Dual Role of the Jak1 FERM and Kinase Domains in Cytokine Receptor Binding and in Stimulation-Dependent Jak Activation

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Jak1 is a tyrosine kinase that noncovalently forms tight complexes with a variety of cytokine receptors and is critically involved in signal transduction via cytokines. Jak1 is predicted to have a 4.1, ezrin, radixin, moesin (FERM) domain at their N terminus. FERM domains are composed of three structurally unrelated subdomains (F1, F2, and F3) which are in close contact to one another and form the clover-shaped FERM domain. We generated a model structure of the Jak1 FERM domain, based on solved FERM structures and the alignments with other FERM domains. To destabilize different subdomains and to uncover their exact function, we mutated specific hydrophobic residues conserved in FERM domains and involved in hydrophobic core interactions. In this study, we show that the structural integrity of the F2 subdomain of the FERM domain of Jak1 is necessary to bind the IFN-γRα. By mutagenesis of hydrophobic residues in the hydrophobic core between the three FERM subdomains, we find that the structural context of the FERM domain is necessary for the inhibition of Jak1 phosphorylation. Thus, FERM domain mutations can have repercussions on Jak1 function. Interestingly, a mutation in the kinase domain (Jak1-K907E), known to abolish the catalytic activity, also leads to an impaired binding to the IFN-γRα when this mutant is expressed at endogenous levels in U4C cells. Our data show that the structural integrity of both the FERM domain and of the kinase domain is essential for both receptor binding and catalytic function/autoinhibition. The Journal of Immunology, 2008, 180: 998–1007.

The molecular mechanism of Jak activation upon cytokine stimulation is not understood in detail. It is still under debate how the predicted structural domains of the Jaks interplay in kinase activation. The originally defined seven Jak homology (JH) regions which were based on sequence similarities between the Jaks only partially match the more recently predicted domain structure. The tyrosine kinase domain (JH1) is flanked by the pseudokinase domain (JH2) that may play a regulatory role (4, 5). The N-terminal half of the Jaks contains a predicted FERM domain (FERM standing for: band-4.1 protein, ezrin, radixin, and moesin) (6) and a putative SH2 domain, the borders of which do not correspond to the JH3 to JH7 domains. The SH2 domain (C-terminal to the FERM domain) of Jak1 has been shown to have a nonclassical function for binding of the oncostatin M (OSM) receptor and the promotion of its surface expression (7). The FERM domain is involved in constitutive binding of Jak1 to the cytokine receptors (8, 9) and the resulting tight complex behaves (in many aspects) like a receptor tyrosine kinase (1, 10, 11). Published x-ray structures of FERM domains (12–16) show that these domains consist of three separate subdomains arranged in a clover shape. The N-terminal F1 subdomain has a ubiquitin-like fold, the F2 subdomain is rich in α-helices and shows structural similarity to the acyl-CoA-binding protein, and the F3 subdomain has a pleckstrin homology domain fold.

We are interested in the activation mechanism of Jaks, in particular Jak1, following cytokine stimulation. We previously described a structure-based model of the F1 domain of Jak1 (8) which has proven useful for mutagenesis studies. By a similar approach of structure-based site-directed mutagenesis, we have now identified the F2 subdomain in the Jak1 FERM domain to be crucial for interaction with the IFN-γ receptor. This approach also revealed that the conformational context of the FERM domain is important for the inhibition of Jak1 phosphorylation and that

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FERM domain mutations can have repercussions on Jak1 function. Furthermore, we describe that a mutation in the kinase domain leads to an impaired cytokine receptor binding which was unexpected in the view of previous studies.

Materials and Methods

Structural alignment and molecular modeling of the Jak1 FERM domain

For molecular modeling and graphic representation of protein structures, the programs WHAT IF (17) and Ribbons (18) were used. The structure of the FERM domain of focal adhesion kinase (FAK), Brookhaven Data Bank entry code 2A6L (15), was used as a template for the model structure of Jak1 (15) and was implicated in homology modeling and molecular modeling with the GROMOS program library (W. F. van Gunsteren, distributed by BI OMS Biomolecular Software, Laboratory of Physical Chemistry, University of Groningen, Groningen, The Netherlands).

The initial alignment of the FERM domain sequences of human and murine Jak1, human Jak2, human Jak3, and human Tyk2 with the sequences of the structurally explored FERM domains of chicken FAK, human moesin, and human merlin was performed by the use of the basic local alignment search tool program (19). Modifications were then introduced to meet structural requirements derived from the known FERM structures. The sequential alignment of the known structures is based on the superposition of their backbone coordinates. Secondary structure predictions were performed using the Predict Protein Server (20). Brookhaven Data Bank entry codes for the used structures are: 2A6L for chicken FAK (15); 1JEF for human moesin (12); 1G7C for murine radixin (21) (the murine and human radixin sequences are identical in the amino acid range represented in Fig. 1); and 1H4R for human merlin (16). The Swiss-Prot accession numbers for the used Jak sequences used are: P23458 (hJak1), NP666257 (nJak1), O60674 (hJak2), P29597 (hTyk2), and P52333 (hJak3).

Cell culture and transfection

U4C, y2A, and 2C4 cells (human fibrosarcoma cells provided by Dr. I. M. Kerr, Cancer Research U.K., London, U.K.) as well as COS-7 cells (simian kidney cells; American Type Culture Collection CRL1619) were maintained in DMEM (Cambrex). All media were supplemented with 10% FCS, 100 mg/L streptomycin, and 60 mg/L penicillin. The U4C-Jak1, U4C-Jak1-K907E, U4C-Jak1-L158A/F159A, U4C-Jak1-L166A/I167A, U4C-Jak1-Y270A, U4C-Jak1-Y281A, U4C-Jak1-Y112A, and U4C-Jak1-K133E cells were grown in 100 ml/mL hydrocortisone. Cells were grown at 37°C in a water-saturated atmosphere at 5% CO2.
y2A-Flip-In cells were generated according to the manufacturer’s protocol (Invitrogen Life Technologies) using Superfect reagent (Qiagen) for transfection. U4C-Flip-In cells (1) stably expressing Jak1, Jak1-K907E, Jak1-L158A/F159A, Jak1-L166A/I167A, Jak1-Y270A, Jak1-Y281A, Jak1-L166A/I167A, Jak1-Y112A, Jak1-K133E, and y2A-Flip-In cells stably expressing Jak2 and Jak2-Y112A were generated using the pFlip-In system from Invitrogen Life Technologies according to the manufacturer’s recommendations. IFN-α, IFN-γ, and OSM were obtained from PeproTech. Cells were stimulated with 20 ng/mL OSM, 1000 U/mL IFN-γ, or IFN-α. Jak inhibitor I was purchased from Calbiochem.

Generation of Jak1 mutant constructs

Standard cloning procedures were performed throughout this study. The mutations (resulting in amino acid substitutions L158A/F159A, L166A/I167A, Y270A, Y281A, Y112A, and K133E) were introduced by PCR amplification and subcloned into pcDNA5/FRT/TO digested with XhoI and AvrII. Subsequently, the amplified fragments were inserted into EcoRI digestion of pcDNA5/FRT/TO and ligated between the FRT-loxP elements of the pSV-L-Jak2 and subcloned into pcDNA5/FRT/TO digested with XhoI/BamHI. The fragment carrying the mutation resulting in amino acid substitution K882E was excised from pRKS-Jak2-K882E (22) using AvrII/NheI and introduced into pcDNA5/FRT/TO-Jak2 digested in the same way. The integrity of all constructs was verified by DNA sequencing using the ABI PRISM 310 Genetic Analyzer (PerkinElmer). The pcDNA5/FRT-plasmid Jak1-K907E was excised previously (7).

Cell lysis, immunoprecipitation, and Western blot analysis

All steps of cell lysis and immunoprecipitation were performed at 4°C using ice-cold buffers. Cells were lysed on the dish with lysis buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 10 mM PMSF, 1 mM benzamidine, 5 μg/ml aprotinin, 3 μg/ml pepstatin, 5 μg/ml leupeptin, and 1 mM EDTA. After incubation of the cleared lysates with Abs, the immunoprecipitates were collected with protein A-Sepharose for 1 h, washed several times and analyzed further by SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech) and probed with the respective Abs. Anti-Jak1 (Santa Cruz Biotechnology) and -IFN-α/κ (Santa Cruz Biotechnology) were used for immunoprecipitation. Anti-Jak1 (BD Transduction Laboratories), anti-phosphotyrosine (Santa Cruz Biotechnology), anti-IFN regulatory factor 1 (IRF1; Santa Cruz Biotechnology), anti-calcinein (BD Transduction Laboratories), and anti-Fit3 (BD Transduction Laboratories) were used for downstream analysis. The HRP-conjugated secondary Abs were purchased from DakoCytomation. Signals were detected using an ECL solution containing 2.5 mM luminol, 2.6 mM hydrogen peroxide, 100 mM Tris-HCl (pH 8.8), and 0.2 mM paracoumaric acid (23).

Flow cytometry

Cells were resuspended in cold PBS supplemented with 5% FCS and 0.1% sodium azide (PBS/azide). A total of 5 × 10^5 to 1 × 10^6 cells in 100 μl of PBS/azide were incubated with 1 μg/ml monoclonal anti-MHC class I (MHC-I; Sigma-Aldrich) for 30 min at 4°C. Cells were then washed with cold PBS/azide. To visualize the bound Abs, the cells were subsequently incubated with a 1/100 dilution of an RPE-conjugated anti-mouse IgG-Fab (Dianova) for 30 min at 4°C. Cells were again washed with cold PBS/azide and then 10^4 cells per sample were analyzed by flow cytometry using an Epics XL (Coulter) equipped with a 488 nm argon laser.

Polyinosinic-polyctydilic acid (PIC) and IFN-γ treatment and Annexin V<sup>FITC</sup> apoptosis detection

The different Jak-mutant cells were seeded at a density of 1 × 10^6 cells/well in 6-well plates in complete medium; after 3 h, cells were incubated for 48 h in complete medium in the presence of recombinant human IFN-γ (1000 U/ml; PeproTech) and then 10^4 cells per sample were analyzed by flow cytometry using an Epics XL (Coulter) equipped with a 488 nm argon laser.

Results

Structure-based alignment of the Jak FERM domains and model structure of Jak1

Mutations of residues involved in hydrophobic core interactions often result in a local destabilization of the structure and in loss-of-function of the corresponding protein domain. This approach helped previously to investigate the function of the F1 subdomain of Jak1 (8). We wanted to investigate the function of the F2 domain and of the central hydrophobic core of the FERM domain which stabilizes the three subdomains F1, F2, and F3 in a clover shape. Because no structural data are available on this region of Jak, we identified the candidate residues involved in hydrophobic core interactions by performing a sequence alignment of the predicted FERM sequence of Jak2 with the sequences of structurally explored FERM domains (12, 13, 15, 16) (Fig. 1).

The alignment shows that the FERM domain of the Jak is a divergent FERM domain which is only remotely related to the classical FERM domains found in ezrin, radixin, and moesin, as described previously by Girault et al. (6). The FERM domains of Jak1 and FAK only show low sequence identity (8–9% for Jak1 and 13% for FAK) with these three classical FERM domains which share 66–89% sequence identity. Similarly, the murine Jak1 FERM domain is rather distantly related to the FAK FERM domain (~9% sequence identity). As illustrated in the alignment shown in Fig. 1A, the Jaks contain several large insertions (insertions 1–4) which are not present in the other FERM domains. These insertions seem to be mostly unstructured and show little homology between the different family members. However, many
FIGURE 1. The structure-based alignment of the Jaks and model of the Jak1 FERM domain. A, FERM domain alignment of Jak1, Jak2, Jak3, and Tyk2 with other FERM domains. The sequences of the Jak FERM domains of different origin (h, human; m, mouse) were aligned with the FERM domain of human moesin (12), human radixin (13), human merlin (16), and chicken FAK (15). Secondary structure characteristics are given below following the common nomenclature (S, strand; H, helix; 3, 310-helix). Residues that are highly conserved among the Jak sequences are highlighted in blue. Residues conserved in classical ezrin, radixin, moesin (ERM) proteins are marked in red. If these residues were conserved in FAK or most of the Jaks the corresponding residues were also marked in red in these sequences. Residues conserved in the divergent FERM domains of FAK and Jaks are highlighted in green. Mutated residues are highlighted in yellow. ssFK represents the secondary structure of cFAK. pssJ1 and pssJ2 represent the secondary structure prediction of Jak1 and Jak2, respectively. Insertions 1–3 within the Jaks are indicated.

B, Model structure of the Jak1 FERM domain illustrating the position of the hydrophobic residues that were selected for mutagenesis. The location of insertions 1–4 is indicated. C, Positions of the mutated amino acids in solved structures of the radixin and FAK FERM domains. Representation of the amino acids that were chosen for the Jak1 mutagenesis study in the solved FERM domain structures of radixin (13) and FAK (15).
structurally important residues are well-conserved between Jaks, FAK, and the classical FERM domain proteins. To better evaluate the effect of introduced mutations in Jak1, we generated a model structure of the Jak1 FERM domain based on the solved structure of the FAK FERM domain (15) (Fig. 1B). The large insertions present in Jak1 were not included in the model as there is no rationale for a possible conformation. We chose to mutate specific conserved hydrophobic core residues extremely conserved in FERM domains to investigate which function of Jak1 is lost upon mutation. We introduced two double mutations, L158A/F159A and L166A/I167A, as well as single mutations of Y270, Y112, and Y281 to alanine (highlighted in Fig. 1A and B). The residues L158, Y112, and Y281 are involved in hydrophobic core interactions between the F1 and F2 domain while the residues F159, L166, I167, and Y270 are part of the hydrophobic core of the F2 subdomain. Substitution of K133 to glutamate was a negative control which was not supposed to have any effect because this residue is part of a hydrophilic region. Another negative control was the Jak1-K907E mutant, which is known to render the kinase inactive (22), but has been described not to interfere with binding to cytokine receptors, at least in overexpression systems (24). Fig. 1B shows the location of the introduced FERM mutations in the model structure of Jak1. A comparison of the FERM domain of radixin with the distantly related FERM domain of FAK (Fig. 1C) illustrates that the location of these residues is highly conserved in FERM domains. To further evaluate the importance of the mutated hydrophobic residues within a FERM domain structure, we investigated the degree of conservation of the mutated residues in an additional sequence alignment of the predicted FERM domain of Jaks with 95 other FERM domain sequences of 37 different proteins of different species (excluding Jaks) that was generated by the Multalign software (version 5.4.1) (Ref. 25; data not shown). We found the hydrophobic residues L158, F159, L166, Y270, and Y281 to be conserved to 100% and the residues I167 and Y112 to be conserved to 97 and 96%, respectively.

Mutations in the FERM domain of Jak1 impair IFN-γ-induced protein expression

Taking advantage of the Flp-In system, we introduced the Jak1 mutants stably expressing WT Jak1 or the Jak1 mutants were stimulated with IFN-γ for 1 or 2 days or left untreated. Cells were lysed and subjected to Western blot analysis. The signals were detected using the ECL system. B, U4C cells stably expressing WT Jak1 or the Jak1 mutants were stimulated for 3 days with IFN-γ before MHC-I expression was monitored by FACS analysis using an MHC-I-specific Ab. Histograms from unstimulated cells are shown as a solid line; those from cells treated with IFN-γ are depicted in gray.

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FIGURE 2. Selected Jak1 mutants show impaired gene expression upon IFN-γ stimulation. A, U4C cells stably expressing WT Jak1 or the Jak1 mutants were stimulated with IFN-γ for 1 or 2 days or left untreated. Cells were lysed and subjected to Western blot analysis. The blots were counterstained with Abs against control proteins Fin13 and calnexin (CNX), two proteins not regulated upon IFN-γ stimulation. The signals were detected using the ECL system. B, U4C cells stably expressing WT Jak1 or the Jak1 mutants were stimulated for 3 days with IFN-γ before MHC-I expression was monitored by FACS analysis using an MHC-I-specific Ab. Histograms from unstimulated cells are shown as a solid line; those from cells treated with IFN-γ are depicted in gray.

FIGURE 3. Certain Jak1 mutants show an impaired signal transduction through the Jak/STAT pathway upon IFN-γ stimulation. A, U4C cells stably expressing WT Jak1 or the Jak1 mutants were stimulated with IFN-γ for 1 or 2 days or left untreated. Cells were lysed and subjected to Western blot analysis. The blots were detected with a phosphospecific STAT1 Ab and reprobed with a STAT1 Ab and an Ab against calnexin (CNX) which is not regulated upon IFN-γ stimulation. B, U4C cells stably expressing WT Jak1 or the Jak1 mutants were stimulated with IFN-γ for different periods of time (as indicated) and Western blots of total cellular lysates were prepared. The blots were detected with a phosphospecific STAT1 Ab and reprobed with a STAT1 Ab.
important not to overexpress Jak1 because we have shown previously that overexpression of Jak1 can lead to artifacts in cytokine receptor binding (26). In addition, Jak overexpression leads to unphysiological autoactivation and an altered localization compared with Jaks expressed at endogenous levels (1, 10).

To investigate whether the point mutations of Jak1 have repercussions on gene transcription upon IFN-γ/H9253 stimulation, the stable U4C transfectants were treated with IFN-γ/H9253 for 1 or 2 days, before the expression of several IFN-γ-responsive genes was monitored. The expression of STAT1, IRF1, and lung resistance protein (LRP) (22, 27) was investigated by Western blot (Fig. 2A). Fin13 and calnexin were monitored as control proteins which are not regulated by IFN-γ/H9253. Compared with wild-type (WT) Jak1, the mutants Jak1-L158A/F159A and Jak1-Y281A showed no or only slight differences in target gene expression. However, IRF1, STAT1, and LRP were not up-regulated in U4C-Jak1-L166A/I167A and U4C-Jak1-Y270A cells while they were reduced in U4C-Jak1-Y112A cells. In the stable U4C transfectants expressing Jak1-K907E, we still found low-level expression of IRF1 and STAT1 upon IFN-γ/H9253, as described previously (22). Interestingly, the effect of the mutants Jak1-L166A/I167A and Jak1-Y270A was equal to or even more pronounced than the one of Jak1-K907E. Because it has been shown that the presence of kinase-inactive Jak1 in the IFN-γ-signaling complex is important for a residual gene expression (22), the before-mentioned observation could mean that the two mutants do not bind the IFN-γRα anymore. Fig. 2B shows a reduced MHC-I expression, analyzed using flow cytometry, of those mutants showing reduced target gene expression in Western blots. The up-regulation of MHC-I surface expression in U4C-Jak1-L166A/I167A and U4C-Jak1-Y270A in response to IFN-γ was strongly impaired compared with cells expressing Jak1-WT, -L158A/F159A, and -Y281A (Fig. 2B) while again cells expressing Jak1-Y112A exhibited a reduced expression of the target protein.

Mutations in the FERM domain of Jak1 impair IFN-γ-induced STAT activation

The stable U4C transfectants were stimulated with IFN-γ for 1 or 2 days, cells were lysed and the STAT1 activation was monitored by detection of Western blots with a phosphospecific STAT1 Ab. U4C-Jak1-L166A/I167A and U4C-Jak1-Y270A cells revealed no STAT1 activation after 1 or 2 days of continuous stimulation. U4C-Jak1-Y281A and U4C-Jak1-L158A/F159A cells showed no significantly reduced STAT1 activation. U4C-Jak1-Y112A cells, however, presented clearly reduced STAT1 activation (Fig. 3A). The stable U4C cells were also stimulated with IFN-γ for different periods of time (5–90 min) to investigate possible differences in short-term kinetics. Lysates were prepared, separated by SDS-PAGE, subjected to Western blot analysis and STAT1 phosphorylation was monitored using a phosphotyrosine-specific Ab for detection. The Jak1 mutants L166A/I167A and Y270A led to a strongly impaired STAT phosphorylation upon IFN-γ stimulation (Fig. 3B) as did the kinase-negative control mutant Jak1-K907E. Again, U4C-Jak1-Y112A cells exhibited reduced STAT1 activation. The activation kinetics of this mutant, however, did not seem to be influenced. The same observations were made when investigating IFN-α stimulation kinetics, which uses Jak1 and Tyk2 in its signal transduction cascade (data not shown).

Mutations in the FERM domain of Jak1 impair IFN-γ-induced apoptosis of PIC-treated cells

PIC is a synthetic dsRNA that simulates a viral-infected state in cells. It has been described that treatment of cells with PIC and IFNs can induce apoptosis (28). We tested those Jak1 mutant cell lines that displayed a reduced gene induction and STAT1 activation for IFN-γ-induced apoptosis upon PIC treatment.

**FIGURE 4.** Mutations in the FERM domain of Jak1 impair IFN-γ-induced apoptosis of PIC-treated cells. A, Stable U4C and y2A transfectants were stimulated with etoposide, PIC, IFN-γ, and PIC/IFN-γ in combination or were left untreated for 48 h. Apoptosis was monitored by flow cytometry as described in Materials and Methods. Etoposide treatment was used as a positive control for apoptosis induction. The results are represented as percent apoptotic cells. B, U4C-Jak1, U4C-Jak1-K907E, U4C-Jak1-L166A/I167A, U4C-Jak1-Y270A, and U4C-Jak1-Y112A cells were treated with etoposide, PIC/IFN-γ, or were left untreated for 48 h. Apoptosis was monitored as described in A.
The stable U4C transfectants were stimulated with etoposide, PIC, IFN-γ/H9253, and PIC/IFN-γ/H9253 in combination or were left untreated for 48 h. Apoptosis was monitored by flow cytometry as described in Materials and Methods. Etoposide treatment was used as a positive control for apoptosis induction. Fig. 4A shows that only γ2A-Jak2, but not γ2A-Jak2-K882E, cells underwent apoptosis effectively when treated with IFN-γ and PIC. Treatment with IFN-γ or PIC alone had little effect on apoptosis induction. Exactly the same effect could be observed in the reconstituted Jak1-deficient U4C cells. U4C-Jak1 but not U4C-Jak1-K907E cells underwent apoptosis effectively when treated with IFN-γ and PIC. Thus, both Jak1 and Jak2 are necessary for the induction of apoptosis upon treatment with IFN-γ and PIC.

Then, U4C-Jak1, U4C-Jak1-K907E, U4C-Jak1-L166A/I167A, U4C-Jak1-Y270A, and U4C-Jak1-Y112A cells were treated with etoposide, the combination of PIC and IFN-γ or were left untreated for 48 h (Fig. 4B). U4C-Jak1-L166A/I167A and U4C-Jak1-Y270A cells showed no induction of apoptosis compared with U4C-Jak1-K907E while the effect of U4C-Jak1-Y112A was intermediate. Thus, the mutants behave in the same way in the IFN-γ/PIC-mediated apoptosis assay as they do in the experiments shown in Figs. 2 and 3 where IFN-γ-mediated STAT activation and gene induction were measured.

Mutations in the FERM domain of Jak1 impair binding to the IFN-γRα

We performed coprecipitation studies to investigate whether the impaired signaling capacity of the Jak1 mutants results from impaired IFN-γRα association. We tested the Jak1 mutants for their ability to

![FIGURE 5. Association of the Jak1 mutants to the endogenous IFN-γRα. Stable U4C transfectants expressing the Jak1 mutants were lysed and subjected to immunoprecipitation using an IFN-γRα Ab. Western blots of the immunoprecipitates and lysates were analyzed using Abs against IFN-γRα, Jak1, and Fin13. IP, Immunoprecipitation; Lys, lysates.](image)

![FIGURE 6. The Jak1-Y112A mutant is constitutively phosphorylated. A, U4C cells stably expressing WT Jak1 or the Jak1 mutants were starved for 6 h or left untreated. Nonstarved cells were then stimulated with IFN-γ for 20 min or left untreated. Cells were lysed and subjected to immunoprecipitation using a Jak1 Ab. The Western blot of the immunoprecipitates was detected with a phosphotyrosine Ab (PY) and reprobed with a Jak1 Ab. B, U4C cells stably expressing WT Jak1 or the Jak1-Y112A mutant were stimulated with IFN-γ for 20 min or left untreated, as indicated. Cells were lysed and subjected to immunoprecipitation using a Jak2 Ab. Western blots of the immunoprecipitates were then detected with a PY and reprobed with a Jak2 Ab. C, U4C cells stably expressing WT Jak1 or the Jak1-Y112A mutant were incubated with Jak inhibitor I (1 μM) for 30 min and stimulated with IFN-γ for 20 min or left untreated, as indicated. Western blots of the lysates were detected with a phosphospecific STAT1 Ab and reprobed with a STAT1 Ab. D, U4C cells stably expressing WT Jak1 or the Jak1-Y112A mutant were incubated with Jak inhibitor I (1 μM) for 3 h. Cells were lysed and the lysate was subjected to immunoprecipitation using a Jak1 Ab. The Western blot of the immunoprecipitates was detected with a PY and reprobed with a Jak1 Ab.](image)
bind to the endogenous IFN-γRα of the stable U4C transfectants. After lysis of the cells, the IFN-γR was precipitated with an anti-IFN-γRα Ab and Jak1 binding was assessed by immunodetection of Western blots (Fig. 5). All the mutants, except for the negative control Jak1-K133E, revealed an impaired association to the IFN-γRα. Jak1-L166A/I167A and Jak1-Y270A showed no residual binding at all. The binding of the other mutants, Jak1-L158A/F159A, -Y281A, and -Y112A, was reduced. The reduced binding of the latter mutants could be due to denaturation effects by the use of the detergent-containing lysis buffer. Interestingly, this result shows that even those Jak1 mutants, which in living cells do not (L158A/F159A; Y281A) or only moderately disturb (Y112A) the function, can be destabilized by the presence of detergent, indicating that these amino acids are indeed involved in hydrophobic core interactions.

**Jak1-Y112A shows increased constitutive Jak1 phosphorylation**

To investigate phosphorylation of the Jak1 mutants, cells were stimulated with IFN-γ and total cellular lysates were prepared. Part of each lysis was used for an immunoprecipitation with a Jak1 Ab. The immunoprecipitates and the lysates were then subjected to SDS-PAGE and Western blotting (Fig. 6A). Cells expressing a kinase-negative Jak1 mutant Jak1-K907E were used as negative control. We also compared starved cells and nonstarved cells to those which were stimulated with IFN-γ. Interestingly, a strongly enhanced constitutive phosphorylation of the Jak1-Y112A mutant was always observed in the unstimulated lanes. In U4C-Jak1-Y281A cells, a minor constitutive phosphorylation could also be observed but not as consistently as in U4C-Jak1-Y112A cells. However, no constitutive STAT phosphorylation could be observed in the corresponding lysates (data not shown, see also unstimulated lanes in Fig. 3). Thus, mutation of Y112 to alanine leads to a constitutive Jak1 phosphorylation without, however, promoting constitutive STAT1 phosphorylation.

Interestingly, phosphorylation of Jak2, the other kinase present in the IFN-γ-signaling complex, was reduced in IFN-γ-stimulated U4C-Jak1-Y112A in comparison to cells expressing Jak1-WT (Fig. 6B). This is consistent with and explains the reproducibly lower STAT1 activation and target gene expression observed in IFN-γ-stimulated U4C-Jak1-Y112A cells (Figs. 2 and 3).

To test whether the Jak1-Y112A mutant is still inhibited by Jak inhibitor I, we stimulated U4C-Jak1-WT and U4C-Jak1-Y112A with IFN-γ in the presence or absence of a Jak-specific inhibitor (Jak inhibitor I) and we observed that the STAT1 phosphorylation was effectively repressed by this inhibitor (Fig. 6C). To investigate whether the constitutive phosphorylation of Jak1-Y112A was mediated by Jaks or other kinases, we left U4C-Jak1-Y112A cells untreated and incubated them in presence or absence of Jak inhibitor I for 3 h. Western blots of Jak1-Y112A immunoprecipitates from so treated cells showed that the phosphorylation of Jak1-Y112A is reduced upon pretreatment with Jak inhibitor I (Fig. 6D). Thus, the constitutive phosphorylation of this Jak1 mutant is likely due to Jak activity.
**Table I. Overview of the effects of the different Jak1 mutants in reconstituted U4C cells**

| Mutated in Jak1 | Localization in the FERM Domain | Corresponding Amino Acid | Gene Induction upon IFN-γ | STAT Activation upon IFN-γ | Apoptosis upon IFN-γ and PIC | Binding in IFN-γ-Rx Complex | Constitutive Jak1 Phosphorylation upon IFN-γ |
|----------------|----------------------------------|--------------------------|--------------------------|----------------------------|----------------------------|-----------------------------|----------------------------------|
| Y112A          | Central hydro. core              | F85 L129                 | ++                       | +                          | +                          | +                           | +                                |
| L158A          | Central hydro. core              | F102 F147                | ++++                     | ++++                       | /                          | +                           | −                                |
| F159A          | F2 hydro. core                   | F103 Y148                | −                        | −                          | −                          | −                           | −                                |
| L166A          | F2 hydro. core                   | I110 Y155                | −                        | −                          | −                          | −                           | /                                |
| I167A          | F2 hydro. core                   | L111 M156                | −                        | −                          | −                          | −                           | −                                |
| Y270A          | F2 hydro. core                   | Y191 F243                | −                        | −                          | −                          | −                           | /                                |
| Y281A          | Central hydro. core              | F201 F253                | ++++                     | ++++                       | /                          | +                           | ±                                |
| WT             | Not mutated                      | F281                      | ++++                     | ++++                       | ++++                       | ++++                        | ++++                            |
| K907E          | Kinase-deficient mutant          | −                        | −                        | −                          | −                          | −                           | −                                |

*Not detected.

**Mutation of the kinase domain of Jak1 has repercussions on IFN-γ-Rx binding**

The Jak1-K907E mutation was described not to interfere with binding to cytokine receptors in overexpression systems (24). Surprisingly, we found that coinmunoprecipitation of Jak1-K907E with IFN-γ-Rx was reduced in comparison to Jak1-WT (Fig. 7A). To confirm the activation status of the Jak1 mutants, cells were stimulated with IFN-γ and total cellular lysates were prepared. Part of each lysate was used for an immunoprecipitation with a Jak1 Ab. The immunoprecipitates and the lysates were then subjected to SDS-PAGE and Western blotting (Fig. 7B). As expected, cells expressing the kinase-negative Jak1 mutant Jak1-K907E showed no activation of STAT1 after OSM and IFN-γ. OSM was included here because it induces a stronger Jak1 phosphorylation than IFN-γ. Thus, the kinase-inactivating mutation K907E abrogates Jak1 signaling and clearly reduces receptor association when expressed at near-endogenous levels.

Surprisingly, Jak1-K907E is not phosphorylated upon stimulation by OSM or IFN-γ (Fig. 7B), in contrast to a previous report with reconstituted Jak1-negative U4A fibrosarcoma cells (22). To investigate the activation status of Jak2, the U4C-Jak1-K907E, U4C-Jak1-WT, U4C, and γ2A cells (Jak2-deficient) were stimulated with IFN-γ and Jak2 immunoprecipitates were analyzed by immunodetection of Western blots (Fig. 7C). Interestingly, Jak2 is not phosphorylated in U4C-Jak1-K907E cells upon stimulation by IFN-γ, which is also in contrast to the previous report (22). To further elucidate the role of Jak1 and Jak2 in IFN-γ signal transduction we reconstituted Jak2-defective γ2A-FRT cells with Jak2-WT and kinase-deficient Jak2-K882E. Interestingly, the kinase-deficient Jak2-K882E could not prevent stimulation-dependent Jak1 phosphorylation, although Jak1 phosphorylation was lower compared with cells reconstituted with WT Jak2 (Fig. 7D).

Thus, Jak2 could not be phosphorylated in presence of kinase-dead Jak1 while Jak1 became phosphorylated in the presence of kinase-dead Jak2. These data suggest a primary role for Jak1 in initiating/triggering the IFN-γ response, which is in contrast to the previous model (22). This discrepancy might be explained by lower expression levels as in the present study Jak1 and Jak2 were expressed at near-endogenous levels in U4C-FRT and γ2A-FRT cells.

**Discussion**

Jaks contain a divergent FERM domain that is only remotely related to classical FERM domains and to the FAK FERM domain (6). Because structures of classical FERM domains (ezrin, radixin, moesin . . . ) and of the divergent FERM domain of FAK have been published, we generated a structure-based alignment of these FERM domains with those of the Jaks. This alignment (Fig. 1A) shows that hydrophobic core residues within the three subdomains, and between them, are highly conserved and that the Jaks contain several large insertions (insertions 1–4) not present in the other FERM domains. Several small insertions, some of which containing secondary structure elements such as 310 helices and an additional α-helix in the F2 domain not found in classical FERM domains, are present in the FERM domain of FAK (15). However, these insertions do not match the insertions found in the Jaks. Here, the insertions seem to be mostly unstructured and show little homology between the different family members so that no reliable prediction concerning their structure and function is possible. In a previously generated model structure of Jak2 (29), these regions were modeled by using loop-searching techniques. However, a similar approach would not have generated any reliable prediction in the case of Jak1, as the insertions are much larger than in Jak2.

The most striking difference between the structurally solved domains of FAK and classical FERM domain proteins such as radixin is the orientation of the F3 subdomain (15). Ceccarelli et al. (15) found the F3 lobe to be rotated and shifted with respect to the F1 and F2 subdomains so that the F3 subdomain is less tightly associated with the F2 subdomain whereas it is more intimately linked to the F1 lobe (Fig. 1C). This divergence in structure very likely reflects a difference in function, as the FAK FERM domain, in contrast to the FERM domain of radixin, cannot bind phosphoinositides in the cleft between the subdomains F1 and F3. In the Jaks, the basic residues contacting phosphoinositides in classical FERM domains are equally poorly conserved, suggesting that the orientation of the F3 lobe in Jak2 could rather resemble the orientation found in FAK than the one seen in the classical FERM domains. However, it is conceivable that the F3 domain of the Jaks could adopt yet another orientation. Nevertheless, predictions concerning the structurally important residues can be made from the model as these show a high degree of conservation even between distantly related proteins (see Fig. 1, B and C).

We mutated highly conserved hydrophobic core residues to investigate which function of Jak1 is lost upon mutation, because such mutations can impair or destabilize the structure of the corresponding subdomain. This strategy has proven to be useful as shown for the F1 subdomain of the Jak1 FERM domain previously (8). In a model structure supported by a mutagenesis study, residues of structural importance as well as surface-exposed residues involved in cytokine receptor interactions could be identified in Jak1 (8). With this Jak1-F1 model, we were also able to explain effects of mutations in the F1 subdomain described for other Jak proteins (Refs. 30 and 31; discussed in Refs. 8 and 32). We used the same approach to investigate the functions of the F2-FERM...
subdomain and the function of the central hydrophobic core between the F1, F2, and F3 domains which stabilizes the FERM domain subunits in their clover-shaped orientation. Jak mutants were expressed at slightly subendogenous levels in Jak1-negative U4C-Flp-In cells allowing expression of heterologous proteins from the same genomic site (1). Jak2 and Jak2-K882E were expressed in Jak2-deficient γ2A-Flp-In cells also using the Flp-In system at near-endogenous levels (for a summary of the results, see Tables I and II).

The mutants Jak1-L158A/F159A, -Y112A, and -Y281A showed a reduced binding to the IFN-γRα (Fig. 4), although the introduction of these mutants does not have any or only moderate repercussions on expression of target genes (Figs. 2 and 3) and PIC/IFN-γ-induced apoptosis (Fig. 4). Thus, even though these mutations do not influence Jak function in intact cells, they clearly destabilize the protein complex in detergent-containing binding assays as the coprecipitation assay (Fig. 5), arguing in favor of an involvement of those residues in hydrophobic core interactions as predicted by the structure-based Jak1-FERM domain model. The amino acids Y112, L158, and Y281 are involved in hydrophobic core interactions between the F1 and F2 subdomains (Fig. 1, B and C) while F159 is involved in the same type of interactions in the F2 domain. Interestingly, two other mutants Jak1-L166A/I167A and Jak1-Y270A showed a slightly reduced expression in the stable transfectants (Fig. 5) which is an indication for a reduced stability due to a perturbation of the structural integrity by mutation of the hydrophobic core residues. These two Jak1 mutants do not sustain IFN-γ-induced signaling and gene expression (Figs. 2 and 3) nor do they bind to the IFN-γRα (Fig. 5). These amino acids (L166, I167, and Y270) are involved in hydrophobic core interactions within the F2 subdomain (Fig. 1, B and C). Thus, mutations in the hydrophobic core of the F2 subdomain seem to have a more deleterious effect on Jak1 binding to the IFN-γRα and subsequent ligand-dependent signaling events than mutations in the central hydrophobic core between F1 and F2 (Y112, L158, and Y281). Together with our previous finding that mutations in the hydrophobic core of the F1 subdomain abrogate Jak1 binding to various cytokine receptors, this means that both the F1 and the F2 subdomain contain regions which are crucial for Jak1 binding to cytokine receptors and that the orientation of the F1 and F2 subdomains toward each other or the receptor which is stabilized by the central hydrophobic core is of lesser importance to cytokine receptor binding.

Strikingly, in U4C-Jak1-Y112A cells IFN-γ-induced biologic responses as PIC and IFN-γ-induced apoptosis, gene expression, and signal transduction events (STAT1 tyrosine phosphorylation, Jak2 phosphorylation) were reproducibly reduced in comparison to Jak1-WT (Figs. 2, 3, and 6) cells while this Jak1 mutant shows a constitutive Jak1 tyrosine phosphorylation (Fig. 6). However, no constitutive STAT activation was observed (Figs. 2 and 3). This amino acid substitution which is located at the center of the central hydrophobic core between F1 and F2 thus has an impact on kinase function. We conclude that the central hydrophobic core is involved in orienting the FERM subdomains in a manner that prevents “inhibitory” phosphorylation of Jaks. For other Jaks, mutation of the corresponding residue (Y105 in Jak3 and Y119 in Jak2) performed in a different context than ours show varying effects. For Jak3, no effect on receptor binding could be found when overexpressing both receptor and Jak3-Y105A (33). For Jak2-Y119E, a constitutive phosphorylation but no concomitant STAT5 phosphorylation and an impaired signal transduction was reported (34).

A constitutive phosphorylation with an associated inhibition of Jak activity and no constitutive STAT phosphorylation has earlier been described for mutations in the kinase-like domain of Jaks (5, 35). Thus, both the FERM domain and the kinase-like domain seem to be involved in keeping the protein in a low phosphorylated state, and Jaks with mutations leading to constitutive phosphorylation have defects in stimulation-dependent signal transduction.

Surprisingly, the kinase-defective Jak1-K907E mutant bound less well to IFN-γRα compared with Jak1-WT in stably transfected U4C cells expressing the kinases at near-endogenous levels (Fig. 7A). In contrast, Jak1-K907E and Jak1-WT bound equally well to a chimeric cytokine receptor in an overexpression system (24). We have described before that the expression level of a Jak can drastically influence its apparent binding behavior to cytokine receptors (26) and its localization (1, 10). In addition, it is known that overexpressed Jaks autoactivate themselves cytokine independently (36, 37), so one has to be cautious with the interpretation of experiments with overexpressed Jaks. Thus, different expression levels may also be the reason for our current findings that upon IFN-γ stimulation 1) in U4C-Jak1-K907E cells neither this Jak1 mutant nor Jak2 was phosphorylated, and 2) that in γ2A-Jak2-K882E cells this Jak2 mutant was not phosphorylated whereas a weak phosphorylation of Jak1 could be observed (Fig. 7). These data are in contrast to an earlier study with higher expression levels in reconstituted fibrosarcoma cells which lead to a model giving Jak2 the primary role in initiating/triggering the IFN-γ response through Jak activation (22). We conclude from our present data on a mutation in the kinase domain can influence not only the enzymatic activity, but can also have repercussions on the binding behavior of the kinase to cytokine receptors.

Small molecule kinase inhibitors like staurosporine have also been described to lead to conformational changes upon binding to the catalytic cleft of kinase domains (38) and Jak3 binding to the common γ-chain was found to be impaired by addition of staurosporine to an in vitro-binding assay (33). In fact, the ATP-binding pocket is in direct proximity to the site where K907 in Jak1 is situated. However, upon preincubation of stable U4C-Jak1 cells with the broad spectrum inhibitor staurosporine or with the specific Jak inhibitor I and subsequent coinmunoprecipitation of Jak1 with the IFN-γRα, we found that both inhibitors abolished Jak-mediated signaling without interfering with the Jak/receptor interaction (data not shown).
events upon cytokine stimulation as has been described before for the pseudokinase domain. In contrast, also mutations in the kinase domain of Jak1 can have repercussions on IFN-γRα binding. Thus, there seems to be a complex interplay between the FERM domain and the kinase domain to ensure “correct” receptor association and stimulation-dependent catalytic activity.

Knowledge of the Jak/receptor-binding interface can be helpful in the design of low molecular mass inhibitors of cytokine signaling of potential therapeutic value. Such compounds could be used in combination with ATP-competitive inhibitors to achieve a greater selectivity for a certain cytokine/receptor complex.

Disclosures

The authors have no financial conflict of interest.

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