Tyner, J. W., Loriaux, M., Goss, V. L., Lee, K. A., Eide, C. A., Wong, M. J., Stoffregen, E. P., McGreevey, L., Nardone, J., Moore, S. A., Crispino, J., Boggon, T. J., Heinrich, M. C., Deininger, M. W., Polakiewicz, R. D., Gilliland, D. G., and Druker, B. J. (2006) *Cancer Cell* **10**, 65-75
103. Schindler, C., Levy, D. E., and Decker, T. (2007) *J Biol Chem* **282**, 20059-20063
104. Dumoutier, L., de Meester, C., Tavernier, J., and Renauld, J. C. (2009) *J Biol Chem* **284**, 26377-26384
105. Darnell, J. E., Jr. (1997) *Science* **277**, 1630-1635
106. Renner, E. D., Torgerson, T. R., Rylaarsdam, S., Anover-Sombke, S., Golob, K., LaFlam, T., Zhu, Q., and Ochs, H. D. (2007) *N Engl J Med* **357**, 1667-1668
107. Holland, S. M., DeLeo, F. R., Elloumi, H. Z., Hsu, A. P., Uzel, G., Brodsky, N., Freeman, A. F., Demidowich, A., Davis, J., Turner, M. L., Anderson, V. L., Darnell, D. N., Welch, P. A., Kuhns, D. B., Frucht, D. M., Malech, H. L., Gallin, J. I., Kobayashi, S. D., Whitney, A. R., Voyich, J. M., Musser, J. M., Woellner, C., Schaffer, A. A., Puck, J. M., and Grimbacher, B. (2007) *N Engl J Med* **357**, 1608-1619
108. Renner, E. D., Rylaarsdam, S., Anover-Sombke, S., Rack, A. L., Reichenbach, J., Carey, J. C., Zhu, Q., Jansson, A. F., Barboza, J., Schimke, L. F., Leppert, M. F., Getz, M. M., Seger, R. A., Hill, H. R., Belohradsky, B. H., Torgerson, T. R., and Ochs, H. D. (2008) *J Allergy Clin Immunol* **122**, 181-187
109. Benekli, M., Baer, M. R., Baumann, H., and Wetzler, M. (2003) *Blood* **101**, 2940-2954
110. Bromberg, J. (2002) *J Clin Invest* **109**, 1139-1142
111. Benekli, M., Baumann, H., and Wetzler, M. (2009) *J Clin Oncol* **27**, 4422-4432
112. Buettner, R., Mora, L. B., and Jove, R. (2002) *Clin Cancer Res* **8**, 945-954
113. Lafky, J. M., Wilken, J. A., Baron, A. T., and Maihle, N. J. (2008) *Biochim Biophys Acta* **1785**, 232-265
114. Siavash, H., Nikitakis, N. G., and Sauk, J. J. (2004) *Crit Rev Oral Biol Med* **15**, 298-307
115. Tan, S. H., and Nevalainen, M. T. (2008) *Endocr Relat Cancer* **15**, 367-390
116. Yasui, H., Hideshima, T., Richardson, P. G., and Anderson, K. C. (2006) *Br J Haematol* **132**, 385-397
117. Shih, A. H., and Holland, E. C. (2006) *Cancer Lett* **232**, 139-147
118. Joos, S., Kupper, M., Ohl, S., von Bonin, F., Mechttersheimer, G., Bentz, M., Marynen, P., Moller, P., Pfreundschuh, M., Trumper, L., and Lichter, P. (2000) *Cancer Res* **60**, 549-552
119. Gan, H. K., Kaye, A. H., and Luwor, R. B. (2009) *J Clin Neurosci* **16**, 748-754
120. John, T., Liu, G., and Tsao, M. S. (2009) *Oncogene* **28 Suppl 1**, S14-23
121. Duensing, A., Medeiros, F., McConarty, B., Joseph, N. E., Panigrahy, D., Singer, S., Fletcher, C. D., Demetri, G. D., and Fletcher, J. A. (2004) *Oncogene* **23**, 3999-4006
122. Gilliland, D. G., and Griffin, J. D. (2002) *Curr Opin Hematol* **9**, 274-281
123. Hennighausen, L., and Robinson, G. W. (2008) *Genes Dev* **22**, 711-721
124. Germeshausen, M., Ballmaier, M., and Welte, K. (2007) *Blood* **109**, 93-99

125. Pikman, Y., Lee, B. H., Mercher, T., McDowell, E., Ebert, B. L., Gozo, M., Cuker, A., Wernig, G., Moore, S., Galinsky, I., DeAngelo, D. J., Clark, J. J., Lee, S. J., Golub, T. R., Wadleigh, M., Gilliland, D. G., and Levine, R. L. (2006) *PLoS Med* **3**, e270
126. Wormald, S., and Hilton, D. J. (2004) *J Biol Chem* **279**, 821-824
127. de la Chapelle, A., Traskelin, A. L., and Juvonen, E. (1993) *Proc Natl Acad Sci U S A* **90**, 4495-4499
128. Simoncic, P. D., Lee-Loy, A., Barber, D. L., Tremblay, M. L., and McGlade, C. J. (2002) *Curr Biol* **12**, 446-453
129. Nicholson, S. E., Willson, T. A., Farley, A., Starr, R., Zhang, J. G., Baca, M., Alexander, W. S., Metcalf, D., Hilton, D. J., and Nicola, N. A. (1999) *Embo J* **18**, 375-385
130. Fujimoto, M., and Naka, T. (2003) *Trends Immunol* **24**, 659-666
131. Nicholson, S. E., De Souza, D., Fabri, L. J., Corbin, J., Willson, T. A., Zhang, J. G., Silva, A., Asimakis, M., Farley, A., Nash, A. D., Metcalf, D., Hilton, D. J., Nicola, N. A., and Baca, M. (2000) *Proc Natl Acad Sci U S A* **97**, 6493-6498
132. Shuai, K., and Liu, B. (2005) *Nat Rev Immunol* **5**, 593-605
133. Yoshikawa, H., Matsubara, K., Qian, G. S., Jackson, P., Groopman, J. D., Manning, J. E., Harris, C. C., and Herman, J. G. (2001) *Nat Genet* **28**, 29-35
134. Wang, L. H., Yang, X. Y., Mihalic, K., Xiao, W., Li, D., and Farrar, W. L. (2001) *J Biol Chem* **276**, 31839-31844
135. Lensch, M. W.
136. Kubly Janis, T. J. K., Barbara A. Osborne, Richard A. Goldsby. (2000) *Immunology*, 4 Ed., W.H. Freeman & Company
137. Graux, C., Cools, J., Michaux, L., Vandenberghe, P., and Hagemeijer, A. (2006) *Leukemia* **20**, 1496-1510
138. Greer, J. P., Foerster, J., Rodgers, G. M., Paraskevas, F., Glader, B., Arber, D. A., and Means, R. T. (2008) *Wintrobe's Clinical Hematology, 12th edition*
139. De Keersmaecker, K., Marynen, P., and Cools, J. (2005) *Haematologica* **90**, 1116-1127
140. Pui, C. H., and Jeha, S. (2007) *Nat Rev Drug Discov* **6**, 149-165
141. Mullighan, C. G., and Downing, J. R. (2009) *Leukemia* **23**, 1209-1218
142. Grabher, C., von Boehmer, H., and Look, A. T. (2006) *Nat Rev Cancer* **6**, 347-359
143. Van Vlierberghe, P., Pieters, R., Beverloo, H. B., and Meijerink, J. P. (2008) *Br J Haematol* **143**, 153-168
144. Hanahan, D., and Weinberg, R. A. (2000) *Cell* **100**, 57-70
145. Duncan, A. W., Rattis, F. M., DiMascio, L. N., Congdon, K. L., Pazianos, G., Zhao, C., Yoon, K., Cook, J. M., Willert, K., Gaiano, N., and Reya, T. (2005) *Nat Immunol* **6**, 314-322
146. Maillard, I., Fang, T., and Pear, W. S. (2005) *Annu Rev Immunol* **23**, 945-974
147. Weng, A. P., Ferrando, A. A., Lee, W., Morris, J. P. t., Silverman, L. B., Sanchez-Irizarry, C., Blacklow, S. C., Look, A. T., and Aster, J. C. (2004) *Science* **306**, 269-271
148. Thompson, B. J., Buonamici, S., Sulis, M. L., Palomero, T., Vilimas, T., Basso, G., Ferrando, A., and Aifantis, I. (2007) *J Exp Med* **204**, 1825-1835
149. Ferrando, A. A., Neuberg, D. S., Staunton, J., Loh, M. L., Huard, C., Raimondi, S. C., Behm, F. G., Pui, C. H., Downing, J. R., Gilliland, D. G., Lander, E. S., Golub, T. R., and Look, A. T. (2002) *Cancer Cell* **1**, 75-87
150. Hebert, J., Cayuela, J. M., Berkeley, J., and Sigaux, F. (1994) *Blood* **84**, 4038-4044
151. Bertin, R., Acquaviva, C., Mirebeau, D., Guidal-Giroux, C., Vilmer, E., and Cave, H. (2003) *Genes Chromosomes Cancer* **37**, 44-57

152. Sherr, C. J. (2001) *Nat Rev Mol Cell Biol* **2**, 731-737
153. Graux, C., Cools, J., Melotte, C., Quentmeier, H., Ferrando, A., Levine, R., Vermeesch, J. R., Stul, M., Dutta, B., Boeckx, N., Bosly, A., Heimann, P., Uyttebroeck, A., Mentens, N., Somers, R., MacLeod, R. A., Drexler, H. G., Look, A. T., Gilliland, D. G., Michaux, L., Vandenberghe, P., Wlodarska, I., Marynen, P., and Hagemeijer, A. (2004) *Nat Genet* **36**, 1084-1089
154. Quentmeier, H., Cools, J., Macleod, R. A., Marynen, P., Uphoff, C. C., and Drexler, H. G. (2005) *Leukemia* **19**, 295-296
155. Yokota, S., Nakao, M., Horiike, S., Seriu, T., Iwai, T., Kaneko, H., Azuma, H., Oka, T., Takeda, T., Watanabe, A., Kikuta, A., Asami, K., Sekine, I., Matsushita, T., Tsuchiya, T., Mimaya, J., Koizumi, S., Miyake, M., Nishikawa, K., Takae, Y., Kawano, Y., Iwai, A., Ishida, Y., Matsumoto, K., and Fujimoto, T. (1998) *Int J Hematol* **67**, 379-387
156. Kawamura, M., Kikuchi, A., Kobayashi, S., Hanada, R., Yamamoto, K., Horibe, K., Shikano, T., Ueda, K., Hayashi, K., Sekiya, T., and et al. (1995) *Blood* **85**, 2546-2552
157. von Lintig, F. C., Huvar, I., Law, P., Diccianni, M. B., Yu, A. L., and Boss, G. R. (2000) *Clin Cancer Res* **6**, 1804-1810
158. Palomero, T., Sulis, M. L., Cortina, M., Real, P. J., Barnes, K., Ciofani, M., Caparros, E., Buteau, J., Brown, K., Perkins, S. L., Bhagat, G., Agarwal, A. M., Basso, G., Castillo, M., Nagase, S., Cordon-Cardo, C., Parsons, R., Zuniga-Pflucker, J. C., Dominguez, M., and Ferrando, A. A. (2007) *Nat Med* **13**, 1203-1210
159. Van Etten, R. A. (2007) *Oncogene* **26**, 6738-6749
160. Renauld, J. C., van der Lugt, N., Vink, A., van Roon, M., Godfraind, C., Warnier, G., Merz, H., Feller, A., Berns, A., and Van Snick, J. (1994) *Oncogene* **9**, 1327-1332
161. Merz, H., Houssiau, F. A., Orscheschek, K., Renauld, J. C., Fliedner, A., Herin, M., Noel, H., Kadin, M., Mueller-Hermelink, H. K., Van Snick, J., and et al. (1991) *Blood* **78**, 1311-1317
162. Fischer, M., Bijman, M., Molin, D., Cormont, F., Uyttenhove, C., van Snick, J., Sundstrom, C., Enblad, G., and Nilsson, G. (2003) *Leukemia* **17**, 2513-2516
163. Kelleher, K., Bean, K., Clark, S. C., Leung, W. Y., Yang-Feng, T. L., Chen, J. W., Lin, P. F., Luo, W., and Yang, Y. C. (1991) *Blood* **77**, 1436-1441
164. Demoulin, J. B., Uyttenhove, C., Lejeune, D., Mui, A., Groner, B., and Renauld, J. C. (2000) *Cancer Res* **60**, 3971-3977
165. Knoops, L., Hornakova, T., Royer, Y., Constantinescu, S. N., and Renauld, J. C. (2008) *Oncogene* **27**, 1511-1519
166. Flex, E., Petrangeli, V., Stella, L., Chiaretti, S., Hornakova, T., Knoops, L., Ariola, C., Fodale, V., Clappier, E., Paoloni, F., Martinelli, S., Fragale, A., Sanchez, M., Tavolaro, S., Messina, M., Cazzaniga, G., Camera, A., Pizzolo, G., Tornosello, A., Vignetti, M., Battistini, A., Cave, H., Gelb, B. D., Renauld, J. C., Biondi, A., Constantinescu, S. N., Foa, R., and Tartaglia, M. (2008) *J Exp Med* **205**, 751-758
167. Jeong, E. G., Kim, M. S., Nam, H. K., Min, C. K., Lee, S., Chung, Y. J., Yoo, N. J., and Lee, S. H. (2008) *Clin Cancer Res* **14**, 3716-3721
168. Mullighan, C. G., Zhang, J., Harvey, R. C., Collins-Underwood, J. R., Schulman, B. A., Phillips, L. A., Tasian, S. K., Loh, M. L., Su, X., Liu, W., Devidas, M., Atlas, S. R., Chen, I. M., Clifford, R. J., Gerhard, D. S., Carroll, W. L., Reaman, G. H., Smith, M., Downing, J. R., Hunger, S. P., and Willman, C. L. (2009) *Proc Natl Acad Sci U S A* **106**, 9414-9418
169. Hornakova, T., Staerk, J., Royer, Y., Flex, E., Tartaglia, M., Constantinescu, S. N., Knoops, L., and Renauld, J. C. (2009) *J Biol Chem* **284**, 6773-6781

170. Uze, G., Schreiber, G., Piehler, J., and Pellegrini, S. (2007) *Curr Top Microbiol Immunol* **316**, 71-95
171. Chawla-Sarkar, M., Lindner, D. J., Liu, Y. F., Williams, B. R., Sen, G. C., Silverman, R. H., and Borden, E. C. (2003) *Apoptosis* **8**, 237-249
172. Jonasch, E., and Haluska, F. G. (2001) *Oncologist* **6**, 34-55
173. Kiladjian, J. J., Chomienne, C., and Fenaux, P. (2008) *Leukemia* **22**, 1990-1998
174. Luo, H., Hanratty, W. P., and Dearolf, C. R. (1995) *Embo J* **14**, 1412-1420
175. Xiang, Z., Zhao, Y., Mitaksov, V., Fremont, D. H., Kasai, Y., Molitoris, A., Ries, R. E., Miner, T. L., McLellan, M. D., DiPersio, J. F., Link, D. C., Payton, J. E., Graubert, T. A., Watson, M., Shannon, W., Heath, S. E., Nagarajan, R., Mardis, E. R., Wilson, R. K., Ley, T. J., and Tomasson, M. H. (2008) *Blood* **111**, 4809-4812
176. Xie, H. J., Bae, H. J., Noh, J. H., Eun, J. W., Kim, J. K., Jung, K. H., Ryu, J. C., Ahn, Y. M., Kim, S. Y., Lee, S. H., Yoo, N. J., Lee, J. Y., Park, W. S., and Nam, S. W. (2009) *Neoplasma* **56**, 136-140
177. Asnafi, V., Le Noir, S., Lhermitte, L., Gardin, C., Legrand, F., Vallantin, X., Malfuson, J. V., Ifrah, N., Dombret, H., and Macintyre, E. (2009) *Br J Haematol*
178. Rich, B. E., Campos-Torres, J., Tepper, R. I., Moreadith, R. W., and Leder, P. (1993) *J Exp Med* **177**, 305-316
179. Bercovich, D., Ganmore, I., Scott, L. M., Wainreb, G., Birger, Y., Elimelech, A., Shochat, C., Cazzaniga, G., Biondi, A., Basso, G., Cario, G., Schrappe, M., Stanulla, M., Strehl, S., Haas, O. A., Mann, G., Binder, V., Borkhardt, A., Kempfski, H., Trka, J., Bieltorei, B., Avigad, S., Stark, B., Smith, O., Dastugue, N., Bourquin, J. P., Tal, N. B., Green, A. R., and Izraeli, S. (2008) *Lancet* **372**, 1484-1492
180. Mullighan, C. G., Collins-Underwood, J. R., Phillips, L. A., Loudin, M. G., Liu, W., Zhang, J., Ma, J., Coustan-Smith, E., Harvey, R. C., Willman, C. L., Mikhail, F. M., Meyer, J., Carroll, A. J., Williams, R. T., Cheng, J., Heerema, N. A., Basso, G., Pession, A., Pui, C. H., Raimondi, S. C., Hunger, S. P., Downing, J. R., Carroll, W. L., and Rabin, K. R. (2009) *Nat Genet* **41**, 1243-1246
181. Hertzberg, L., Vendramini, E., Ganmore, I., Cazzaniga, G., Schmitz, M., Chalker, J., Shiloh, R., Iacobucci, I., Shochat, C., Zeligson, S., Cario, G., Stanulla, M., Strehl, S., Russell, L. J., Harrison, C. J., Bornhauser, B., Yoda, A., Rechavi, G., Bercovich, D., Borkhardt, A., Kempfski, H., Kronnie, G. T., Bourquin, J. P., Domany, E., and Izraeli, S. (2009) *Blood*

Appendix

ORIGINAL ARTICLE

JAK kinases overexpression promotes *in vitro* cell transformationL Knoops^{1,2,3}, T Hornakova^{1,2}, Y Royer^{1,2}, SN Constantinescu^{1,2} and J-C Renauld^{1,2}¹Ludwig Institute for Cancer Research, Brussels Branch, Brussels, Belgium; ²de Duve Institute, Université catholique de Louvain, Brussels, Belgium and ³Division of Hematology, Cliniques Universitaires Saint-Luc, Brussels, Belgium

Constitutive activation of the JAK-STAT pathway is frequent in cancer and contributes to oncogenesis. Here, we took advantage of the Ba/F3 cell line, a murine proB cell line dependent on IL-3 for growth, to analyse mechanisms of constitutive STAT activation *in vitro*. Cytokine-independent and tumorigenic Ba/F3 cell lines were derived from a two-step selection process. Cells transfected with a defective IL-9 receptor acquire IL-9 responsiveness during a first step of selection, and progress after a second selection step to autonomously growing tumorigenic cells. Microarray analysis pointed to JAK1 overexpression as a key genetic event in this transformation. Overexpression of JAK1 not only increased the sensitivity to IL-9 but also allowed a second selection step toward cytokine-independent growth with constitutive STAT activation. This progression was dependent on a functional FERM and kinase JAK1 domain. Similar results were observed after JAK2, JAK3 and TYK2 overexpression. All autonomous cell lines showed an activation of STAT5, ERK1–2 and AKT but only TYK2-overexpressing cell lines showed a constitutive activation of STAT3. Thus, JAK overexpression can be considered as one of the oncogenic events leading to the constitutive activation of the JAK-STAT pathway.

Oncogene (2008) 27, 1511–1519; doi:10.1038/sj.onc.1210800; published online 17 September 2007

Keywords: interleukin-9; Janus kinases; transforming gene; overexpression

Introduction

The JAK-STAT pathway is responsible for signal transduction for a large number of cytokines. STATs (signal transducers and activators of transcription) are a family of seven proteins: STAT1, 2, 3, 4, 5a, 5b and 6. JAKs (Janus kinases) represent a family of four non-receptor tyrosine kinases, JAK1, JAK2, JAK3 and TYK2 (Kisseleva *et al.*, 2002). These kinases are associated to cytokine receptors and are activated upon

ligand binding. Activated JAKs phosphorylate STATs that dimerize and migrate to the nucleus to regulate the transcription of target genes. Constitutive activation of the JAK-STAT pathway is frequently associated to cancer. Persistent STAT3 phosphorylation is described in solid tumors, while STAT5 activation is essentially found in myeloid and lymphoid malignancies (Bromberg, 2002). Constitutive STAT activation is typically dependent upon the dysregulation of tyrosine kinases such as Src, v-Abl, BCR-Abl and NPM-Alk (Bowman *et al.*, 2000). The Tel-JAK2 fusion protein found in acute lymphoblastic leukemias (ALL) with the t(9;12) translocation leads to the constitutive activation of JAK2 and STAT5 (Lacronique *et al.*, 1997). Recently, an activating mutation in JAK2 (V617F) was found in the majority of patients with polycythemia vera and a significant proportion of patients with other myeloproliferative disorders such as essential thrombocythemia and idiopathic myelofibrosis (Baxter *et al.*, 2005; James *et al.*, 2005; Kralovics *et al.*, 2005; Levine *et al.*, 2005). When JAK2 V617F was transfected into factor-dependent cells, these cells became hypersensitive to cytokines and rapidly acquired cytokine independence, with constitutive activation of STAT5, the ERK/MAP kinase and the PI3/AKT pathways. JAK2 V617F homologous mutations in JAK1 and TYK2 also lead to constitutively active kinases (Staerk *et al.*, 2005).

IL-9 is a multifunctional TH2 cytokine implicated in asthma (Renauld and Van Snick, 2003). Its activities are mediated by a specific receptor chain that forms a heterodimeric receptor with the γ c common chain also involved in IL-2, -4, -7, -15 and -21 signaling. The IL-9 receptor and γ c associate with JAK1 and JAK3, respectively, and trigger the STAT1, 3, 5, IRS and Ras-MAPK pathways (Knoops and Renauld, 2004). Beside the role of IL-9 during immune responses, its growth factor and anti-apoptotic activities on transformed cells suggest a potential role in tumorigenesis. Indeed, IL-9 overexpression induced thymic lymphomas in mice (Renauld *et al.*, 1994), and IL-9 production is associated with Hodgkin's lymphoma (Merz *et al.*, 1991; Fischer *et al.*, 2003) and HTLV-I transformed T cells (Kelleher *et al.*, 1991) in humans. Moreover, *in vitro*, dysregulation of the IL-9 response is associated with autonomous cell growth and malignant transformation of lymphoid cells showing constitutive activation of the JAK-STAT pathway. Freshly isolated murine T cells do not respond to IL-9, but IL-9-dependent cell lines can be observed after long term *in vitro* culture. This acquisition

Correspondence: Professor J-C Renauld, Ludwig Institute for Cancer Research and Experimental Medicine Unit, Université catholique de Louvain, Avenue Hippocrate, 74, Brussels B-1200, Belgium.
E-mail: renauld@bru.lir.org

Received 7 February 2007; revised 31 July 2007; accepted 15 August 2007; published online 17 September 2007

of IL-9 responsiveness is associated with a transformed phenotype (Uyttenhove *et al.*, 1991). Interestingly, cytokine-independent cells with a constitutive STAT3 and STAT5 activation can sporadically arise from these IL-9-dependent T-cell clones (Renauld *et al.*, 1995).

Another model linking IL-9 responsiveness and *in vitro* transformation stemmed from the study of factor-dependent Ba/F3 cells transfected with a defective IL-9 receptor (Demoulin *et al.*, 2000). Ba/F3 is a cell line derived by culturing Balb/c bone marrow B-cell progenitors in the presence of IL-3 (Palacios and Steinmetz, 1985). Although these cells can grow without limitation in the presence of IL-3, they usually never generate cytokine-independent subpopulations. When Ba/F3 cells were transfected with an IL-9-receptor mutant that lacks the STAT-binding site (tyr116phe), IL-9 almost completely failed to induce STAT activation and proliferation (Demoulin *et al.*, 1996). However, a small number of cells managed to survive and even proliferated, and IL-9-dependent cell lines could be derived. In contrast to parental cells, those IL-9-selected cells progressed to cytokine-independent tumorigenic clones at low but significant frequencies. Again, these cytokine-independent cells showed a constitutive activation of STAT factors and particularly of STAT5. Those cells, in contrast to the parental cells, were highly tumorigenic when injected in mice (Demoulin *et al.*, 2000). To better understand mechanisms leading to spontaneous tumorigenesis harboring constitutive STAT activation, we studied genes expressed in transformed Ba/F3 cells to identify potential molecular mechanisms involved in this model. Our results show that overexpression of JAK kinases can be one of the steps leading to constitutive STAT activation and cytokine-independent growth.

Results

JAK1 is overexpressed in Ba/F3-Phe116-derived autonomous cells

Ba/F3 cells transfected with a mutated hIL-9R (Ba/F3-Phe116), defective in STAT recruitment and activation, poorly respond to IL-9. However, after selection with this factor, we obtained a cell line, designated Ba/F3-Phe116/9, which proliferated well in IL-9. After cytokine withdrawal, those cells, in contrast to the parental cells, gave rise to autonomous Ba/F3 cell lines, which were highly tumorigenic *in vivo* and were named Ba/F3-A4 or -A6 (Demoulin *et al.*, 2000). This selection process is schematically represented in Figure 1a. To dissect the mechanisms involved in this cellular transformation, we compared, using cDNA microarrays, the genes expressed in one of these lines, Ba/F3-A6, to the genes expressed in another autonomous Ba/F3 cell line obtained independently by transfection with an oncogenic mutant of M-Ras (Louahed *et al.*, 1999). Microarray analysis data indicated a 6.8 upregulation of JAK1 in Ba/F3-A6 as compared to Ba/F3-M-Ras. To confirm this overexpression at the protein level, we performed a

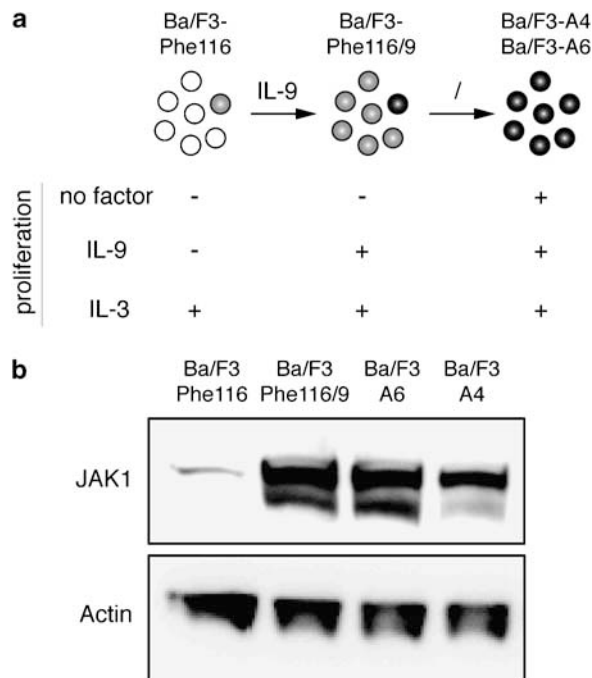


Figure 1 JAK1 expression in Ba/F3 cell lines selected *in vitro* with IL-9 or without cytokine. **(a)** *In vitro* model of tumorigenesis: Ba/F3 cells were transfected with a defective hIL-9R in which Tyr116 was mutated in Phe (Ba/F3-Phe116). Culture in IL-9 led to the selection of a few cells able to proliferate in response to this cytokine (Ba/F3-Phe116/9). These cells could give rise to autonomous cell lines highly tumorigenic *in vivo* (Ba/F3-A4, Ba/F3-A6), while parental cells were not. **(b)** JAK1 expression was assessed in these different cell lines by western blot, with a specific anti-JAK1 antibody. As a control, the membrane was reprobed with an anti-actin antibody.

western blot with an anti-JAK1 antibody. As shown in Figure 1b, JAK1 was overexpressed in Ba/F3-A6 as compared to the parental Ba/F3-Phe116 cell line, and was also overexpressed in another autonomous cell line, Ba/F3-A4. The same overexpression was found in the Ba/F3-Phe116/9 cell line, showing that JAK1 upregulation was a genetic event associated with the first step of selection, namely the selection of IL-9-responsive Ba/F3-Phe116 cells.

Ectopic JAK1 overexpression increases sensitivity to IL-9

To test if JAK1 overexpression could explain the growth advantage in response to IL-9, we stably transfected Ba/F3-Phe116 cells with a construction encoding the wild-type murine JAK1. JAK1 level was increased 5- to 10-fold in bulk transfectants as compared to wild-type Ba/F3 (western blot, data not shown). This level was similar to the level found in Ba/F3-Phe116/9 and autonomous clones. We stimulated those cells with increasing concentrations of IL-9 and measured proliferation after 2 days. As shown in Figure 2, while Ba/F3-Phe116 cells poorly responded to IL-9, JAK1 overexpression dramatically increased the responsiveness to this cytokine. Thus, JAK1 overexpression can explain the better response of

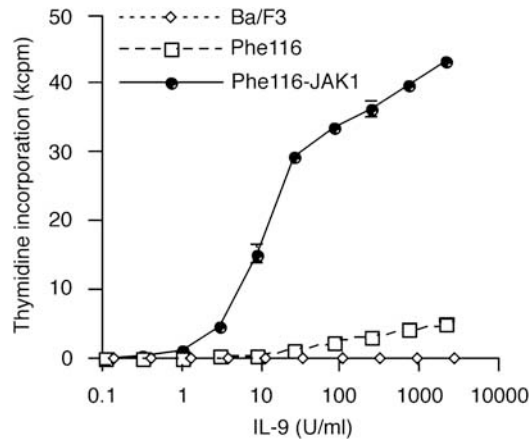


Figure 2 IL-9 response of Ba/F3-Phe116 cells after ectopic JAK1 overexpression. Ba/F3 cells, Ba/F3 cells expressing a defective hIL-9R (Phe116) and transfected with an empty vector or Ba/F3-Phe116 cells overexpressing JAK1 (Phe116-JAK1) were washed and cultured at 3000 cells per well in quadruplicates in 96-well culture plates with increasing concentration of IL-9. After 48 h, ^3H -thymidine was added to the culture, and incorporated ^3H -thymidine was counted after 5 h.

Ba/F3-Phe116/9 cells to IL-9, and was likely the key genetic event associated with the first step of selection.

We next tested if the growth advantage resulting from JAK1 overexpression was restricted to the mutated form of the IL-9 receptor, or could also be observed in Ba/F3 cells expressing the wild-type IL-9R (Ba/F3-h9R). As shown in Figure 3, JAK1 overexpression in Ba/F3-h9R increased about 100 $\times$ the sensitivity to IL-9, while the maximal response appeared slightly decreased. This effect was specific for IL-9, since the response to IL-3, for which JAK2 mediates the signal, was not modified (data not shown). Taken together, these results show that overexpression of JAK1 increases the sensitivity to IL-9.

JAK1 overexpression induces autonomous Ba/F3 cell growth

Ba/F3-Phe116 cells selected in IL-9, and overexpressing JAK1, gave rise to autonomous cell lines after cytokine withdrawal. To see if JAK1 overexpression alone could explain this characteristic, we assessed the rate of autonomous lines after washing Ba/F3-Phe116 cells stably transfected with JAK1 and culturing them without cytokine. As shown in Table 1, Ba/F3-Phe116 cell clones ectopically overexpressing JAK1 gave rise to a significant number of autonomous cell lines, as compared to the absence of autonomous lines arising from control cells, transfected with an empty vector. The same results were obtained with three clones, and cumulative numbers of autonomous lines are represented in Table 1. Altogether, 77 autonomous lines were obtained from 150×10^6 JAK1-transfected cells tested, a rate that was comparable to the 35 cell lines obtained from 186×10^6 tested Ba/F3-Phe116/9 cells overexpressing JAK1 (Demoulin *et al.*, 2000). These observations confirm the hypothesis that both the better response to IL-9 and the generation of autonomous cell lines result from JAK1 overexpression.

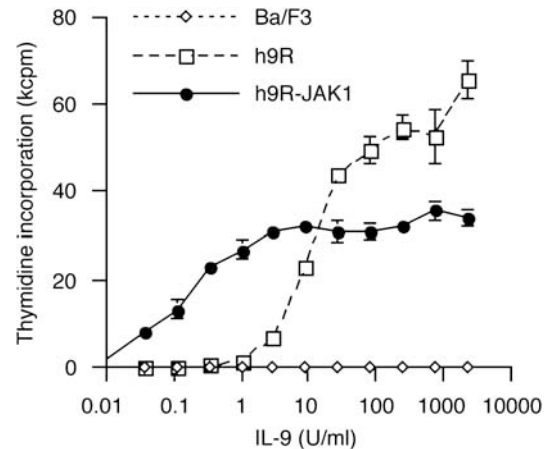


Figure 3 IL-9 response of Ba/F3-h9R cells after ectopic JAK1 overexpression. Ba/F3 cells, Ba/F3 cells expressing the hIL-9R (h9R) and transfected with an empty vector or Ba/F3-h9R cells overexpressing JAK1 (h9R-JAK1) were washed and cultured at 3000 cells/well in quadruplicates in 96-well culture plates with increasing concentrations of IL-9. After 48 h, ^3H -thymidine was added to the culture, and incorporated ^3H -thymidine was counted after 5 h.

To test whether the generation of autonomous cell lines might be dependent on the ectopic overexpression of the IL-9 receptor, we transfected wild-type Ba/F3 cells with JAK1. As shown in Table 1, JAK1 transfectants gave rise to autonomous cells, demonstrating that the phenomenon was IL-9R-independent.

To overcome the potential bias resulting from the utilization of transfected cell clones, we took advantage of bicistronic retroviral vectors, coding for both CD2 and JAK1, allowing to sort cell populations that overexpressed JAK1 based on CD2 expression. JAK1 expression level was similar to the level obtained in stably transfected clones (data not shown). As shown in Table 2, the overexpression of JAK1 by this method also gave rise to autonomous cell lines, while retroviral transduction of the gamma chain or of the IL-9 receptor, used as control, gave no autonomous cells.

JAK1-induced autonomous cell transformation is dependent on the kinase and FERM domains

Two domains of JAK1 are necessary for signal transduction. The kinase domain is responsible for the autophosphorylation, and for phosphorylation of the receptor and the STAT factors. The FERM domain was essentially shown to mediate receptor association (Kohlhuber *et al.*, 1997). To assess if the generation of autonomous Ba/F3-JAK1 cell lines depended on the integrity of those two domains, we took advantage of mutated forms of JAK1. To create a kinase-dead JAK1 (JAK1-KD), a point mutation in the conserved ATP-binding domain was introduced (K907A) that rendered JAK1 inactive (data not shown). A mutation in the FERM domain, Y107A, was shown to abolish the capacity of JAK1 to bind to gp130 (Haan *et al.*, 2001). As shown in Tables 1 and 2, Ba/F3 cells overexpressing

Table 1 Numbers of autonomous Ba/F3 lines obtained from Ba/F3 transfectants stably overexpressing the different JAK family members

Ba/F3 transfectants (number of clones tested)	Autonomous lines	Cells tested (million)	Frequency of autonomous lines/ 10 ⁸ cells tested
Phe116 JAK1 (3)	77	150	51.3
Phe116 mock	0	154	<0.7
JAK1 (4)	986	301	327.8
JAK1 KD (4)	2	200	1.0
JAK1 Y107A (4)	0	200	<0.5
JAK2 (6)	359	600	59.8
JAK3 (5)	9	250	3.6
TYK2 (4)	125	400	31.3
Mock	0	1206	<0.1

Abbreviation: KD, kinase death. Different Ba/F3 cell clones obtained after electroporation of constructions encoding the JAKs were grown in IL-3, washed and cultured at 50 000 cells/well, without cytokines, in 96-well plates. After 3 weeks, numbers of autonomous growing lines were counted. The number of clones tested per JAK family member is shown under brackets. Mock corresponds to the empty vector, for which bulk populations were used. *Y107A*: mutation in the FERM domain abolishing interaction with the receptor.

JAK1-KD gave 300-fold less autonomous cells than the wild-type JAK1. When the FERM domain was mutated, no autonomous cells could be obtained. This demonstrates that the generation of autonomous lines is dependent on the tyrosine kinase activity of JAK1 and on a functional FERM domain.

Overexpression of all JAK family members gives rise to autonomous Ba/F3 cell proliferation

We showed that JAK1 overexpression confers a growth advantage and leads to the autonomous transformation of Ba/F3 cells. To check if this observation could be extended to the other JAK family members, we ectopically overexpressed the other JAKs in Ba/F3 cells, using both clones obtained after electroporation (Table 1) and cell populations obtained after retroviral transduction (Table 2). Overexpression of TYK2 led to the autonomous proliferation of Ba/F3 in both experimental systems, demonstrating that high TYK2 levels can promote the autonomous growth of Ba/F3. For JAK2 and JAK3, we obtained significant numbers of autonomous clones (369 for JAK2 and 28 for JAK3, compared to none in control Ba/F3 cells). For JAK2, the difference between transfected and transduced cells can be explained by a different JAK2 expression level obtained with the two methods. As assessed by quantitative PCR, the transfected clones expressed from 11 to 49 times the level of JAK2 obtained for control cells, while transduced lines expressed from 7 to 9 times the endogenous level (data not shown). Nevertheless, these results indicate that overexpression of all JAK members could lead to the autonomous transformation of Ba/F3 cells.

Activated signaling pathways in autonomous clones

For Ba/F3-Phe116/9 cells, which were selected for growth in the presence of IL-9, progression toward

Table 2 Autonomous Ba/F3 lines obtained from Ba/F3 cells overexpressing JAKs by retroviral transduction

Ba/F3 population	Autonomous lines	Cells tested (million)	Frequency of autonomous lines/10 ⁸ cells tested
JAK1	380	174	218.4
JAK1 KD	1	155	0.6
JAK1 Y107A	0	79	<1.3
JAK2	10	245	4.1
JAK3	19	140	13.6
TYK2	> 1632	134	> 1217.9
Mock	0	252	<0.4

Abbreviation: KD, kinase death. Different Ba/F3 cell populations were grown in IL-3, washed and cultured at 50 000 cells/well, without cytokines, in 96-well plates. After 3 weeks, numbers of autonomous growing clones were counted. Negative control cells consist of Ba/F3 cells transduced with the wild-type h9R or γ c. *Y107A*: mutation in the FERM domain abolishing interaction with the receptor.

cytokine-independent tumorigenic clones was associated with a strong constitutive activation of STAT5 (Demoulin *et al.*, 2000). We therefore examined if autonomous cell lines obtained after ectopic JAK1 overexpression shared such a constitutive STAT5 activation. As shown in Figure 4, JAK1 overexpression by itself did not induce STAT5 phosphorylation, but JAK1-overexpressing autonomous cells (Ba/F3-JAK1A) showed a constitutive STAT5 activation, similar to the Ba/F3-A4 line. STAT3, in contrast, was not constitutively activated. We next tested the other signaling pathways activated by IL-3 in Ba/F3. Cytokine-independent growth was associated with a constitutive activation of Erk1–2 and Akt, mimicking to some extent the activation induced by IL-3. These results were reproduced on more than five JAK1 autonomous cell lines tested (data not shown), demonstrating that cytokine-independent progression after JAK1 overexpression is associated with the constitutive activation of the STAT5, MAP kinase and PI3kinase-PKB pathways.

We next analysed the activation of those pathways in autonomous Ba/F3 cells obtained after overexpression of the other JAK family members. As shown in Figure 5 by FACS analysis of cells labeled with anti-phospho-STAT and -Erk antibodies, all autonomous cell lines showed a constitutive activation of STAT5 and Erk1/2. This was confirmed by western blot, as for the constitutive activation of Akt in all autonomous cell lines (data not shown). STAT3 constitutive phosphorylation varied depending on the overexpressed JAK. Phosphorylated STAT3 was reproducibly observed in autonomous clones overexpressing TYK2. STAT3 was also weakly activated in autonomous clones obtained after JAK2 overexpression but not JAK1 or JAK3 overexpression (five autonomous clones tested for each JAK family member). Interestingly, autonomous Ba/F3 cells obtained by the overexpression of a constitutively phosphorylated fusion protein composed of STAT5, the transactivation domain of VP16 and the kinase domain of JAK2 (Demoulin *et al.*, 2000) showed no significant Erk1–2 phosphorylation.

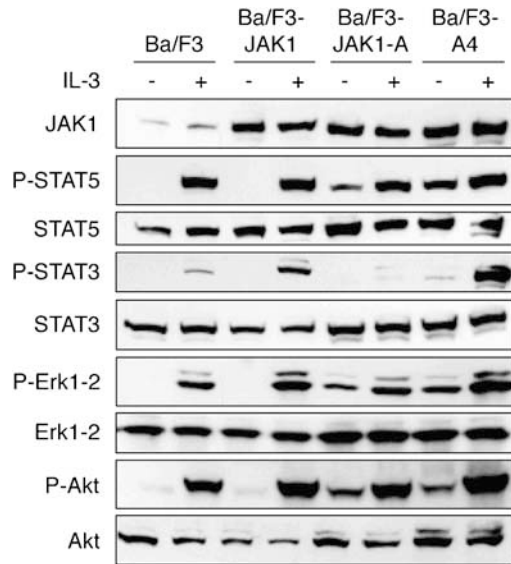


Figure 4 Activated signaling pathways in Ba/F3 cell lines overexpressing JAK1. Ba/F3 cells, Ba/F3 cells overexpressing JAK1 (Ba/F3-JAK1), autonomous cells obtained from Ba/F3-JAK1 (Ba/F3-JAK1 A) and the spontaneously obtained autonomous Ba/F3-A4 cell line were grown in IL-3, washed and starved for 4 h before stimulation with or without IL-3 for 15 min. Cellular extracts were prepared, and western blots were performed as described in the 'Materials and methods' section with anti-JAK1 and anti-phosphorylated and anti-total STAT5, STAT3, ERK1-2 and Akt.

JAK-overexpressing autonomous cells are dependent on JAK activity for survival

To check if the autonomous lines obtained after JAK overexpression were dependent on their JAK activity for survival, we treated three autonomous cell lines obtained after JAK1, 2 or 3 overexpression with a pan-JAK inhibitor. As a control, we used autonomous Ba/F3 cells obtained by the overexpression of Pim1. As shown in Figure 6, after 24 h, the JAK inhibitor killed many JAK-overexpressing autonomous cells, while Pim1 autonomous cells were totally resistant. Similar results were obtained for two additional autonomous JAK-overexpressing cell lines and with a JAK3 inhibitor. Taken together, these results show that autonomous JAK-overexpressing Ba/F3 cells have a constitutive activation of the JAK-STAT pathway and that this activation is necessary for the survival of the autonomous cells.

Discussion

Here, we show that spontaneous overexpression of wild-type JAK kinases promotes the transformation of Ba/F3 cells into cytokine-independent cell lines with constitutive STAT activation. Activation of JAK2 has been reported as the result of a translocation leading to a Tel-JAK2 fusion protein in a few cases of acute lymphoblastic leukemia (Lacronique *et al.*, 1997) and more frequently, as the result of the JAK2 V617F mutation in the majority of polycythemia vera patients and other

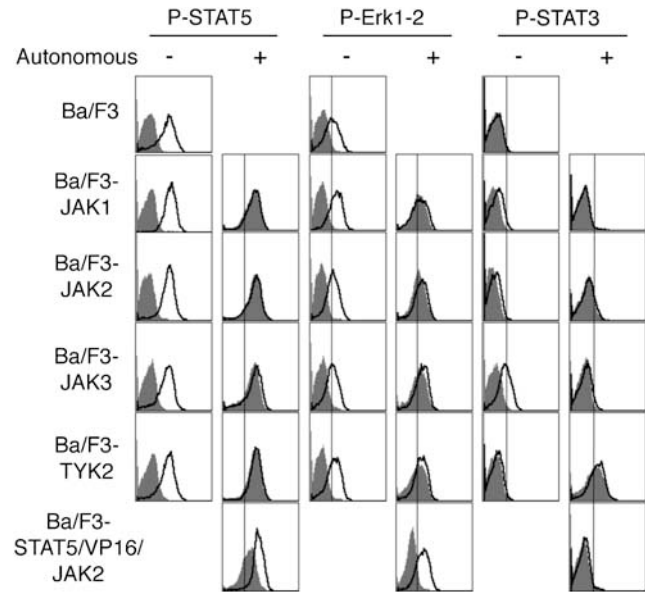


Figure 5 Activated signaling pathways in Ba/F3 cells overexpressing JAK1, JAK2, JAK3 or TYK2. Ba/F3 cells, Ba/F3 cells overexpressing the four JAK family members, autonomous Ba/F3 cell lines subsequently obtained and Ba/F3 cells transfected with the STAT5/VP16/JAK2 construction were grown in the presence of IL-3, washed and starved for 4 h. After 15 min of stimulation with or without IL-3, cells were fixed in paraformaldehyde, permeabilized with methanol and stained with Alexa fluor 647-anti phospho-STAT3, -STAT5 or -Erk1-2 (BD Biosciences). Cell fluorescence was assessed by flow cytometry. Gray histograms represent cells without stimulation, while black lines represent cells stimulated with IL-3.

MPDs (Baxter *et al.*, 2005; James *et al.*, 2005; Kralovics *et al.*, 2005; Levine *et al.*, 2005). However, numerous malignancies remain where the origin of constitutive STAT activation is not explained. Our observations point to a possible role of dysregulated activation of overexpressed non-mutated JAK kinases in constitutive STAT activation. In human disease, this hypothesis is difficult to demonstrate compared to a specific and recurrent mutation. Nevertheless, JAK2 overexpression was described in primary mediastinal large B-cell lymphoma and might explain the constitutive STAT6 activation (Guiter *et al.*, 2004). Moreover, amplification of the JAK2 gene was observed in Hodgkin's lymphoma (Joos *et al.*, 2000, 2003), were STAT3 and STAT5 are often phosphorylated (Kube *et al.*, 2001; Cochet *et al.*, 2006), and in some cases of acute myeloid leukemia (Rucker *et al.*, 2006).

Overexpression of JAKs is not sufficient to fully transform BaF3 cells, but a significant number of JAK-overexpressing cells can progress toward this stage, which can never be reached spontaneously by parental cells. This contrasts with the mutated JAK2 V617F, for which a majority of transfected BaF3 cells progress to cytokine-independent growth (James *et al.*, 2005; Lu *et al.*, 2005). In our system, autonomous transformation remains a rare event, which became detectable only when JAK-overexpressing cells were selected and expanded from the bulk parental Ba/F3 population.

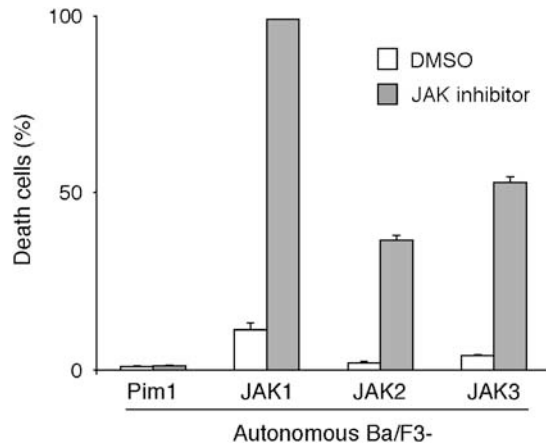


Figure 6 Survival of autonomous JAK-overexpressing Ba/F3 cells is dependent on JAK activity. Autonomous Ba/F3 cells obtained after Pim1 or JAK overexpression were treated with DMSO as a control, or a JAK inhibitor (556 nM). The number of dead cells was counted after 24 h by propidium iodide staining and FACS analysis. Mean and s.d. from triplicates are shown. This experiment is representative of a total of four experiments.

As a consequence, it is likely that progression to growth-factor independence after an isolated genetic event causing the overexpression of a JAK in a single cell *in vivo* would require that JAK-overexpressing cells have a selective advantage versus normal cells. Interestingly, as for JAK2 V617F and the response to EPO (James *et al.*, 2005), we show that JAK overexpression increases the responsiveness to cytokines, at least for IL-9. So, *in vivo*, JAK overexpression could provide a growth advantage and favor the expansion of JAK-overexpressing cells in the presence of limiting cytokine concentrations. After this first step of selection, progression to a cytokine-independent stage could occur as the result of a second genetic hit. A typical example of such *in vivo* progression is multiple myeloma. In this disease and in murine myeloma models, proliferation of malignant cells is initially IL-6-dependent. After cytokine withdrawal, like after the administration of IL-6-blocking antibodies, the growth of myeloma cells is decreased, but invariably some IL-6-independent clones arise and proliferate (Tripathi *et al.*, 2003). STAT3, the STAT factor activated by IL-6, was constitutively activated in such autonomous myeloma cells (Rawat *et al.*, 2000). Whether JAK overexpression is involved in this pathology is an important question that needs further investigation.

The mechanisms involved in the increased cytokine responsiveness could include increased receptor cell-surface expression upon JAK overexpression. This was demonstrated for JAK2 and Erythropoietin, as well as for JAK1 and Oncostatin M. JAK overexpression was associated with a better transport of the receptor to the cell surface (Huang *et al.*, 2001; Radtke *et al.*, 2002). For the Interferon receptor I, TYK2 was reported to increase cell-surface expression by inhibiting the endocytosis of the receptor (Ragimbeau *et al.*, 2003). However, the increased cytokine sensitivity could also be explained by other mechanisms. This was illustrated by studies of fusion proteins composed of dimeric-coiled

coil and the cytosolic part of the EPO receptor, which allow to identify active and inactive conformation of the receptor dimer. Overexpression of JAK2 allowed the activation of cell signaling with constructs in suboptimal conformation (Seubert *et al.*, 2003). Higher efficiency of signal transduction due to higher concentration of JAK molecules could also increase cytokine responsiveness.

The way JAK kinases autonomously activate signaling pathways remains elusive. The low level of autonomous lines arising from Ba/F3 cells transfected with JAK1-KD and the high toxicity of the JAK inhibitor on autonomous JAK-overexpressing cells indicate that JAK kinase activity is required for the phenomenon. Absence of autonomous lines arising from cells transfected with a FERM domain-mutated JAK1 suggests that JAKs need to associate to a receptor to mediate constitutive activation of the JAK-STAT pathway. However, the FERM domain was also described as necessary to maintain the integrity of the kinase domain (Zhou *et al.*, 2001). The requirement of receptor binding is therefore not formally demonstrated by this experiment. Moreover, autonomous progression was comparable in Ba/F3 cells overexpressing JAK1 transfected or not with the IL-9 receptor, which binds JAK1. To our knowledge, with the exception of interferon receptors, there are no JAK1-binding receptors expressed at the surface of Ba/F3 cells, but the presence of such receptors cannot be excluded. So, the need for receptor binding for JAK1 autonomous activity remains possible. The same question stands for JAK2 V617F. Lodish and colleagues demonstrated that transformation of Ba/F3 by the mutant JAK occurred only if an homodimeric type I receptor was co-expressed (Lu *et al.*, 2005), while for others this was not a prerequisite (James *et al.*, 2005). Whether constitutively active JAK kinases are free in the cytoplasm or need receptor binding to be active need further attention.

Whatever the mechanism responsible for its constitutive activation, STAT5 is likely responsible for the autonomous growth of Ba/F3 cells, since ectopic expression of constitutively activated STAT5 variants was sufficient for Ba/F3 growth (Demoulin *et al.*, 2000). However, in all autonomous clones tested, STAT5 activation was associated with constitutive activation of Erk1–2 and AKT, like for JAK2 V617F expressing autonomous Ba/F3 (Staerk *et al.*, 2005). STAT5, Erk1–2 and Akt are typically activated by IL-3. Inappropriate JAK activation could directly activate regulators of the MAP kinase and the PI3 kinase pathways, like IRS or SHP2. An alternative explanation is that phosphorylation of unknown receptors by active JAKs could mimic cytokine-induced signaling pathways. It is for instance possible that JAK1, in excess, phosphorylate the IL-3 receptor to induce an IL-3-signaling pathway. Another striking observation is the constitutive activation of STAT3, which was observed in all TYK2-overexpressing autonomous clones, sometimes in the JAK2- and never in the JAK1- or JAK3-overexpressing autonomous clones. This could represent unforeseen specificity of the different JAKs, or could reflect the binding to different receptors to induce constitutive activation of the signal.

Nevertheless, overexpression of non-mutated JAK or expression of JAK2 V617F (James *et al.*, 2005) is in itself not sufficient to lead to autonomous transformation of Ba/F3 cells. We were able to detect an IL-3-autocrine loop in a minority of our autonomous cell lines (data not shown), but it explains less than 10% of the transformation events. For the vast majority of the clones, unknown secondary genetic events are necessary for constitutive activation of the pathway. Such events could include a gain of kinase function allowing JAK-constitutive activation, or the loss of negative regulators of the JAK-STAT pathway. Whether overexpression of JAK kinase could increase the rate of novel activating mutations is speculative. Inactivation of phosphatases such as SHP1 or SHP2, which dephosphorylate JAKs and STATs, might explain JAK deregulation. Other hypotheses include inactivation of negative regulators of the JAK-STAT pathway, such as the SOCS proteins, that were shown to be transcriptionally repressed by hypermethylation in different types of cancer (He *et al.*, 2003; Nagai *et al.*, 2003). However, we did not detect any decrease in SOCS1, 2, 3 and CISH expression in the five autonomous clones tested (data not shown). We also looked for other potential transcriptional modifications by comparing parental and autonomous cells with Affymetrix microarrays. In the seven autonomous clones tested, there were no differentially expressed genes that could explain the transformation. Nevertheless, other experiments comparing non-autonomous cells overexpressing JAK1 and the derived autonomous cell lines could unravel molecular mechanisms involved in this JAK-STAT constitutive activation, and could be used as a tool to find candidate cofactors involved in the constitutive activation of wild-type or mutated JAKs such as JAK2 V617F.

Materials and methods

Cells and cell culture

Pro-B Ba/F3 cells were cultured in Dulbecco's modified Eagle's medium with fetal bovine serum (10%) and IL-3 (100 U ml⁻¹), which was produced by transfected CHO cells. The generation of Ba/F3 cells transfected with the hIL-9 receptor (Ba/F3-h9R) or with the mutated IL-9R (Ba/F3-Phe116) was described previously (Demoulin *et al.*, 2000). Recombinant human IL-9 was produced in the baculovirus system in our laboratory and purified as previously described (Druez *et al.*, 1990). The frequency of autonomous clones was assessed as previously described (Demoulin *et al.*, 2000). Transfected or control Ba/F3 cells were grown in the presence of IL-3, and cultured at 1.25×10^5 ml⁻¹ 24 h before the experiment. One day later, cells were washed twice with PBS pH 7.4 and 2% FCS, resuspended at 2.5×10^5 ml⁻¹ in culture medium without IL-3, and cultured in microtiter plates 5×10^4 washed cells per well. Plates were checked for proliferating clones during 3 weeks. Clones were subsequently cultured in the same conditions as those described for Ba/F3 cells, but without IL-3.

Plasmid construction, stable DNA transfections and analysis of transfected cells

Wild-type and mutated murine JAK1, murine JAK2, human JAK3 and human TYK2 cDNAs were inserted into the

pEFbos/puro plasmid (Demoulin *et al.*, 1996), which contains a resistance gene to puromycin. Ba/F3 cells were transfected by electroporation (300 V, 1200 μ F, 74 Ω) with 50 μ g of DNA and selected with puromycin (2.5 μ g ml⁻¹, Sigma, Bornem, Belgium). Bulk-transfected cultures and clones were used as specified in the experiments. Overexpression of JAKs was controlled by western blot. For retroviral expression, wild-type and mutated mJAK1, mJAK2, hJAK3 and hTYK2 cDNAs were subcloned into the pMX-IRES-CD2 or pMX-IRES-CD4 bicistronic retroviral vector upstream of the IRES (Liu *et al.*, 2000). Retroviral supernatants were generated by transient transfection of the BOSC packaging cell line with bicistronic vectors encoding the gene of interest and CD2 or CD4 as markers, and used for infection, as described (Liu *et al.*, 2000; Seubert *et al.*, 2003). Populations of cells expressing CD2 or CD4 above a predetermined level (top 25%) were isolated by FACS sorting.

Proliferation assays

A total of 3000 Ba/F3-transfected cells were seeded in 96-well plates, and stimulated by hIL-9 at different concentrations. After 48 h, tritiated thymidine was added to the cultures for 5 h. Cells were then collected on microfilter plates and thymidine incorporation was measured, after addition of liquid scintillant, with a Top Count microplate scintillation counter (Canberra-Packard, Meriden, CT, USA).

Viability assay

A total of 30 000 autonomous Ba/F3 cells were seeded in 96-well plates with JAK inhibitor I (Calbiochem, La Jolla, CA, USA; 556 nM), JAK3 inhibitor II, JAK3 inhibitor IV or DMSO alone. After 24 h, cells were incubated for 20 min in propidium iodide (60 μ g ml⁻¹) at room temperature before FACS analysis with a BD FACSCalibur flow cytometer. Under these conditions dead cells are brightly stained while live cells are not. A total of 5000 cells were counted per sample.

Western blots

Ba/F3 cells were washed and placed for 4 h in medium without IL-3 before stimulation. A total of 10^6 cells were left unstimulated or stimulated with IL-3 (100 U ml⁻¹) for 15 min, lysed in 250 μ l of Laemmli buffer (BioRad, Hercules, CA, USA) and boiled for 3 min before loading 25 μ l on 8% pre-cast sodium dodecyl sulfate-PAGE polyacrylamide gels (Invitrogen, Merelbeke, Belgium) and electrophoretical transfer to nitrocellulose membranes (Hybond-C; Amersham, Buckingham, England). Membranes were then blocked in 5% non-fat dry milk, washed and probed using antibodies specific for JAK1, JAK3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), JAK2 (Upstate Biotechnology, Lake Placid, NY, USA), TYK2 (Biosource, Camarillo, CA, USA) or phosphorylated STAT3-Y705, STAT5-Y694, AKT-S473, ERK1-2-T202/Y204 (Cell Signaling, Beverly, MA, USA). Blots were reprobbed with anti- β -actin antibodies (Sigma, Bornem Belgium), anti-STAT3 or anti-STAT5 monoclonal antibodies (Transduction Laboratories, Lexington, KY, USA), as a control. A Super-Signal West Pico detection kit (Pierce, Rockford, IL, USA) was used for detection.

FACS analysis

Ba/F3 transfectants were cultured in the presence of IL-3, washed and starved for 4 h. A total of 2×10^5 cells were left unstimulated or stimulated with IL-3 (100 U ml⁻¹) for 15 min at 37 °C. Cells were fixed in 2% paraformaldehyde in PBS 10 min at 37 °C and then permeabilized in methanol 90%, 30 min on ice. Cells were washed twice with Hank's medium

and stained with anti-phosphorylated STAT3-Y705, STAT5-Y694 and ERK1-2-T202/Y204 (BD Pharmingen, San Jose, CA, USA). Cells were analysed on a BD FACSCalibur flow cytometer.

Microarray

Total RNA was extracted from autonomous growing Ba/F3-A6 (Demoulin *et al.*, 2000) and Ba/F3-M-Ras 71K (Louahed *et al.*, 1999), using the Trizol method as recommended by the manufacturer (Life Technologies, Gent, Belgium). The RNA quality was checked by agarose gel electrophoresis. Total RNA (50 µg) was labeled in reverse transcription reactions (Superscript II kit, Invitrogen) with dCTP-Cy5 for one Ba/F3 cell line and dCTP-Cy3 for the other (Amersham, Buckinghamshire, UK). In every second replicate experiment, the fluorescent deoxynucleotides were swapped. cDNA probes were purified with AutoSeq G-50 columns (Amersham, Buckinghamshire, UK) following the manufacturer's instructions. cDNA labeled with Cy3 and Cy5 were mixed per pair, precipitated and resuspended in hybridization buffer (5 × SSC, 6 × Denhardt's solution, 60 mM Tris-HCl pH7.6, 0.12% sarkosyl, 48% formamide). Hybridization to cDNA microarray chips (mver1.1.1) from the Sanger Institute/LICR/CRUK consortium was carried at 47 °C for 16 h. These arrays contained about 15 000 cDNA, with up to 50% derived from novel genes. Hybridizations were done in quadruplicate for each condition. After washing, in 2 × SSC, 0.1 × SSC and 0.1% sodium dodecyl sulfate, and finally in 0.1 × SSC, chips were scanned in a Scannarray Express HT (Packard Bioscience, PerkinElmer, Wellesley, MA, USA) and spot intensities were measured using the QuantArray software (histogram method with background subtraction). Normalization and statistical analysis of the quadruplicate data sets were performed using GeneSpring analysis software (Silicon Genetics). A Lowess non-linear normalization was applied and the median of the

ratio distribution for each array was set to 1. For each gene, expression level in Ba/F3-A6 was compared to the level in Ba/F3-M-Ras 71K cells, used as control (Louahed *et al.*, 1999).

Reverse transcription-quantitative PCR

Total RNA was extracted from 5 × 10⁶ cells using the TriPure isolation reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Reverse transcription was performed on 2 µg of total RNA with an oligo-(dT) primer (Roche) and M-MLV RT (Invitrogen). Quantitative PCR was performed using primers sets corresponding to murine JAK2 with qPCR Mastermix for SYBR Green I (Eurogentec, Seraing, Belgium). The sequences of the primers (final concentration: 300 nM) were: 5'-CAGCAGCTTGGCAAAGG TAACTTC-3' (forward) and 5'-TCAGTGCTGTGCTGGAG TTTCTTC-3' (reverse). Samples were first heated 2 min at 50 °C then 10 min at 95 °C. cDNA was amplified as follows: 40 cycles of a two-step PCR program at 95 °C for 15 s and 60 °C for 1 min. Melting point analysis was conducted by heating the amplicon from 60 to 95 °C. For β-actin, similar cycling conditions were used with the addition of an internal TaqMan oligonucleotide probe. Primers for β-actin were as follows: 5'-TCCTGAGCGCAAGTACTCTGT-3' (forward), 5'-CTGATCCACATCTGCTGGAAG-3' (reverse) and 5'-ATCGGTGGCTCCATCCTGGC-3' (Taqman probe).

Acknowledgements

This work was supported in part by the Belgian Federal Service for Scientific, Technical, and Cultural Affairs, by the Actions de Recherche Concertées of the Communauté Française de Belgique and the operation Télévie. LK is a postdoctoral researcher and SC a research associate with the Fonds National de la Recherche Scientifique, Belgium.

References

- Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S *et al.* (2005). Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet* **365**: 1054–1061.
- Bowman T, Garcia R, Turkson J, Jove R. (2000). STATs in oncogenesis. *Oncogene* **19**: 2474–2488.
- Bromberg J. (2002). STAT proteins and oncogenesis. *J Clin Invest* **109**: 1139–1142.
- Cochet O, Frelin C, Peyron JF, Imbert V. (2006). Constitutive activation of STAT proteins in the HDLM-2 and L540 Hodgkin lymphoma-derived cell lines supports cell survival. *Cell Signal* **18**: 449–455.
- Demoulin JB, Uyttenhove C, Lejeune D, Mui A, Groner B, Renaud JC. (2000). STAT5 activation is required for interleukin-9-dependent growth and transformation of lymphoid cells. *Cancer Res* **60**: 3971–3977.
- Demoulin JB, Uyttenhove C, Van Roost E, DeLestre B, Donckers D, Van Snick J *et al.* (1996). A single tyrosine of the interleukin-9 (IL-9) receptor is required for STAT activation, antiapoptotic activity, and growth regulation by IL-9. *Mol Cell Biol* **16**: 4710–4716.
- Druez C, Coulie P, Uyttenhove C, Van Snick J. (1990). Functional and biochemical characterization of mouse P40/IL-9 receptors. *J Immunol* **145**: 2494–2499.
- Fischer M, Bijman M, Molin D, Cormont F, Uyttenhove C, Van Snick J *et al.* (2003). Increased serum levels of interleukin-9 correlate to negative prognostic factors in Hodgkin's lymphoma. *Leukemia* **17**: 2513–2516.
- Gutier C, Dusanter-Fourt I, Copie-Bergman C, Boulland ML, Le Gouvello S, Gaulard P *et al.* (2004). Constitutive STAT6 activation in primary mediastinal large B-cell lymphoma. *Blood* **104**: 543–549.
- Haan C, Is'harc H, Hermanns HM, Schmitz-Van De Leur H, Kerr IM, Heinrich PC *et al.* (2001). Mapping of a region within the N terminus of JAK1 involved in cytokine receptor interaction. *J Biol Chem* **276**: 37451–37458.
- He B, You L, Uematsu K, Zang K, Xu Z, Lee AY *et al.* (2003). SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. *Proc Natl Acad Sci USA* **100**: 14133–14138.
- Huang LJ, Constantinescu SN, Lodish HF. (2001). The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor. *Mol Cell* **8**: 1327–1338.
- James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C *et al.* (2005). A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature* **434**: 1144–1148.
- Joos S, Granzow M, Holtgreve-Grez H, Siebert R, Harder L, Martin-Subero JI *et al.* (2003). Hodgkin's lymphoma cell lines are characterized by frequent aberrations on chromosomes 2p and 9p including REL and JAK2. *Int J Cancer* **103**: 489–495.
- Joos S, Kupper M, Ohl S, von Bonin F, Mechttersheimer G, Bentz M *et al.* (2000). Genomic imbalances including amplification of the tyrosine kinase gene JAK2 in CD30+ Hodgkin cells. *Cancer Res* **60**: 549–552.
- Kelleher K, Bean K, Clark SC, Leung WY, Yang-Feng TL, Chen JW *et al.* (1991). Human interleukin-9: genomic sequence, chromosomal location, and sequences essential for its expression in human T-cell

- leukemia virus (HTLV)-I-transformed human T cells. *Blood* **77**: 1436–1441.
- Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. (2002). Signaling through the JAK/STAT pathway, recent advances and future challenges. *GENE* **285**: 1–24.
- Knoops L, Renauld JC. (2004). IL-9 and its receptor: from signal transduction to tumorigenesis. *Growth Factors* **22**: 207–215.
- Kohlhuber F, Rogers NC, Watling D, Feng J, Guschin D, Briscoe J *et al.* (1997). A JAK1/JAK2 chimera can sustain alpha and gamma interferon responses. *Mol Cell Biol* **17**: 695–706.
- Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR *et al.* (2005). A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med* **352**: 1779–1790.
- Kube D, Holtick U, Vockerodt M, Ahmadi T, Haier B, Behrmann I *et al.* (2001). STAT3 is constitutively activated in Hodgkin cell lines. *Blood* **98**: 762–770.
- Lacronique V, Boureux A, Valle VD, Poirel H, Quang CT, Mauchauffe M *et al.* (1997). A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. *Science* **278**: 1309–1312.
- Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ *et al.* (2005). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* **7**: 387–397.
- Liu X, Constantinescu SN, Sun Y, Bogan JS, Hirsch D, Weinberg RA *et al.* (2000). Generation of mammalian cells stably expressing multiple genes at predetermined levels. *Anal Biochem* **280**: 20–28.
- Louahed J, Grasso L, De Smet C, Van Roost E, Wildmann C, Nicolaides NC *et al.* (1999). Interleukin-9-induced expression of M-Ras/R-Ras3 oncogene in T-helper clones. *Blood* **94**: 1701–1710.
- Lu X, Levine R, Tong W, Wernig G, Pikman Y, Zarnegar S *et al.* (2005). Expression of a homodimeric type I cytokine receptor is required for JAK2V617F-mediated transformation. *Proc Natl Acad Sci USA* **102**: 18962–18967.
- Merz H, Houssiau FA, Orscheschek K, Renauld JC, Flidner A, Herin M *et al.* (1991). Interleukin-9 expression in human malignant lymphomas: unique association with Hodgkin's disease and large cell anaplastic lymphoma. *Blood* **78**: 1311–1317.
- Nagai H, Naka T, Terada Y, Komazaki T, Yabe A, Jin E *et al.* (2003). Hypermethylation associated with inactivation of the SOCS-1 gene, a JAK/STAT inhibitor, in human hepatoblastomas. *J Hum Genet* **48**: 65–69.
- Palacios R, Steinmetz M. (1985). IL-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes *in vivo*. *Cell* **41**: 727–734.
- Radtke S, Hermanns HM, Haan C, Schmitz-Van De Leur H, Gascan H, Heinrich PC *et al.* (2002). Novel role of Janus kinase 1 in the regulation of oncostatin M receptor surface expression. *J Biol Chem* **277**: 11297–11305.
- Ragimbeau J, Dondi E, Alcover A, Eid P, Uze G, Pellegrini S. (2003). The tyrosine kinase Tyk2 controls IFNAR1 cell surface expression. *EMBO J* **22**: 537–547.
- Rawat R, Rainey GJ, Thompson CD, Frazier-Jessen MR, Brown RT, Nordan RP. (2000). Constitutive activation of STAT3 is associated with the acquisition of an interleukin 6-independent phenotype by murine plasmacytomas and hybridomas. *Blood* **96**: 3514–3521.
- Renauld JC, Kermouni A, Vink A, Louahed J, Van Snick J. (1995). Interleukin-9 and its receptor: involvement in mast cell differentiation and T cell oncogenesis. *J Leukoc Biol* **57**: 353–360.
- Renauld JC, van der Lugt N, Vink A, van Roon M, Godfraind C, Warnier G *et al.* (1994). Thymic lymphomas in interleukin 9 transgenic mice. *Oncogene* **9**: 1327–1332.
- Renauld JC, Van Snick J. (2003) In: Thompson A (ed). *The Cytokine Handbook*. Academic Press: London, , pp 347–358.
- Rucker FG, Bullinger L, Schwaenen C, Lipka DB, Wessendorf S, Frohling S *et al.* (2006). Disclosure of candidate genes in acute myeloid leukemia with complex karyotypes using microarray-based molecular characterization. *J Clin Oncol* **24**: 3887–3894.
- Seubert N, Royer Y, Staerk J, Kubatzky KF, Moucadet V, Krishnakumar S *et al.* (2003). Active and inactive orientations of the transmembrane and cytosolic domains of the erythropoietin receptor dimer. *Mol Cell* **12**: 1239–1250.
- Staerk J, Kallin A, Demoulin JB, Vainchenker W, Constantinescu SN. (2005). JAK1 and Tyk2 activation by the homologous polycythemia vera JAK2 V617F mutation: cross-talk with IGF1 receptor. *J Biol Chem* **280**: 41893–41899.
- Trikha M, Corringham R, Klein B, Rossi JF. (2003). Targeted anti-interleukin-6 monoclonal antibody therapy for cancer: a review of the rationale and clinical evidence. *Clin Cancer Res* **9**: 4653–4665.
- Uyttenhove C, Druetz C, Renauld JC, Herin M, Noel H, Van Snick J. (1991). Autonomous growth and tumorigenicity induced by P40/interleukin 9 cDNA transfection of a mouse P40-dependent T cell line. *J Exp Med* **173**: 519–522.
- Zhou YJ, Chen M, Cusack NA, Kimmel LH, Magnuson KS, Boyd JG *et al.* (2001). Unexpected effects of FERM domain mutations on catalytic activity of JAK3: structural implication for Janus kinases. *Mol Cell* **8**: 959–969.

Ligand-independent Homomeric and Heteromeric Complexes between Interleukin-2 or -9 Receptor Subunits and the γ Chain*

Received for publication, April 23, 2008, and in revised form, September 18, 2008 Published, JBC Papers in Press, October 1, 2008, DOI 10.1074/jbc.M803125200

Yaniv Malka[‡], Tekla Hornakova^{§1,2}, Yohan Royer^{§1,3}, Laurent Knoops[§], Jean-Christophe Renaud[§], Stefan N. Constantinescu^{§4}, and Yoav I. Henis^{‡5}

From the [‡]Department of Neurobiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel and the

[§]Ludwig Institute for Cancer Research and de Duve Institute, Université Catholique de Louvain, Brussels B-1200, Belgium

Signaling via interleukin-2 (IL-2) and interleukin-9 receptors (IL-2R and IL-9R) involves heteromeric interactions between specific interleukin receptor subunits, which bind Janus kinase 1 (JAK1) and the JAK3 binding common γ chain (γ_c). The potential existence and roles of homomeric and heteromeric complexes before ligand binding and their modulation by ligand and JAK3 are unclear. Using computerized antibody-mediated immunofluorescence co-patching of epitope-tagged receptors at the surface of live cells, we demonstrate that IL-2R β , IL-9R α , and γ_c each display a significant fraction of ligand-independent homomeric complexes (24–28% co-patching), whereas control co-patching levels with unrelated receptors are very low (7%). Heteromeric complex formation of IL-2R β or IL-9R α with γ_c is also observed in the absence of ligand (15–30%). Ligand binding increases this hetero-oligomerization 2-fold but does not affect homo-oligomerization. Co-expression of IL-2R α does not affect the hetero-oligomerization of IL-2R β and γ_c . Recruitment of γ_c into heterocomplexes is partly at the expense of its homo-oligomerization, suggesting that a functional role of the latter may be to keep the receptors inactive in the absence of ligand. At the same time, the preformed complexes between γ_c and IL-2R β or IL-9R α promote signaling by the JAK3 A572V mutant without ligand, supporting a pathophysiological role for the constitutive oligomerization in triggering ligand-independent activation of JAK3 (and perhaps other JAK mutants) mutants identified in several human cancers.

Interleukin-2 and -9 (IL-2 and IL-9)⁶ signaling pathways and receptors (IL-2R and IL-9R) are of high medical relevance in view of their important roles in the immune response and the frequent involvement of their loss or mutation in immunodeficiency and pathological autoimmune conditions (1–5). IL-2 is critically involved in regulating T cell proliferation (6). Loss of IL-2 (3), IL-2R α (4), IL-2R β (5), or of the STAT5 transcription factor (2, 7) results in autoimmune diseases due to ineffective induction of anergy in peripheral T cells. IL-9 is known to induce proliferation and differentiation of mast cells as well as stimulation of murine T cell lymphomas (1). It also stimulates the proliferation of the B1 subset of B lymphocytes and of erythroid progenitors (8) and has been implicated in the induction of certain forms of asthma (9).

IL-2 and IL-9 act through binding to specific cell-surface receptors. The high affinity IL-2R is comprised of three separate chains, termed α (CD25), β (CD122), and γ (γ_c , CD132), which is a Janus tyrosine kinase 3 (JAK3)-interacting chain common to many cytokine receptors, including IL-2 and IL-9 receptors (10–15). IL-2R α by itself has only low affinity to IL-2 and is not directly involved in signal transduction (16). On the other hand, in the absence of IL-2R α , the IL-2R β/γ_c heterocomplex is sufficient to support IL-2 binding and signaling (17, 18). We, therefore, focused on the interactions of the latter subunits. In contrast, the IL-9R has only one ligand binding chain (designated IL-9R α), which interacts with γ_c for signaling via activation of JAK3 (8, 19–21). Thus, the lack of a “modulatory” chain equivalent to IL-2R α makes it interesting to compare the interactions between the subunits of the IL-9R and those of the IL-2R system.

Complex formation among the subunits of these receptors is an essential feature of their signaling, as they function as heteromeric complexes between the ligand binding receptor chain and the common γ_c that recruits JAK3 to the complex (20–22). Early fluorescence resonance energy transfer-based studies were conducted on IL-2R but not on IL-9R. In that study (23) the existence of preassembled heterocomplexes between α/β ,

* This research was supported in part by a grant from the Israeli Ministry of Health Chief Scientist's Office (to Y. I. H.). This work was also supported by the Fonds National de la Recherche Scientifique (Belgium), the Salus Sanguinis Foundation, the Action de Recherche Concertée MEXP31C1 (Université Catholique de Louvain), the Fondation Contre le Cancer (Brussels), PAI Program BCHM61B5 (Belgium), and the Atlantic Philanthropies, New York (to S. N. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ These authors were equal contributors.

² Supported by Télévie.

³ Supported by a Mandat d'Aspirant of the Fonds National de la Recherche Scientifique, Belgium.

⁴ A Research Associate of the Fonds National de la Recherche Scientifique, Belgium. To whom correspondence may be addressed: Ludwig Institute for Cancer Research, Brussels B-1200, Belgium. Tel.: 32-2-764-7540; Fax: 32-2-764-6566; E-mail: stefan.constantinescu@bru.lir.org.

⁵ An incumbent of the Zalman Weinberg Chair in Cell Biology. To whom correspondence may be addressed. Tel.: 972-3-640-9053; Fax: 972-3-640-7643; E-mail: henis@post.tau.ac.il.

⁶ The abbreviations used are: IL, interleukin; IL-2R, IL-2 receptor; HA, influenza hemagglutinin; HBSS, Hanks' balanced salt solution; γ_c , γ chain; JAK, Janus tyrosine kinase; TpoR, thrombopoietin receptor; T β RIL, type II transforming growth factor- β receptor; LHRE, lactogenic hormone response element; IRES, internal ribosome entry site; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; BSA, bovine serum albumin; STAT, signal transducers and activators of transcription; MESF, molecules of equivalent soluble fluorochrome; JAK1, Janus kinase 1.

β/γ_c , or α/γ_c of the IL-2R, which were modulated by ligand binding, was reported. However, the experiments were conducted mostly on cells expressing a high excess of IL-2R α over the other subunits and did not explore homomeric complexes. Recent crystallographic studies on the quaternary structure of the soluble ectodomains of IL-2R subunits suggested that IL-2 first binds to IL-2R α , enhancing IL-2 binding to IL-2R β , followed by recruitment of γ_c to the IL-2/IL-2R β complex (14, 15).

Despite the functional importance of IL-2R and IL-9R oligomerization, many aspects of the interaction between the subunits comprising these receptors and their potential modulation by ligand binding and/or JAK1 and JAK3 are still unclear, especially in their native milieu (the plasma membrane of live cells). We tackled these questions by combining computerized immunofluorescence co-patching (24, 25) to quantify both homomeric and heteromeric complex formation of IL-2R and IL-9R subunits with signaling assays in live cells. Our data demonstrate that the signaling subunits of both receptors (IL-2R β , IL-9R α , and γ_c) display a subpopulation of preassembled homomeric complexes, which is not altered by ligand. On the other hand, hetero-oligomerization of both IL-2R β and IL-9R α with γ_c existed before ligand binding but was significantly augmented by the relevant ligands, and IL-2R β/γ_c complex formation was insensitive to co-expression of IL-2R. The hetero-oligomerization of IL-2 and IL-9 receptor subunits with γ_c , even in the presence of JAK3, did not result in activation unless ligand was present, suggesting that oligomerization *per se* does not suffice for activation. However, we hypothesized that such preformed complexes could serve as scaffolds for activation of mutated JAK proteins, such as those recently described for JAK1 (26) and JAK3 (27). Indeed, we show that preformed complexes of IL-2R β or IL-9R α and γ_c support signaling by the constitutively active JAK3 A572V.

EXPERIMENTAL PROCEDURES

Cell Culture—BOSC23 cells (derived from HEK293T cells and expressing the gag, pol, and env proteins of the Moloney leukemia ecotropic virus (28)) and HEK293 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Biological Industries Beit Haemek or Invitrogen) as described (29, 30). Ba/F3 cells (IL-3-dependent mouse pro-B cells) were maintained and transduced as described (31). BETT cells (human T-ALL cells) were maintained in RPMI medium containing 10% fetal calf serum.

Plasmids and Recombinant Virus Vectors—The human IL-2R β , IL-9R α , and γ_c cDNAs were cloned in the pMX-IRES-GFP1.1 retroviral vector (32). The cDNAs encoding these proteins were cloned upstream of the internal ribosome entry site (IRES) followed by a GFP construct, such that transfection can be monitored by low level expression (at a level that does not interfere with regular immunofluorescence studies) of GFP (32, 33). Each construct was tagged extracellularly with either the 12CA5 influenza hemagglutinin (HA) tag (YPYDVPDYA) or the 9E10 Myc tag (EQKLISEEDL), with tags being introduced downstream of the predicted signal sequence cleavage site as described earlier (30). Thus, in IL-2R β the tags were inserted between residues Thr-31 and Ser-32, in IL-9R α between residues Gln-26 and Gly-27 (designating the Met followed immediately

by the signal sequence as 1, where the actual translation starts), and in γ_c between residues Ile-27—Leu-28. IL-2R α cDNA (untagged) and human wild-type or JAK3 A572V mutant cDNAs were subcloned in pREX-IRES-CD4 (34). FLAG-tagged murine thrombopoietin receptor (TpoR) containing the IL-7R signal sequence (35) was cloned in pMX-IRES-GFP1.1 (30). Myc-tagged type II transforming growth factor- β (T β R2) in pcDNA3 was described (36).

Generation of Stable Cell Lines—High titer replication-defective retroviral supernatants were generated from BOSC23 cells (serving as a packaging cell line) after transfection with the retroviral bicistronic vectors encoding the constructs mentioned above as described (32). The viruses thus produced were used to transduce Ba/F3 cells by centrifugation in the presence of 4 μ g/ml Polybrene (Sigma). The efficiency of infection was usually 40–50%. Populations of cells expressing the GFP marker above a predetermined level (top 10%) were isolated by FACS sorting.

Immunofluorescence Co-patching—BOSC23 cells were plated on glass coverslips in 6-well dishes (2×10^5 cells/dish). After 24 h, they were co-transfected with the different epitope-tagged IL-R subunits at various combinations using 1 μ g of DNA of each construct complemented to 6 μ g by empty vector. In some cases expression vectors for untagged IL-9R α , IL-2R β , or IL-2R α were also included (see Fig. 3 legend). Experiments were performed 44–48 h after transfection with calcium phosphate (Sigma). To measure receptor oligomerization, we employed antibody-mediated immunofluorescence co-patching (24, 37). Cells expressing pairs of receptors carrying different epitope tags (e.g. Myc-IL-9R α together with HA-IL-9R α) were washed twice with serum-free Dulbecco's modified Eagle's medium and incubated 30 min at 37 °C to allow digestion of serum-derived ligands. After washing twice with cold Hanks' balanced salt solution (HBSS) supplemented with 20 mM Hepes (pH 7.4) and 2% BSA (HBSS/Hepes/BSA; all from Sigma), the cells were incubated with normal goat γ globulin (Jackson ImmunoResearch Laboratories; 200 μ g/ml, 30 min, 4 °C) to block nonspecific binding. This was followed by successive incubation in the cold (to avoid internalization) in HBSS/Hepes/BSA with primary anti-tag IgGs (20 μ g/ml each, 45 min); that is, rabbit HA.11 against the HA tag (anti-HA) together with 9E10 mouse anti-Myc (both from Covance Research Products) followed by labeling/patching with secondary Alexa594-goat anti-rabbit and Alexa488-goat anti-mouse IgG (Invitrogen-Molecular Probes; 20 μ g/ml each, 30 min, 4 °C). In some control experiments using a FLAG-tagged construct, mouse anti-FLAG IgG (M2; Sigma) was employed. The cells were washed and fixed in methanol (5 min, –20 °C) and acetone (3 min, –20 °C) and mounted in Prolong Antifade (Invitrogen-Molecular Probes). In experiments where ligands were present, human IL-9 (750 units/ml; produced in baculovirus as described in Renauld *et al.* (38)) or IL-2 (500 units/ml; Sigma) was added at 4 °C 30 min before labeling with IgGs and maintained during all subsequent incubations. For Ba/F3 cells, a similar protocol was employed, except that incubations with IgGs were carried out on cells in 100 μ l of suspension. For washing, Ba/F3 cells were centrifuged for 1 min and resuspended in 100 μ l of HBSS/Hepes/BSA. After antibody labeling,

the Ba/F3 cells were mounted onto poly-L-lysine-coated coverslips using cytospin centrifuge (5 min, medium speed) as described (34) followed by fixation in methanol and acetone.

Fluorescence digital images were recorded using a CCD camera as described (25). The Alexa488 (green) and Alexa594 (red) images were exported in TIFF format to Image-Pro Plus (Media Cybernetics) and subjected to quantitative analysis of the extent of co-patching using an algorithm we have recently developed (25). Briefly, the program segments the patches in a user-defined region of interest, subtracts the background, and identifies the center of each object in the green and red images. This is followed by nearest-neighbor analysis, calculating the distances from the center of green patch to that of the nearest red patch (and vice versa). Patches whose nearest neighbor is within one optical resolution unit (up to 0.2 μm) are considered colocalized (25). The % co-patching of e.g. green with red patches is given by dividing the number of the green patches colocalized with red patches by the total number of green patches. 20–25 cells were analyzed in each case.

Dual-luciferase Reporter Assay—HEK293 cells grown in 24-well plates were transfected using Lipofectamine 2000 (Invitrogen) with vectors encoding the IL-R ligand binding subunits and γ_c as well as wild-type or mutant JAK3 A572V where indicated (200 ng DNA/well each vector, replacing JAK3 vector with empty vector in controls). To test for STAT transcriptional activation, the STAT3-responsive or STAT5-responsive luciferase constructs (250 ng/well) were co-transfected into the cells together with 100 ng/well of a control vector (pRL-TK, expressing constitutively active Renilla luciferase; Promega) to calibrate for transfection efficiency. STAT5-mediated transcription was evaluated with the pLHRE-luc reporter gene constructs harboring tandem copies of the STAT5-inducible lactogenic hormone response element (LHRE) of the rat β -casein gene promoter, inserted upstream a luciferase gene (39). STAT3-mediated transcription was evaluated with pGL3-pap1-luc plasmid containing the luciferase gene under the control of the STAT3-inducible rat *Pap1* (pancreatitis-associated protein-1) promoter (40). After 4 h the cells were stimulated with 100 units/ml IL-2 or 100 units/ml IL-9 for 20 h and lysed with passive lysis buffer as described (31). Firefly (STAT reporter) and Renilla (control vector) luciferase activities were measured with the dual-luciferase reporter kit (Promega). To normalize for transfection efficiency, the results of firefly luciferase activity were divided by those of the respective Renilla luciferase activity.

FACS Analysis—BETT (T-ALL) cells or Ba/F3 cells expressing HA-tagged human IL-2R β and Myc-tagged human γ_c were washed in phosphate-buffered saline containing 0.1% fetal calf serum. 3×10^5 cells were stained by monoclonal anti-human IL-2R β (R&D systems) or anti-human γ_c (BD Biosciences) antibody coupled with phycoerythrin, according to manufacturer's recommendations (1 h, 4 °C). These antibodies detect both untagged endogenous receptor subunits and transduced tagged receptors and, thus, provide a means to compare cell surface levels. After washing by phosphate-buffered saline (PBS), cells were resuspended in 200 μl of PBS with 0.1% fetal calf serum, and cell surface expression of human IL-2R β and human γ_c was analyzed on 10^4 cells by a BD FACSCaliburTM flow cytometer

(BD Biosciences). An aliquot of non-stained cells was used as autofluorescence control. We employed quantitative fluorescence cytometry to measure absolute levels of receptors at the cell surface expressed as molecules of equivalent soluble fluorochrome (MESF values). To the obtained MESF values Sphero Rainbow Calibration particles were used to calibrate the flow cytometer scale. The mean/median relative fluorescence intensity channel value for each bead population was calculated using Cell Quest software. A calibration curve was constructed, and subsequent data from the analyzed samples obtained from mean/median relative fluorescence intensity were converted to MESF for each antibody. All data were corrected for autofluorescence quantified in the same way.

RESULTS

Cell-surface IL-2R β , IL-9R α , and γ_c Form Ligand-independent Homomeric Complexes—In view of the proposed importance of the oligomeric state of IL-2 and IL-9 receptors for their activation, we conducted experiments to determine whether the ligand binding chains of these receptors (IL-2R β and IL-9R α) form stable homo-oligomers before ligand binding or only in the presence of ligand (ligand-induced oligomerization). To detect the formation of homomeric complexes of the intact receptor chains in their natural environment (the plasma membrane of live cells), we employed a computerized digital analysis version of immunofluorescence co-patching (Ref. 25; see "Experimental Procedures"). This method, which we have formerly used to demonstrate homo- and hetero-oligomerization of several transmembrane receptors (24, 30, 41), is based on the expression at the surface of live cells of two receptors tagged with different epitope tags at their extracellular termini. One receptor is forced into micropatches by a double layer of bivalent IgGs using a fluorescent (e.g. green) secondary IgG. The co-expressed receptor, which carries a different epitope tag, is patched/labeled by anti-tag primary IgG from another species and secondary antibodies coupled to another fluorophore (red). Receptors in mutual oligomers are swept into mutual micropatches, which appear yellow when the two images are overlapped. To measure the homo-oligomerization of IL-2R β , IL-9R α , or γ_c , we co-expressed Myc and HA-tagged versions of each protein in BOSC23 cells and subjected them to immunofluorescence co-patching studies in the absence or presence of ligand. Co-patching was determined by algorithms written for Image-Pro Plus, defining green and red patches as overlapping if their intensity peaks were separated by less than 0.2 μm (25). To evaluate the level of random co-patching (uncorrelated overlap of patches) in the same image (which depends on the surface density of the patches), a "randomized" region of interest was created by overlaying the green image of a given region of interest upon the red image of an identically sized neighboring region (see Fig. 1, C and G). This way, any co-localization reflects random overlap of patches due to their finite density at the cell surface. These randomized values can then be subtracted to obtain the actual co-patching (% co-patching). Typical images showing the co-patching between differently tagged forms of the same receptor chain (IL-9R α or IL-2R β) in the absence of ligand are shown in Fig. 1. Similar experiments were conducted in the presence of IL-2 or IL-9.

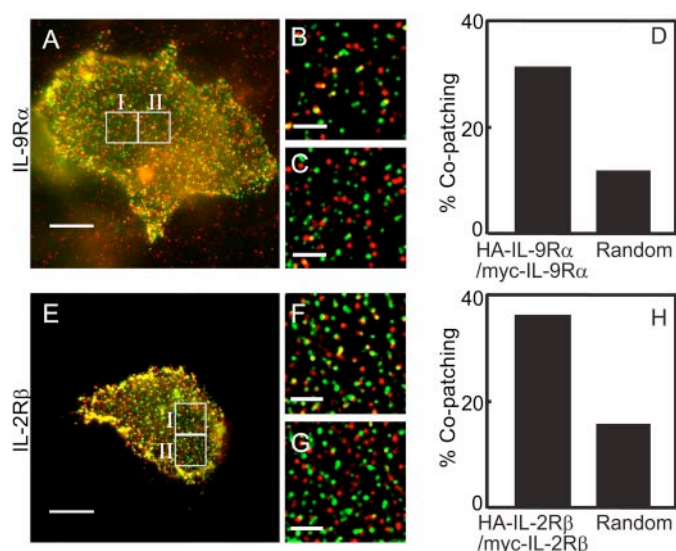


FIGURE 1. Typical immunofluorescence co-patching images of homo-oligomer complex formation between IL-2R β or IL-9R α subunits in the absence of ligand. BOSC23 cells were co-transfected with pairs of pMX-IRES-GFP1.1 expression vectors encoding HA- and Myc-tagged versions of IL-9R α or IL-2R β . After 44–48 h, live cells were labeled consecutively in the cold by a series of antibodies to mediate patching and fluorescent labeling, as described under “Experimental Procedures.” The patching/labeling protocol resulted in Alexa 594-labeled HA-tagged receptors (red fluorescent patches) and Alexa 488-labeled Myc-tagged receptors (green patches). A and E, typical images of cells subjected to co-patching. The white borders depict regions of interest that were analyzed for co-patching (panels B and F) and for randomized co-localization (panels C and G). Bars, 10 μ m in panels A and E and 2 μ m in panels B, C, F, and G. B and F, zoomed images of region I in panels A and E, respectively. Using an algorithm written for Image-Pro Plus, a green and red patch were defined as overlapping (co-patched) if their centers were separated by less than 0.2 μ m (25). C and G, zoomed randomized images to evaluate random overlap between the red and green patches (control for occasional overlap due to finite density of patches). Random images were created from two adjacent regions of interest by merging the green channel of region I with the unrelated red channel of region II. D and H, computer analysis results of the specific images shown for total and randomized % co-patching of IL-2R β (panel D) or IL-9R α (panel H).

Quantitative average data derived from many such experiments with IL-2R β , IL-9R α , or γ_c both in the absence and presence of ligands are depicted in Fig. 3A. These studies demonstrate that 23–29% of each of the receptor subunits examined are already co-patched in the absence of ligand. It should be noted that if one assumes that the homo oligomers are dimeric, the percentage of homodimerization is higher than the percentage of co-patching by a factor of 3/2; as discussed by us earlier (30, 37), this occurs because homodimers containing identically tagged receptors may form but would not be swept into mutual patches. Interestingly, for all the receptor subunits examined, the homo-oligomerization did not increase significantly upon ligand binding. In control experiments (an HA-tagged IL-R chain co-expressed with an unrelated receptor-Myc-T β R β II or FLAG-TpoR) the co-patching level was very low (5–10%; Fig. 3A).

Hetero-oligomers of IL-2R β or IL-9R α with γ_c Form before Ligand Binding and Are Enhanced by Ligand—Because IL-2 and IL-9 signal transduction is mediated via hetero-oligomerization between the ligand binding chain and γ_c in the presence of ligand (8, 17–19), we proceeded to measure the hetero-oligomerization of IL-2R β or IL-9R α with the common γ_c chain. BOSC23 cells were co-transfected by HA-IL-9R α or HA-IL-

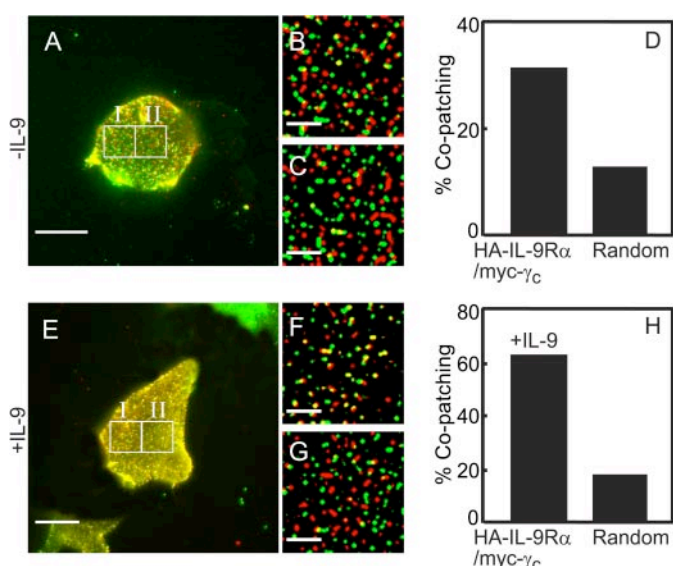


FIGURE 2. Representative immunofluorescence co-patching images showing heterocomplex formation between IL-9R α and γ_c . The experiments were conducted as in Fig. 1 on cells co-expressing HA-IL-9R α and Myc- γ_c . The IgG labeling/patching protocol resulted in HA-IL-9R α in red patches and Myc- γ_c in green patches. A and E, typical images of cells subjected to co-patching in the absence (panel A) or presence (panel E) of IL-9. In the latter case the ligand (750 units/ml) was added for 30 min at 4 °C before antibody labeling and maintained during all subsequent incubations. The white borders depict regions of interest that were analyzed for co-patching (panels B and F) and for randomized co-localization (panels C and G). Bars, 10 μ m in panels A and E and 2 μ m in panels B, C, F, and G. B and F, zoomed images of region I in panels A and E, respectively, processed for co-patching by Image-Pro Plus as described in Fig. 1. C and G, zoomed randomized images to evaluate random overlap between red and green patches, constructed from the green channel of region I and the unrelated red channel of region II. D and H, computer analysis results of the specific images in the absence (B and C; shown in panel D) or presence (F and G; shown in panel H) of ligand.

2R β together with Myc- γ_c . The cells were then subjected to immunofluorescence co-patching studies as in Fig. 1 in the presence or absence of the respective ligands. Fig. 2 shows representative results of co-patching experiments obtained for HA-IL-9R α heterocomplex formation with γ_c ; analogous results were obtained for the association of HA-IL-2R β with γ_c . The average data of many such experiments are summarized in Fig. 3A. As shown in these figures, both IL-2R β and IL-9R α had a subpopulation of receptors (15% for IL-2R β , 25% for IL-9R α) residing in preformed heteromeric complexes with γ_c before ligand binding. However, unlike the situation encountered for homodimerization of the ligand binding subunits, the addition of the respective ligands enhanced the heterodimerization measured on live cells by nearly 100%, demonstrating ligand-mediated heterocomplex formation.

In view of the role IL-2R α chain has in enhancing ligand binding to IL-2R β , we also examined whether overexpression of IL-2R α enhances IL-2R β / γ_c hetero-oligomerization. As shown in Fig. 3A, no significant effects were observed either in the absence or presence of ligand, suggesting that the basic interactions between IL-2R β and γ_c are largely unaffected.

A large fraction of the purified γ_c ectodomain was reported to exist in homo-oligomers, which were proposed to dissociate during its association into heterocomplexes with the other IL-2R chains (15). We, therefore, examined in our system whether co-expression with untagged IL-2R β or IL-9R α

nomenon, namely a certain degree of pre-existing hetero-oligomerization, which significantly increases upon ligand binding, is reproducible in stable cell lines and is not due to excessive overexpression.

Preformed Oligomers of IL-9R α or IL-2R β and γ_c Promote Signaling by Constitutively Active JAK3 A572V Mutant—Cytokine receptors lack intrinsic enzymatic activity and use cytosolic JAK proteins to phosphorylate tyrosines on the cytokine receptors themselves, STAT proteins, and additional proteins involved in signaling (43). This induces transcriptional activation of many genes (44, 45). JAK3 is recruited to interleukin receptor complexes via γ_c (20–22). We compared signaling by IL-2 and IL-9 receptor complexes in the presence and absence of JAK3 and the constitutively active JAK3 A572V mutant, which was recently described to be oncogenic and constitutively active (27). We co-transfected HEK293 cells (which are devoid of JAK3 but express the ubiquitous JAK1) with various combinations of cDNAs coding for JAK3, the ligand binding chains, and γ_c . To measure STAT transcriptional activation, two luciferase reporter constructs were included, one responsive mainly to STAT3 (pGL3-pap1-luc) and the other to STAT5 (pLHRE-luc), as well as the constitutively active Renilla luciferase plasmid pRL-TK. As shown in Fig. 5, A and B, JAK3 co-expression was necessary for transcriptional activation response to either IL-2 or IL-9; co-expression of the receptors in the absence of JAK3 was not sufficient to produce increased signaling unless JAK3 was also expressed. Signaling by IL-2 and IL-9 was marginally increased by providing extra-JAK1 to the endogenous levels in these cells, whereas under these conditions there was no detectable signaling in the absence of ligands (co-expression of specific chains, γ_c , JAK1, and JAK3 (empty bars, right-most columns of Fig. 5, A and B).

We then tested the effect of replacing JAK3 with the constitutively active A572V mutant. Like JAK2 V617F, which contains an intact FERM domain and which requires interaction with cytokine receptors for signaling, JAK3 A572V is predicted to interact with γ_c . In Fig. 5, C and D, we show that co-expression of JAK3 A572V with specific chains and JAK1 leads to significant constitutive signaling both in the case of IL-2R β and γ_c and IL-9R α and γ_c complexes. Interestingly, preformed heteromeric complexes are required for constitutive signaling by JAK3 A572V, as no transcriptional activity was detected when only specific chains or only γ_c were expressed. These data suggest that preformed heteromeric oligomerization might provide an important support for mutated JAKs to induce signaling in the absence of ligand.

DISCUSSION

In the studies reported here we investigated homomeric and heteromeric complex formation among IL-2 or IL-9 receptor chains situated at the plasma membrane of intact cells. Our key finding is that all the IL-R subunits examined (IL-2R β , IL-9R α , and γ_c) display a fraction of homomeric complexes at the cell surface independent of ligand binding. Heteromeric complexes between IL-9R α or IL-2R β and γ_c are also formed in the absence of ligand, but unlike the insensitivity of the homomeric complexes to ligand, heteromeric complex formation was

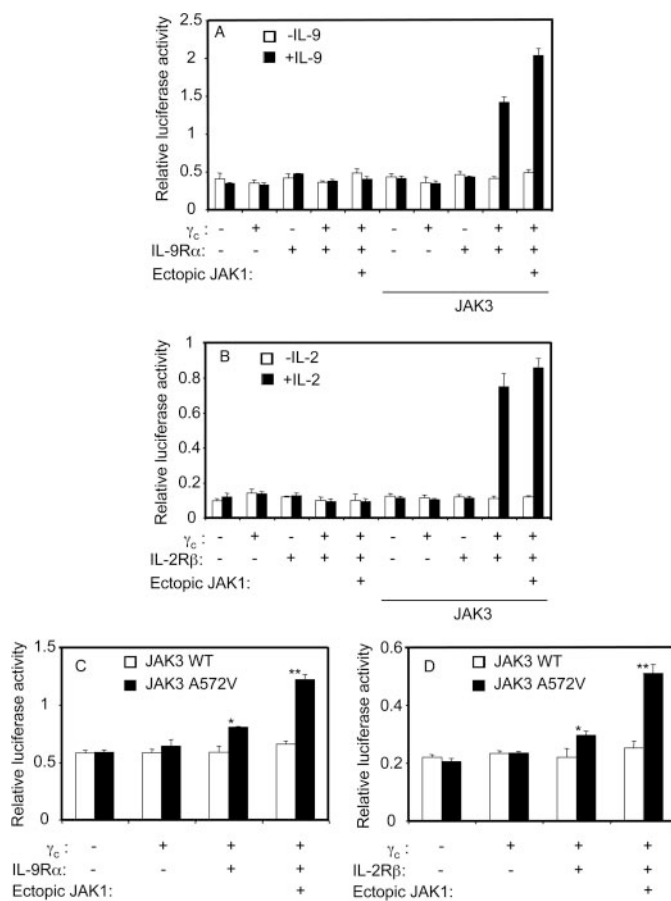


FIGURE 5. Luciferase transcriptional activation assay demonstrating that JAK3 expression is required for IL-2 and IL-9 response and that preformed heteromeric complexes promote constitutive signaling by JAK3 A572V. HEK293 cells, which do not express endogenous JAK3, were co-transfected with pMX-IRES-GFP1.1 vectors encoding HA-tagged IL-2R β or IL-9R α in various combinations with HA- γ_c , JAK3, JAK1, JAK3 A572V, the STAT-responsive luciferase reporter vectors pGL3-pap1-luc (for STAT3 in the case of IL-9R α , panel C), pLHRE-luc (for STAT5 in the case of IL-2R β , panel D), and the pRL-TK constitutively active Renilla luciferase vector. A and B, 4 h post-transcription, the cells were incubated overnight with ligands (solid bars) or with vehicle control (empty bars) and subjected to luciferase assays ("Experimental Procedures"). Results are the mean $\pm$ variation of duplicate samples, which are representative of three independent experiments. C and D, HEK293 cells were transfected with cDNA coding for HA-tagged IL-2R β or IL-9R α in various combinations with HA- γ_c along with cDNAs coding for JAK3 (empty bars), JAK3 A572V (solid bars), and when indicated the cDNA coding for JAK1. Shown are the means $\pm$ S.E. of two experiments, each performed in duplicate. C, *, $p < 0.02$; **, $p < 3 \times 10^{-4}$. D, *, $p < 0.04$; **, $p < 3 \times 10^{-4}$ (indicate a significant increase in the luciferase activity).

increased by 100% after ligand binding. The latter findings in transiently transfected BOSC23 cells were also validated in Ba/F3 cells stably expressing near-physiologic levels of tagged IL-R subunits. Importantly, the need for ligand binding to activate even the preformed heteromeric complexes of IL-2R α or IL-9R β with γ_c suggests that the oligomeric interfaces between IL-9R α or IL-2R β and γ_c must differ between the unliganded and liganded receptor complexes. Although strictly inactive, we propose that the preformed heteromeric complexes may act to prime signaling by low levels of ligands. This is reminiscent of the case of the erythropoietin receptor, where preformed dimers were also detected by immunofluorescence co-patching (30, 37) and by fluorescence resonance energy transfer (46). These preformed dimers require a conformational change for

activation (47) in order to impose a productive dimeric interface necessary to activate JAK2.

The immunofluorescence co-patching studies on homo-oligomerization (Figs. 1 and 3) demonstrate that significant fractions of IL-2R β , IL-9R α , or γ_c reside in homomeric complexes before ligand binding. The co-patching observed for the homomeric interactions of each chain was rather similar (23–29%; Figs. 1 and 3). As discussed earlier (30, 37), the % co-patching is identical to the percentage of dimerization in the case of heterodimers (where each interacting chain is necessarily labeled by a different tag) but not in the case of homo-oligomers. Thus, for a pure homodimeric population, the statistical prediction is for 66.6% (2/3) co-patching, as homodimers containing identically tagged chains may also form but would not be swept into mutual patches with similar chains carrying the other tag. For a homodimer, the fraction of “same tag” complexes is $\frac{1}{3}$, leaving $\frac{2}{3}$ as differently tagged dimers. Thus, 23–29% co-patching would actually reflect a proportion of 37–44% of receptor chains in homodimers. This level is significant, especially because it is obtained after the subtraction of the randomized co-localization of patches, which is enabled by the new computerized approach.

Importantly, for all the above receptor chains, the homomeric co-patching levels were not affected by ligands (IL-2 or IL-9), demonstrating that the homomeric complexes are ligand-independent. This situation is different from that reported for the epidermal growth factor receptor, where dimerization is ligand-dependent (48). However, it is in accord with earlier reports by us and by others, where the unliganded erythropoietin receptors were found to form homodimers at high efficiency (30, 47, 49, 51). Moreover, our present finding that a significant fraction of γ_c is in homo-oligomers (Fig. 3) is in accord with a recent report that the purified ectodomain of γ_c forms homo-oligomers, which may dissociate and incorporate into heteromeric signaling complexes upon incubation with the other IL-2 receptor chains in the presence of ligand (15). Indeed, co-expression with IL-2R β or IL-9R α reduced the homo-oligomerization of γ_c (Fig. 3B), suggesting that the formation of heteromeric signaling complexes is at the expense of γ_c homo-oligomerization. Because heterocomplex formation is selectively increased in the presence of ligand (see below), γ_c homo-oligomerization may serve a physiological role by reducing the potential for activation in the absence of ligand.

A different picture was observed for the heteromeric association of IL-2R β and IL-9R α with γ_c . Although detectable levels of heteromeric complexes were also observed before ligand binding both in transiently expressing BOSC23 cells and in stably expressing Ba/F3 cells (Figs. 2–4), the effect of ligand was strikingly different, inducing a significant increase (2-fold) in the percentage of heteromeric co-patching. This suggests that the binding of IL-2 or IL-9 to their respective ligand-binding subunits results in ligand-mediated increase in the association of these subunits with γ_c . These findings, obtained on full-length receptor chains situated at the plasma membrane of live cells, are in agreement with crystallographic studies on the quaternary structure of IL-2 in complex with IL-2R α , IL-2R β , and γ_c (14, 15), where a large IL-2R β / γ_c interface was proposed to undergo stabilization following ligand binding. They are also in

agreement with fluorescence resonance energy transfer studies (23) which reported the existence of some IL-2R β / γ_c complexes before ligand binding and their modulation in the presence of IL-2. Interestingly, the crystallographic studies on heterocomplexes comprised of the IL-2R subunits (14, 15) showed that IL-2R α lacks contact with IL-2R β or γ_c , in accord with our demonstration (Fig. 3) that overexpression of IL-2R α fails to affect IL-2R β / γ_c heteromeric complex formation. The insensitivity of IL-R homomeric complex formation to IL-2 or IL-9 binding contrasts with the significant augmentation mediated by the ligands in the formation of heteromeric complexes with γ_c . This implies that ligand binding alters the balance between homomeric and heteromeric complexes containing γ_c in favor of the latter. This notion is in accord with the reported shift of the ectodomain of γ_c from homotrimers into IL-2-containing quaternary complexes with the other receptor in the presence of ligand (15). Moreover, formation of IL-2R β / γ_c or IL-9R α / γ_c heterocomplexes, even in the presence of JAK3, is not sufficient for signaling, which still requires ligand binding. This suggests that the oligomeric interfaces within the heteromeric complex differ between the unliganded and ligand-activated complexes. Furthermore, the requirement for JAK3 for signaling indicates that the ligand-induced conformation in the quaternary complex may be important for the proper positioning of JAK1 and JAK3 for cross-phosphorylation.

Recently, several point mutations have been identified in Janus kinases, especially in pseudokinase domains, which render them constitutively active for signaling. Such mutations have been genetically linked to different malignancies, *i.e.* myeloproliferative syndromes for JAK2 mutants (V617F and exon 12 mutations) (52–56), adult T-acute lymphoblastic leukemia (JAK1 mutations) (26), and acute megakaryocytic leukemia (JAK3 mutations) (27). JAK2 V617F, the model for such mutant JAKs, and homologous mutants of JAK1 and Tyk2 are constitutively active when overexpressed (57). However, at low physiologic levels of expression, dimerization of a cytokine receptor before ligand binding (preformed dimers) was shown to be necessary for activation of JAK2 V617F (50). Our results show that subunits of IL-2R and IL-9R exhibit ligand-independent homomeric complexes as well as both preformed and ligand-induced heteromeric complexes. We tested whether, in an analogous manner with the described effect of preformed erythropoietin receptor dimers on JAK2 V617F (50), signaling by the constitutively active JAK3 A572V mutant is promoted by homomeric γ_c complexes or by heteromeric complexes between IL-2R β or IL-9R α and γ_c . These data suggest that although preformed heteromeric complexes are strictly inactive in the presence of ligand in the case of wild-type JAK proteins, they promote signaling by JAK3 A572V. We suggest that preformed heteromeric complexes of IL-9R α and γ_c in megakaryocytic progenitors may explain the paradox where JAK3, the unique Janus kinase specific for γ_c , triggers megakaryocytic leukemia (27).

In contrast, homo-oligomerization of JAK3 A572V by γ_c homomeric complexes does not induce signaling, emphasizing the need for JAK1 and the cytosolic tyrosine residues of the other receptor chains for STAT signaling. These results point to a novel difference between the activation and signaling

mechanisms of JAK3 A572V and mutants of the other three JAKs. Previously, it has been noted that introduction of a phenylalanine at the homologous V617 position activated all JAKs except JAK3 (57).

In conclusion our study provides evidence that in the absence of ligand, homomeric and heteromeric complexes are formed between the subunits of the IL-2 and IL-9 receptor complexes. Homomeric complexes of γ_c are formed at the expense of heteromeric complexes with IL-2R β and IL-9R α . All preformed complexes are strictly inactive in the absence of ligand. We also show that signaling by an oncogenic mutant of JAK3 (A572V) is promoted by preformed heteromeric complexes of IL-2R β or IL9R α and γ_c . We suggest that preformed oligomers of cytokine receptors might have a more general role in supporting ligand-independent oncogenic signaling by constitutively active forms of Janus kinases.

REFERENCES

- Demoulin, J. B., and Renauld, J. C. (1998) *Cytokines Cell. Mol. Ther.* **4**, 243–256
- Moriggl, R., Sexl, V., Piekorz, R., Topham, D., and Ihle, J. N. (1999) *Immunity* **11**, 225–230
- Schorle, H., Holtschke, T., Hunig, T., Schimpl, A., and Horak, I. (1991) *Nature* **352**, 621–624
- Willerford, D. M., Chen, J., Ferry, J. A., Davidson, L., Ma, A., and Alt, F. W. (1995) *Immunity* **3**, 521–530
- Suzuki, H., Kundig, T. M., Furlonger, C., Wakeham, A., Timms, E., Matsuyama, T., Schmits, R., Simard, J. J., Ohashi, P. S., Griesser, H., Taniguchi, T., Paige, C. J., and Mak, T. W. (1995) *Science* **268**, 1472–1476
- DiSanto, J. P., Certain, S., Wilson, A., MacDonald, H. R., Avner, P., Fischer, A., and de Saint Basile, G. (1994) *Eur. J. Immunol.* **24**, 3014–3018
- Moriggl, R., Topham, D. J., Teglund, S., Sexl, V., McKay, C., Wang, D., Hoffmeyer, A., van Deursen, J., Sangster, M. Y., Bunting, K. D., Grosveld, G. C., and Ihle, J. N. (1999) *Immunity* **10**, 249–259
- Knoops, L., and Renauld, J. C. (2004) *Growth Factors* **22**, 207–215
- Steenwinckel, V., Louahed, J., Orabona, C., Huaux, F., Warnier, G., McKenzie, A., Lison, D., Levitt, R., and Renauld, J. C. (2007) *J. Immunol.* **178**, 3244–3251
- Leonard, W. J., Depper, J. M., Uchiyama, T., Smith, K. A., Waldmann, T. A., and Greene, W. C. (1982) *Nature* **300**, 267–269
- Sharon, M., Klausner, R. D., Cullen, B. R., Chizzonite, R., and Leonard, W. J. (1986) *Science* **234**, 859–863
- Teshigawara, K., Wang, H. M., Kato, K., and Smith, K. A. (1987) *J. Exp. Med.* **165**, 223–238
- Takeshita, T., Asao, H., Ohtani, K., Ishii, N., Kumaki, S., Tanaka, N., Munakata, H., Nakamura, M., and Sugamura, K. (1992) *Science* **257**, 379–382
- Wang, X., Rickert, M., and Garcia, K. C. (2005) *Science* **310**, 1159–1163
- Stauber, D. J., Debler, E. W., Horton, P. A., Smith, K. A., and Wilson, I. A. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 2788–2793
- Wang, H. M., and Smith, K. A. (1987) *J. Exp. Med.* **166**, 1055–1069
- Nelson, B. H., Lord, J. D., and Greenberg, P. D. (1994) *Nature* **369**, 333–336
- Nakamura, Y., Russell, S. M., Mess, S. A., Friedmann, M., Erdos, M., Francois, C., Jacques, Y., Adelstein, S., and Leonard, W. J. (1994) *Nature* **369**, 330–333
- Kimura, Y., Takeshita, T., Kondo, M., Ishii, N., Nakamura, M., Van Snick, J., and Sugamura, K. (1995) *Int. Immunol.* **7**, 115–120
- Leonard, W. J., Shores, E. W., and Love, P. E. (1995) *Immunol. Rev.* **148**, 97–114
- Nelson, B. H., and Willerford, D. M. (1998) *Adv. Immunol.* **70**, 1–81
- Hofmann, S. R., Lam, A. Q., Frank, S., Zhou, Y. J., Ramos, H. L., Kanno, Y., Agnello, D., Youle, R. J., and O'Shea, J. J. (2004) *Mol. Cell. Biol.* **24**, 5039–5049
- Damjanovich, S., Bene, L., Matko, J., Alileche, A., Goldman, C. K., Sharrow, S., and Waldmann, T. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 13134–13139
- Constantinescu, S. N., Keren, T., Russ, W. P., Ubarretxena-Belandia, I., Malka, Y., Kubatzky, K. F., Engelman, D. M., Lodish, H. F., and Henis, Y. I. (2003) *J. Biol. Chem.* **278**, 43755–43763
- Lachmanovich, E., Shvartsman, D. E., Malka, Y., Botvin, C., Henis, Y. I., and Weiss, A. M. (2003) *J. Microsc.* **212**, 122–131
- Flex, E., Petrangeli, V., Stella, L., Chiaretti, L., Hornakova, T., Knoops, L., Ariola, C., Fodale, V., Clappier, E., Paoloni, F., Martinelli, S., Fragale, A., Sanchez, M., Tavolaro, S., Messina, M., Cazzaniga, G., Camera, A., Pizzolo, G., Tornesello, A., Vignetti, M., Battistini, A., Cavé, H., Gelb, B. D., Renauld, J.-C., Biondi, A., Constantinescu, S. N., Foà, R., and Tartaglia, M. (2008) *J. Exp. Med.* **205**, 751–758
- Walters, D. K., Mercher, T., Gu, T. L., O'Hare, T., Tyner, J. W., Loriaux, M., Goss, V. L., Lee, K. A., Eide, C. A., Wong, M. J., Stoffregen, E. P., McGreevey, L., Nardone, J., Moore, S. A., Crispino, J., Boggon, T. J., Heinrich, M. C., Deininger, M. W., Polakiewicz, R. D., Gilliland, D. G., and Druker, B. J. (2006) *Cancer Cell* **10**, 65–75
- Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 8392–8396
- Keren, T., Roth, M. G., and Henis, Y. I. (2001) *J. Biol. Chem.* **276**, 26356–26363
- Constantinescu, S. N., Keren, T., Socolovsky, M., Nam, H. S., Henis, Y. I., and Lodish, H. F. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 4379–4384
- Seubert, N., Royer, Y., Staerk, J., Kubatzky, K. F., Moucadel, V., Krishnakumar, S., Smith, S. O., and Constantinescu, S. N. (2003) *Mol. Cell* **12**, 1239–1250
- Liu, X., Constantinescu, S. N., Sun, Y., Bogan, J. S., Hirsch, D., Weinberg, R. A., and Lodish, H. F. (2000) *Anal. Biochem.* **280**, 20–28
- Constantinescu, S. N., Liu, X., Beyer, W., Fallon, A., Shekar, S., Henis, Y. I., Smith, S. O., and Lodish, H. F. (1999) *EMBO J.* **18**, 3334–3347
- Royer, Y., Staerk, J., Costuleanu, M., Courtoy, P. J., and Constantinescu, S. N. (2005) *J. Biol. Chem.* **280**, 27251–27261
- Sabath, D. F., Kaushansky, K., and Broudy, V. C. (1999) *Blood* **94**, 365–367
- Henis, Y. I., Moustakas, A., Lin, H. Y., and Lodish, H. F. (1994) *J. Cell Biol.* **126**, 139–154
- Gilboa, L., Nohe, A., Geissendorfer, T., Sebald, W., Henis, Y. I., and Knaus, P. (2000) *Mol. Biol. Cell* **11**, 1023–1035
- Renauld, J. C., Goethals, A., Houssiau, F., Van Roost, E., and Van Snick, J. (1990) *Cytokine* **2**, 9–12
- Gerland, K., Bataille-Simoneau, N., Basle, M., Fourcin, M., Gascan, H., and Mercier, L. (2000) *Mol. Cell. Endocrinol.* **168**, 1–9
- Peelman, F., Iserentant, H., De Smet, A. S., Vandekerckhove, J., Zabeau, L., and Tavernier, J. (2006) *J. Biol. Chem.* **281**, 15496–15504
- Gilboa, L., Wells, R. G., Lodish, H. F., and Henis, Y. I. (1998) *J. Cell Biol.* **140**, 767–777
- Palacios, R., and Steinmetz, M. (1985) *Cell* **41**, 727–734
- Watowich, S. S., Wu, H., Socolovsky, M., Klingmuller, U., Constantinescu, S. N., and Lodish, H. F. (1996) *Annu. Rev. Cell Dev. Biol.* **12**, 91–128
- Darnell, J. E., Kerr, I. M., and Stark, G. (1994) *Science* **264**, 1415–1420
- Ihle, J. N. (2001) *Curr. Opin. Cell Biol.* **13**, 211–217
- Brown, R. J., Adams, J. J., Pelekanos, R. A., Wan, Y., McKinstry, W. J., Palethorpe, K., Seeber, R. M., Monks, T. A., Eidne, K. A., Parker, M. W., and Waters, M. J. (2005) *Nat. Struct. Mol. Biol.* **12**, 814–821
- Remy, I., Wilson, I. A., and Michnick, S. W. (1999) *Science* **283**, 990–993
- Schlessinger, J. (2000) *Cell* **103**, 211–225
- Livnah, O., Stura, E. A., Middleton, S. A., Johnson, D. L., Jolliffe, L. K., and Wilson, I. A. (1999) *Science* **283**, 987–990
- Lu, X., Huang, L. J., and Lodish, H. F. (2008) *J. Biol. Chem.* **283**, 5258–5266
- Kubatzky, K. F., Ruan, W., Gurezka, R., Cohen, J., Ketteler, R., Watowich, S. S., Neumann, D., Langosch, D., and Klingmuller, U. (2001) *Curr. Biol.* **11**, 110–115
- James, C., Ugo, V., Le Couedic, J. P., Staerk, J., Delhommeau, F., Lacout, C., Garcon, L., Raslova, H., Berger, R., Bennaceur-Griscelli, A., Villeval, J. L., Constantinescu, S. N., Casadevall, N., and Vainchenker, W. (2005) *Nature* **434**, 1144–1148
- Baxter, E. J., Scott, L. M., Campbell, P. J., East, C., Fourouclas, N., Swanton, S., Vassiliou, G. S., Bench, A. J., Boyd, E. M., Curtin, N., Scott, M. A., Erber,

- W. N., and Green, A. R. (2005) *Lancet* **365**, 1054–1061
54. Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., Wernig, G., Huntly, B. J., Boggon, T. J., Wlodarska, I., Clark, J. J., Moore, S., Adelsperger, J., Koo, S., Lee, J. C., Gabriel, S., Mercher, T., D'Andrea, A., Frohling, S., Dohner, K., Marynen, P., Vandenberghe, P., Mesa, R. A., Tefferi, A., Griffin, J. D., Eck, M. J., Sellers, W. R., Meyerson, M., Golub, T. R., Lee, S. J., and Gilliland, D. G. (2005) *Cancer Cell* **7**, 387–397
 55. Kralovics, R., Passamonti, F., Buser, A. S., Teo, S. S., Tiedt, R., Passweg, J. R., Tichelli, A., Cazzola, M., and Skoda, R. C. (2005) *N. Engl. J. Med.* **352**, 1779–1790
 56. Scott, L. M., Tong, W., Levine, R. L., Scott, M. A., Beer, P. A., Stratton, M. R., Futreal, P. A., Erber, W. N., McMullin, M. F., Harrison, C. N., Warren, A. J., Gilliland, D. G., Lodish, H. F., and Green, A. R. (2007) *N. Engl. J. Med.* **356**, 459–468
 57. Staerk, J., Kallin, A., Demoulin, J. B., Vainchenker, W., and Constantinescu, S. N. (2005) *J. Biol. Chem.* **280**, 41893–41899

Summary

JAK1 is a tyrosine kinase from the Janus protein kinase (JAK) family, which includes also JAK2, JAK3 and TYK2. These protein kinases are associated with cytokine receptors to mediate intracellular signaling upon cytokine binding. JAK kinases control activation of downstream signaling molecules such as STAT transcription factors to regulate proliferation, differentiation and cellular growth of target cells.

We developed a two-step selection *in vitro* model of cellular transformation based on the IL-3-dependent pro-B cell line BaF3 that overcame defects in IL-9 receptor signal transduction by spontaneously overexpressing JAK1 during a first step of selection. We identified 18 activating *de novo* mutations of JAK1 as a second genetic event associated with cytokine-independent proliferation and constitutive activation of the JAK-STAT pathway.

In parallel to these observations, in collaboration with Prof. Marco Tartaglia, we identified activating mutations in JAK1 in 20 % of T-cell acute lymphoblastic leukemia (T-ALL) patients and in 3% of B-ALL patients, confirming the relevance of our *in vitro* model-derived JAK1 mutations for human malignancies.

Focusing on the mechanisms by which the JAK1 mutants might promote the development of these leukemias, we found that they need to associate with JAK1-binding cytokine receptors such as the receptors for IL-2 and IL-9, which play an important role in proliferation and differentiation of normal T lymphocytes. When the JAK1 mutants are present, these receptors are constitutively active, even without their ligand.

In addition, we observed that JAK1 mutants also associate with the interferon (IFN) receptors. Therefore, tumor cells with mutated JAK1 constitutively express IFN target genes. Moreover, cells with JAK1 mutations are hypersensitive to IFN stimulation. This is particularly relevant for patients because IFN is known to have anti-proliferative activity on tumor cells. We have demonstrated the potential application of this finding by using a mouse model of acute lymphoblastic leukemia. In this model, treatment with IFN resulted in prolonged anti-tumoral protection of the mice that had tumors with JAK1 mutations, as compared to the tumors without such mutations. Based on these observations, we think that IFN- α should be considered as a part of the current multi-drug protocol to treat ALL patients positive for JAK1 mutations, and maybe other cancers such as acute myeloid leukemia (AML), where JAK1 mutations occur too.

Résumé

JAK1 est une tyrosine kinase qui appartient, avec JAK2, JAK3 et TYK2, à la famille des Janus kinases (JAKs). Ces protéines sont associées aux récepteurs aux cytokines pour permettre leur signalisation intracellulaire. Les JAKs contrôlent l'activation de molécules de transduction du signal, comme les facteurs de transcription STATs, pour réguler la prolifération, la différenciation et la croissance des cellules cibles.

Nous avons développé un modèle de tumorigenèse *in vitro* en deux étapes, basé sur la lignée cellulaire pro-B BaF3, dépendante de l'IL-3. Ces cellules ont, durant la première étape de sélection, surexprimé JAK1 pour pouvoir répondre à un récepteur de l'IL-9 défectueux. Nous avons pu démontrer que la deuxième étape de sélection, associée à une prolifération cellulaire autonome indépendante des cytokines et à une activation constitutive de la voie JAK-STAT, était liée à la survenue spontanée de mutations dans JAK1. 18 mutations activatrices de JAK1 ont pu être détectées dans différents clones autonomes.

En parallèle à ces observations, en collaboration avec le Prof. Marco Tartaglia, nous avons identifié des mutations activatrices de JAK1 dans 20% des leucémies aiguës lymphoblastiques (LLA) T de l'adulte et dans 3 % des patients atteints de LLA B. Cette observation confirme la pertinence de notre modèle cellulaire *in vitro* pour l'oncogenèse humaine.

En se focalisant sur les mécanismes permettant aux mutants JAK1 de promouvoir la leucémogénèse, nous avons démontré qu'ils devaient s'associer à un récepteur capable de lier JAK1, comme le récepteur de l'IL-2 ou de l'IL-9. Ces récepteurs jouent un rôle important dans la prolifération et la différenciation des lymphocytes T normaux. Quand une mutation dans JAK1 est présente, ces récepteurs s'activent de manière constitutive, même sans ligand.

Nous avons également observé que les mutants JAK1 se lient des récepteurs aux interférons (IFN) de type I. C'est pourquoi les cellules tumorales mutées pour JAK1 expriment constitutivement des gènes cibles des IFN. De plus, ces cellules sont hypersensibles à l'effet des IFNs. Cette observation est particulièrement relevante pour la clinique au vu de l'activité anti-proliférative exercée par les IFNs sur les cellules tumorales. Nous avons démontré l'application potentielle de ces découvertes en utilisant un modèle murin de LLA. Dans ce modèle, le traitement par IFN exerçait un effet anti-tumoral spécifique sur les cellules exprimant une forme mutée de JAK1 par rapport aux tumeurs sans mutation. Sur base de ces observations, nous pensons que l'utilisation d'IFN- α devrait être ajoutée à l'arsenal thérapeutique utilisé pour traiter la LLA chez les patients mutés pour JAK1, et qu'il est envisageable d'utiliser l'IFN dans d'autres tumeurs ou des mutations de JAK1 peuvent survenir, comme dans la leucémie myéloïde aigue.