Mapping of a Region within the N Terminus of Jak1 Involved in Cytokine Receptor Interaction*

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Janus kinase 1 (Jak1) is a cytoplasmic tyrosine kinase that noncovalently associates with a variety of cytokine receptors. Here we show that the in vitro translated N-terminal domains of Jak1 are sufficient for binding to a biotinylated peptide comprising the membrane-proximal 73 amino acids of gp130, the signal-transducing receptor chain of interleukin-6-type cytokines. By the fold recognition approach amino acid residues 36–112 of Jak1 were predicted to adopt a β-grasp fold, and a structural model was built using ubiquitin as a template. Substitution of Tyr107 to alanine, a residue conserved among Jak family proteins, is predicted to impair interaction of the proposed β-grasp domain, abrogating binding of full-length Jak1 to gp130 in COS-7 transfectants. By further mutagenesis we identified the loop 4 region of the Jak1 β-grasp domain as essential for gp130 association and gp130-mediated signal transduction. In Jak1-deficient U4C cells reconstituted with the loop 4 mutants L80A/Y81A and Δ(Tyr81–Ser84), the interferon-γ, interferon-α, and interleukin-6 responses were similarly impaired. Thus, loop 4 of the β-grasp domain plays a role in the association of Jak1 with both class I and II cytokine receptors. Taken together the structural model and the mutagenesis data provide further insight into the interaction of Janus kinases with cytokine receptors.

Cytokines are involved in a variety of biological processes including hematopoiesis and the regulation of the immune system. Many cytokines signal via tyrosine kinases of the Janus family (Jaks) and STAT (signal transducer and activator of transcription) transcription factors. Jaks are large enzymes (molecular mass, 120–140 kDa) that are cytoplasmically pre-associated with signal-transducing cytokine receptor subunits (1). Upon cytokine-induced receptor aggregation, Jaks are activated likely by auto- and transphosphorylation. Tyrosine residues within the cytoplasmic tail of the receptor are subsequently phosphorylated by the kinases, providing docking sites for Src homology 2 domain-containing signaling proteins including STATs, tyrosine phosphatases, and suppressors of cytokine signaling. Tyrosine-phosphorylated STATs homo- or heterodimerize and translocate to the nucleus where they bind to specific DNA sequences in the promoter regions of their respective target genes (2, 3).

Whereas the structure/function relationship for the interaction between cytokines and the extracellular parts of their receptors is reasonably well understood and the structures of STATs bound to enhancer sequences have been solved (4–7), no structural information is available on the interaction of the cytoplasmic parts of the signal-transducing subunits of cytokine receptors with the associated Janus kinases. Structural information on the receptor-kinase complex, however, is crucial to understand the binding specificity and the activation process of Janus kinases, which is the initial event of the intracellular signal transduction cascade after ligand binding to the extracellular part of cytokine receptors. The Jak family of cytoplasmic tyrosine kinases comprises four mammalian members. Three, Jak1, Jak2, and Tyk2, are expressed in a wide variety of tissues, whereas Jak3 expression is restricted to cells of the hematopoietic system. Based on sequence similarities between the Jak family members it has been suggested that seven Jak homology (JH) domains exist in Jaks (8) (see Fig. 1C). The JH1 domain, at the C terminus, is a classical kinase domain. It is N-terminally preceded by the JH2 domain (pseudokinase domain), which has no catalytic activity. The N-terminal half of the Jak family members, domains JH3 to JH7, is involved in binding to cytokine receptors. The minimal binding regions of Tyk2, Jak2, and Jak3 have been further restricted to the JH6 domains (9–12). Patients with a point mutation within the JH7 domain of Jak3, Y100C, suffer from severe combined immunodeficiency (13). Mutagenesis of the region, comprising amino acids 98–102 of Jak3, showed that Tyr100 and the amino acids Leu98 and Ile102 are crucial for Jak3 binding to the common IL-2 receptor γ-chain (12). Despite the existing information the details of the Jak/cytokine receptor interaction are far from understood. No structural information on the N-terminal region of Jaks is available at present, and functional modular binding domains such as Src homology 2 or Src homology 3 domains have not been identified in Janus kinases (14). Recently a part of the N terminus of the Jaks corresponding to the amino acids 24–415 in Jak1 has been reported to share significant sequence similarity with the so-called band 4.1 domain, indicating that the N termini of the Janus kinases might represent divergent members of the classical band 4.1 domain (15).

Our laboratories have been studying signal transduction in

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† The abbreviations used are: Jak, Janus kinase; EMSA, electrophoretic mobility shift assay; FERM, 4.1, ezrin, radixin, moesin; IFN, interferon; IL, interleukin; JH, Jak homology; IL-5R, IL-5 receptor; STAT, signal transducer and activator of transcription; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; SIE, c-Sis-inducible element; IFNaR, IFNa receptor.
response to interleukin-6-type cytokines and the interferons (IFNs). More particularly, we are interested in the interaction of gp130 with Jak1, the kinase essential for gp130 and STAT activation (16, 17). It is known that the membrane-proximal region, including box1 and box2 of gp130, is involved in interaction with Jak1 (18, 19), but the region of Jak1 required for this interaction has not been defined. By a combined approach of molecular modeling and site-directed mutagenesis we have identified a region in the N-terminal domain of Jak1 crucial for its interaction with gp130.

EXPERIMENTAL PROCEDURES

Model of the β-Grasp Domain of Jak1—For fold recognition the program package ProCeryon (a software package for fold recognition and protein structure analysis from King’s Beech Biosoftware, 1999) was used that is based on a knowledge-based force field derived from a set of known protein conformations (20). A library of 4500 protein structures and the N-terminal sequences of the Jakks. All 4500 generated models were evaluated and ranked using different ProSA-II type z-scores based on pair interactions and surface terms (20, 21). With the sequential alignment derived from the fold recognition approach a detailed model of the N-terminal domain of Jak1 was built using the x-ray structure of ubiquitin (PDB accession code 1ub) as the template. Based on this alignment, amino acid residues were exchanged in the template. Insertions and deletions were modeled by using a data base approach included in the software package WHATIF (22). The data base was searched for a peptide sequence of the appropriate length, which was fitted to the template. All loops were selected from the data base so as to give a minimal root mean square distance between the ends of the loops. In the final step the three-dimensional structural models were energy-minimized using the steepest descent algorithm implemented in the GROMOS force field (23). For graphical representation the Ribbons program (24) was used. All programs were run on a Silicon Graphics Indigo work station.

Cell Culture and Transfection—Simian monkey kidney cells (COS-7) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 60 μg/ml penicillin. Cells were grown at 37 °C in a water-saturated atmosphere at 5% CO2. COS-7 cells were transiently transfected using the DEAE-chloroquine transfection method with modifications as described previously (25) or using Fugene (Roche Molecular Biochemicals) according to the manufacturer's instructions.

U4C cells (14) were grown in Dulbecco’s modified Eagle’s medium (BioWhittaker) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 400 μg/ml G418 (Life Technologies, Inc.). Cells were grown at 37 °C in a water-saturated atmosphere at 10% CO2. Transfections were carried out using Superfect (Qiagen) according to the manufacturer’s recommendations.

IL-5 was purchased from Cell Concepts (Umkirch, Germany). IL-6 treatments were carried out with a mixture of IL-6 (0.2 μg/ml) and soluble IL-6 receptor (0.5 μg/ml), both from R&D Systems. IFNα was a highly purified mixture of human subspecies (Wellferron, 1.5 × 108 IU/mg of protein) provided by Wellcome Research Laboratories (Beckenham, Kent, UK). Recombinant IFNγ (4 × 104 IU/mg of protein) was a generous gift from Dr. G. Adolf, Ernst Boehringer Institut für Arzneimittelforschung (Vienna, Austria). Each was used at 1000 IU/ml.

Generation of Jak1 Mutant Constructs—Standard cloning procedures were performed throughout this study. The mutations (resulting in amino acid substitutions L60A, L60A/Y81A, Y81A, ΔTyr3–Ser6, E83K, K86E, Y89A, R104E, and Y107A) were introduced by a polymerase chain reaction technique into pBS-Jak1, a pBluescript derivative containing the cDNA sequence of mJak1. The restriction enzymes EcoRV/Bsp119I were used for exchanging the wild type sequence of pBS-Jak1 with the respective mutated subsequences. pBS-Jak1 constructs were restricted using EcoRV and SmaI. The resulting fragment was inserted into the SmaI-digested eukaryotic expression vector pSVLΔEcorI. The integrity of all constructs was verified by DNA sequencing using the ABI PRISM 310 Genetic Analyzer (PerkinElmer).

Cell Lysis, Immunoprecipitation, and Western Blot Analysis—Cells were lysed on the dish with 500 μl of lysis buffer containing 1% Brij 96 (for coprecipitation studies) or Triton X-100 (for other studies), 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 10 mM PMSF, 1 mM benzamidine, 5 μM/ml aprotinin, 3 μM/ml pepstatin, 5 μM/ml leupeptin, and 1 mM EDTA. Lysates were cleared by centrifugation at 12,000 × g. After overnight incubation at 4 °C with antibodies, the immunoprecipitates were collected with protein A-Sepharose (1 h, 4 °C), washed three times with lysis buffer or, for coprecipitation studies, with washing buffer (0.1% Brij 96, 20 mM Tris/His, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 10 mM PMSF, 1 mM benzamidine, 5 μM/ml aprotinin, 5 μM/ml pepstatin, 1 mM ml leupeptin, and 1 mM EDTA) and analyzed further by SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech) and probed with the respective antibodies, and signals were detected using the ECL system (Amersham Pharmacia Biotech). A polyclonal serum against Jak1 kindly provided by Dr. A. Ziemiecki, Bern, Switzerland, was used as anti-Jak1 polyclonal antiserum (from Dr. A. Ziemiecki) were used for detection. The horseradish peroxidase-conjugated secondary antibodies were detected from Dako.

Electrophoretic Mobility Shift Assays (EMSAs)—COS-7 cells were stimulated 18 h post-transfection with 10 ng/ml IL-5 for 30 min. Protein concentrations of nuclear extracts (prepared as described in Ref. 26) were measured with the Bio-Rad protein assay. A double-stranded mutated S1 oligonucleotide from the c-fos promoter (m6S1E, 5′-GAT CTA CCA TGG TTT ATC TCC ACC CTT TGG TCC-3′) was annealed in the 5′ protruding ends with the Klenow enzyme using [γ-32P]ATP (3,000 Ci/mmol, 10 μCi/ml). Nuclear extracts containing 5 μg of protein were incubated with about 10 fmol (10,000 cpm) of probe in gel shift incubation buffer (10 mM HEPES, pH 7.8, 1 mM EDTA, 5 mM MgCl2, 10% glycerol, 5 mM dithiothreitol, 0.7 mM PMSF, 0.1 mM of poly(dI-dC), and 1 μg/ml bovine serum albumin) for 10 min at room temperature. The protein-DNA complexes were separated on a 4.5% nondenaturing polyacrylamide gel containing 7.5% glycerol in 0.25-fold Tris borate-EDTA at 20 V/cm for 4 h. Gels were fixed in a water solution of 10% methanol and 10% acetic acid for 30 min, dried, and autoradiographed.

U4C cells were stimulated 18–20 h post-transfection with IFNγ, IFNy, or IL-6 for 15 min, washed twice in ice-cold phosphate-buffered saline, and lysed in ice-cold 0.5% Nonidet P-40, 50 mM Tris/HCl, pH 8, 150 mM NaCl, 10% glycerol, 0.1 mM EDTA, 2 mM dithiothreitol, 50 mM NaF, 0.1 mM sodium orthovanadate, 100 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. The sequence of the oligonucleotide probe used corresponded to the high affinity SIE of the c-fos gene (5′-GTC-GACATTTCGCTGAAAATC-3′). Probes were end-labeled with [γ-32P]ATP and aliquots equivalent to ~30,000 cpm/reaction were used in EMSAs (10 μl reactions in 10 mM HEPES, 7.5 mM MgCl2, 0.1 mM EDTA, 5% glycerol, 2.5 mg/ml bovine serum albumin, 0.5 mg/ml RNase A, 4% (v/v) Ficoll (Amersham Pharmacia Biotech). Protein-matched lysates were preincubated for 5 min at room temperature with 150 μg/ml poly(dI-dC) prior to incubation with probe for an additional 20 min at room temperature. Complexes were separated on 6% nondenaturing acrylamide gels in 0.5% Tris-glycine-EDTA and detected by autoradiography of dried gels.

In Vitro Translation of Jak1 Constructs—JH3-4, JH3-5, JH5-7, and JH6-7 domain constructs were cloned from human Jak1 into pET28a by polymerase chain reaction. The JH3-7 construct was cloned into pET14b following a NdeI/BamHI digest of human Jak1. The amino acid residues encoded by the constructs were as follows (numbering is for human Jak1): 351–546 (JH3-5), 33–252 (JH3-7), 33–295 (JH5-7), and 1–655 (JH3-7). For in vitro translation and [35S]methionine labeling of polypeptides, TNT Capped Ribucylcone Lysate Systems (Promega) and Redivue L-[35S]methionine (Amersham Pharmacia Biotech) were used according to the manufacturers’ instructions. T7 RNA polymerase and T3 RNA polymerase were used for the pET constructs (Jak1 wild type and loop 4 mutants), respectively.

gp130 Box1/Box2 Receptor Peptide Pull-Down Assay—The synthetic, biotinylated box1/box2 peptide comprised the first 73 amino acids of the gp130 cytoplasmic domain, while the non-Jak binding mutant was generated by replacing critical proline residues in the box1 motif (FN-EGP) with alanines (see Fig. 2A (18), prepared by Nils-Olaf Reilly, Protein Synthesis Laboratory, ICPR). Biotinylated box1/box2 peptide or mutant box1/box2 peptide or 50–100 μg of an unrelated biotinylated peptide (88-mer) were incubated with 25 μl of in vitro translated Jak1 peptide in 450 μl of binding buffer (0.25% Brij 96, 50 mM Tris/HCl, pH 8, 150 mM NaCl, 10% glycerol, 0.1 mM EDTA, 0.1 mM sodium orthovanadate, 100 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF) overnight at 4 °C. 30 μM of streptavidin-agarose
Mapping of a Receptor Interaction Region in Jak1

Identification of a Putative β-grasp Domain within the N-terminal Region of Jak1—Fold recognition first analyzes which fold in a library of known protein structures would be energetically compatible with a new sequence. To this end a library of 4500 model structures of the N-terminal region of Jak1 was generated using the experimentally derived protein structures from the Protein Data Bank as templates (see “Experimental Procedures”). These model structures were evaluated and ranked using different ProSA II type z-scores based on pair interactions and surface terms (20, 21). This analysis revealed that the N-terminal Jak1 region could be accommodated in the β-grasp fold of ubiquitin with high z-scores for the pair interactions as well as the surface terms. The resultant structural alignment of ubiquitin and the N-terminal region of Jak1 is shown in Fig. 1A.

Furthermore, we subjected the Jak1 sequence to a secondary structure prediction, which exclusively relies on sequence information (27). This prediction yields a succession of secondary structure elements in the N-terminal region of Jak1 compatible with the sequence of structural elements found in ubiquitin (Fig. 1A). This correlation further supports the notion that the N-terminal region of Jak1 shares the fold of a β-grasp domain. To analyze the spatial configuration of the amino acid residues in the N-terminal Jak1 domain we built a detailed three-dimensional molecular model using the ubiquitin structure as template (Fig. 1B, see “Experimental Procedures”).

Mutations in Loop 4 of the Predicted β-grasp Domain of Jak1 Impair Binding to the Cytoplasmic Part of gp130—Since it can be envisaged that Jak1 associate with cytokine receptors in a conserved manner and most often loop regions are involved in protein/protein interactions, we examined these regions in the model for features conserved within the Jak family but differing from the corresponding regions of the ubiquitin β-grasp (Fig. 1A). Interestingly, loop 4 was much longer in the Jak sequence than in ubiquitin. Moreover, within the Jak family members, loop 4 shows considerable sequence differences as would be expected in a region that could promote binding specificity. Using these criteria we identified loop 4 as a promising region for mutagenesis.

We introduced amino acid exchanges into loop 4 and other regions of the Jak1 β-grasp domain and tested the resulting mutants for their ability to bind to the cytoplasmic tail of gp130. As in previous studies, we took advantage of a chimeric receptor consisting of the extracellular part of the IL-5Rβ chain and the transmembrane and cytoplasmic regions of gp130 for which antibodies suitable for immunoprecipitation and Western blot analysis are available (28, 29). Jak1 was coexpressed with IL-5Rβ/gp130 in COS-7 cells, and the interaction was investigated by coprecipitation of the receptor with a Jak1 antibody (Fig. 2) or vice versa by coprecipitation of Jak1 with anti-IL-5Rβ (data not shown).

We deleted a stretch of four amino acids (Tyr81–Ser84) in the loop 4 region (Fig. 1B). This mutation totally abrogated the association of Jak1 with the receptor (Fig. 2, upper right panel, third lane). To analyze further the importance of this predicted loop region for receptor association, point mutations were introduced. The double mutant L80A/Y81A showed a complete loss of receptor binding, while the mutant Y81A/D82A exhibited a significantly impaired receptor association. Minimal effects were observed for the single amino acid exchanges L80A, Y81A, E83K, and K86E. In addition, we exchanged Tyr107 in Jak1 with alanine (106) located in the fourth β-strand to alanine and Arg108 located in the fifth β-strand to glutamate. These mutations outside the predicted loop 4 region did not affect receptor association (Fig. 1B). These data are summarized in Table I.

**FIG. 1. Identification of a β-grasp domain in the N-terminal region of Jak1.** A, alignment of the ubiquitin with the N-terminal Jak/Tyk sequences. For ubiquitin the experimentally derived secondary structure elements are colored in red. The sequences of the Jak/Tyk molecules were aligned according to the results obtained by the fold recognition procedure. The secondary structure elements of the human Jak1, as predicted by the method of Rost and Sander (27), are colored in blue. Overall conserved residues are marked by an asterisk, and residues that are conserved at least in one of the Jak/Tyk sequences compared with the ubiquitin sequence are marked by +. Overall conserved hydrophobic residues are boxed. Amino acids exchanged in this study are shown in bold. B, a Ribbon representation of the N-terminal β-grasp domain of Jak1. C, strip diagram of Jak1. The JH domains, the putative FERM subdomains, and the position of the β-grasp-domain are indicated.

**RESULTS**

We introduced amino acid exchanges into loop 4 and other regions of the Jak1 β-grasp domain and tested the resulting mutants for their ability to bind to the cytoplasmic tail of gp130. As in previous studies, we took advantage of a chimeric receptor consisting of the extracellular part of the IL-5Rβ chain and the transmembrane and cytoplasmic regions of gp130 for which antibodies suitable for immunoprecipitation and Western blot analysis are available (25, 28). Jak1 was coexpressed with IL-5Rβ/gp130 in COS-7 cells, and the interaction was investigated by coprecipitation of the receptor with a Jak1 antibody (Fig. 2) or vice versa by coprecipitation of Jak1 with anti-IL-5Rβ (data not shown).

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**Mutation of the Conserved Tyr107 to Alanine Abrogates Jak1 Binding to gp130—**Tyrosine 107 of Jak1 is conserved within the Jak family of kinases. Interestingly, a single amino acid exchange of the corresponding residue of Jak3 (Tyr106) to cysteine has been identified in a patient with severe combined immunodeficiency (13). This Jak3 mutant is unable to associate with the common γ-chain of the IL-2 receptor complex (12). Based on this information we exchanged Tyr107 in Jak1 with alanine. As shown in Fig. 2 (upper left panel, sixth lane), the Jak1 mutant does not bind to gp130. According to the β-grasp model, this tyrosine residue is located within the fifth β-strand (Fig. 1B) and was found to interact with Leu72, Ile70, and Leu105. Thus, Tyr107 may stabilize the hydrophobic core of the β-grasp domain, and the substitution to alanine very likely destroys the structural integrity of the domain.
Mapping of a Receptor Interaction Region in Jak1

The Ability of the Jak1 Mutants to Mediate Signals via gp130 Parallels Their Receptor Association Behavior—The results of coprecipitation studies can be greatly influenced by experimental parameters; false positive results may arise when working with overexpressed proteins, and harsh detergents as present in “normal” lysis buffers may break up subtle protein interactions. Therefore, we tested the ability of the Jak mutants to mediate ligand-dependent signal transduction events within the cell—phosphorylation of Jak1 and the receptor as well as activation of STAT transcription factors.

COS-7 cells were transiently cotransfected with expression constructs for IL-5Rα/gp130, IL-5Rβ/gp130, and the various Jak1 mutants. After stimulation with IL-5, lysates were prepared, and Jak1 and the IL-5Rβ/gp130 chimera were immunoprecipitated. The immunoprecipitates were separated by SDS-PAGE and subjected to Western blot analysis, and their phosphorylation was monitored using a phosphotyrosine-specific antibody for detection. We also monitored the stimulation-dependent activation of STAT transcription factors by an EMSA.

Jak1 mutants such as Δ(Tyr81–Ser84), L80A/Y81A, and Y107A that did not show receptor binding (Fig. 2) were not tyrosine-phosphorylated upon stimulation with IL-5 and did not mediate phosphorylation of the receptor (Fig. 3A). Y81A/D82A that showed a severely impaired receptor association resulted in phosphotyrosine-specific bands of intermediate intensity, whereas those mutations that did not alter receptor association significantly, such as L80A, Y81A, E83K, and K86E, led to full Jak and receptor phosphorylation as did wild-type Jak1 (Fig. 3A). The results obtained for the various Jak1 mutants in the EMSA assay also closely matched the receptor binding and phosphorylation data (Fig. 3B). Taken together the capacity of the various Jak1 mutants to mediate signals via gp130 closely follows their ability to associate with the receptor as determined by the coprecipitation studies (Table 1).

The N-terminal Domains of Jak1 Mediate Binding to the Box1/Box2 Region of gp130—In an alternative approach, the ability of a series of polypeptides corresponding to different fragments of Jak1 to interact with gp130 was investigated (Fig. 4A1). In vitro translation of appropriate constructs yielded similar amounts of [35S]methionine-labeled Jak1 polypeptides (Fig. 4, B–D). Polypeptides corresponding to JH5–7 and full-length Jak1 interacted comparably (particularly allowing for the difference in methionine content) with a synthetic, biotinylated “box1/box2” peptide representing the first 73 amino acids of the intracellular domain of gp130 (Fig. 4, Aii, B, and C). Essentially identical results were obtained with a JH3–7 polypeptide (data not shown). The interaction was specific; it was not observed with a mutant box1/box2 or unrelated peptide (Fig. 4, A and C) and was efficiently inhibited by the addition of an excess of nonbiotinylated box1/box2 peptide. Despite a higher nonspecific background, clear reproducible binding to the box1/box2 peptide was also observed with the 35S-labeled JH6–7 domain polypeptide (Fig. 4C). Interaction over background was not observed with JH3–5 and JH3–4 polypeptides, which lack the β-grasp domain (Fig. 4D). The introduction of selected mutations, corresponding to the inactivating mutations described above (Fig. 2), into a JH3–7 polypeptide inhibited binding to the box1/box2 peptide. In addition, a fragment corresponding to the N-terminal FERM domain (encompassing JH5–7, Fig. 1C) of Jak1 can confer gp130 binding upon Jak3.2

Taken together these data are consistent first, with a direct interaction of the N-terminal domains of Jak1 with gp130 and second, with the effect of the loop 4 mutations on Jak1 function in the intact cell assays (Figs. 2 and 3) being on this direct interaction rather than through nonspecific mutational disruption of the overall structure of Jak1. Irrespective it can be concluded that residues within the JH6–JH7 region, which contains the putative β-grasp domain, are both necessary and minimally sufficient to mediate an interaction of Jak1 with the box1/box2 domain of gp130. The data do not, of course, exclude a requirement for additional interactions for optimal binding of Jak1 to a full-length native receptor.

Loop 4 Mutants of Jak1 Are Also Defective in the IFN Responses—The effect of the loop 4 mutations on the ability of Jak1 to restore IFN as well as IL-6 responses to the Jak1-deficient cell line U4C was investigated. STAT1 activation following IFNγ stimulation and STAT1/3 activation in response to IL-6 were monitored by EMSA analysis of transiently transfected cells (Fig. 5, upper panels). The low level activation of the STATs by IL-6 in the vector-only-transfected U4C cells (Fig. 5,

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2 Hilkens, C. M. U., Is’harc, H., Lillemoeber, B., Strobl, B., Bates, P. A., Behrmann, I., and Kerr, I. M. (2001) FEBS Lett., in press.
vector) reflects residual signaling through Jak2 and/or Tyk2 in the absence of Jak1 in these cells (16). Expression levels of the different constructs were assessed by anti-Jak1 Western blot (Fig. 5, lower panels). The different mutations exerted equivalent effects on the IFNγ and IL-6 responses (Fig. 5). In addition, essentially identical results were obtained with IFNα (data not shown). The results paralleled those for the COS-7 cell experiments (Figs. 2 and 3 and Table I): Jak1 mutants that did not bind gp130 did not complement U4Cs (L80A, Y81A, and ΔTyr81–Ser84); Jak1 mutants that retained the ability to interact with gp130 were able to restore Jak1-dependent signaling to U4Cs (L80A, Y81A, E83K, and K86E). Importantly the inactivating loop 4 mutations, in contrast to a kinase-inactivating mutation (29), were without effect on the autokinase activity of the transiently transfected Jak1, again arguing against any gross disruption of tertiary structure (data not presented). Thus, despite their highly divergent Jak recruitment motifs (30, 31), Jak1 likely interacts in a conserved manner with type I and type II cytokine receptors, exemplified here by those for IL-6 and the IFNs, respectively.

**DISCUSSION**

Since Jak activation is the initial event in cytokine receptor-dependent signal transduction, there is considerable interest in understanding the interaction and activation mechanism of Janus kinases. To date no structural information exists concerning the Jak/receptor interaction. Here we present the first structural model that helps to understand how Janus kinases associate with cytokine receptors. In the N-terminal region of Jak1 known to mediate receptor association we identified a putative β-grasp domain (amino acids 36–112) by the fold recognition approach, which is a powerful tool to identify a potential fold of a protein with only limited sequence homology to others. Mutational analysis led to the conclusion that loop 4 in the β-grasp domain is essential for association of Jak1 not only with gp130 but also with other cytokine receptors.

**TABLE I**

Summary of the data obtained with the Jak1 mutants

| Jak1 constructs | WT | L80A | L80A/Y81A | Y81A | Y81A/D82A | ΔTyr81–Ser84 | E83K | K86E | Y107A |
|-----------------|----|------|-----------|------|-----------|-------------|------|------|-------|
| Receptor assoc. | + + | + +  | –         | + +  | –         | –           | + +  | + +  | + +   |
| Jak1 YP | + + | ++ + | – – ++ + | + +  | – – ++ +  | – – ++ +    | + +  | + +  | + +   |
| Receptor YP | + + | ++ + | – – ++ + | + +  | – – ++ +  | – – ++ +    | + +  | + +  | + +   |
| EMSA | + + | ++ + | – – ++ + | + +  | – – ++ +  | – – ++ +    | + +  | + +  | + +   |
| EMSA (U4C) IL-6 | ++ | ++ + | – – ++ + | + +  | – – ++ +  | – – ++ +    | + +  | + +  | + +   |
| IFNγ | ++ | ++ + | – – ++ + | + +  | – – ++ +  | – – ++ +    | + +  | + +  | + +   |
| Results | √/√ | Inactive | √/√ | Inactive | √/√ | Inactive |

a Data obtained with chimeric gp130 receptors in COS-7 cells.
We hypothesized that loop 4 of the β-grasp domain could be a region of general importance for Jak binding to cytokine receptors because it proved to be much longer than loop 4 in ubiquitin and is well exposed on the surface of the β-grasp domain. Due to sequence differences among the Jak family members, loop 4 of the β-grasp domain might also have the potential to determine binding specificity. Deletion of amino acids 81–84 totally abrogated receptor association. In this mutant the loop 4 region is shortened to the length of the corresponding loop of ubiquitin. It is therefore unlikely that this deletion affected the structural integrity of the domain but rather indicates that loop 4 is involved in receptor association.

Certain amino acid exchanges in loop 4 interfered with receptor binding and led to reduced Jak1 activation, receptor phosphorylation, and STAT activation in COS-7 transfectants. Two control mutations introduced into regions outside of loop 4, Y89A and R104E, did not interfere with Jak1 binding to gp130. According to the β-grasp model, the hydrophobic residue Tyr89 is not likely to be essential for structural integrity, and Arg104 is located at the outer surface of the domain and should promote solvent contact.

Tyr107 was selected for mutagenesis on the basis of previous data concerning Jak3. This tyrosine residue is conserved among Janus kinases. Interestingly the corresponding residue in Jak3, Tyr109, was found to be exchanged to cysteine in a patient with severe combined immunodeficiency (13). It could

**Fig. 4.** A Jak1 JH6-7 domain polypeptide can interact independently with the box1/box2 region of gp130. **Ai**, schematic representation of the Jak1 JH domain constructs tested. **Aii**, schematic representation of the synthetic, biotinylated gp130 box1/box2 peptide and “mutant box1/box2” peptide. The box1/box2 peptide contains the first 73 amino acids of the gp130 cytoplasmic domain, encompassing the box1 and box2 motifs. In the mutant box1/box2 peptide, proline residues (highlighted) in box1 necessary for Jak recruitment to gp130 (18) have been mutated to alanine. **B–D**, the 35S-labeled, in vitro translated fragments of human Jak1 were tested for their ability to interact with the biotinylated box1/box2 peptide, biotinylated mutant box1/box2 peptide, or an unrelated biotinylated peptide of similar size (see “Experimental Procedures”). Peptide-associated proteins were resolved by SDS-PAGE and visualized by autoradiography. Aliquots of the in vitro translated products were analyzed in parallel; comparable amounts were obtained for each construct. Each construct was tested in at least three independent experiments with essentially identical results.
subsequently be demonstrated that the mutant Jak3–Y100C is severely impaired in its ability to associate with the common γ-chain, which is a signal transducing subunit of the receptor complexes for IL-2, IL-4, IL-7, IL-9, and IL-15 (12). Since exchange of Tyr\textsuperscript{100} of Jak3 to alanine similarly leads to an impaired binding whereas exchange to phenylalanine was without effect, it was suggested that Tyr\textsuperscript{100} in Jak3 is structurally essential residue in a domain that directly contacts the γ-chain and that ablation of the aromatic residue by an alanine or cysteine substitution disrupts the domain fold (12). As shown in the present manuscript, exchange of Tyr\textsuperscript{107} in Jak1 to alanine also impairs receptor association. In our model, Tyr\textsuperscript{107} interacts with the residues Leu\textsuperscript{96}, Ile\textsuperscript{97}, and Leu\textsuperscript{98} within the hydrophobic core of the β-grasp domain. Thus, it can be envisaged that this tyrosine residue is crucial for the structural integrity of the β-grasp domain. Exchange of amino acids in the vicinity of Tyr\textsuperscript{107} of Jak3 revealed that Leu\textsuperscript{98} and Ile\textsuperscript{97} are also important for IL-2Rγ association, while substitution of Leu\textsuperscript{96} to alanine did not affect receptor association (12). According to our alignment, these residues are part of a β-strand (see Fig. 1A), and the Jak1 residue Leu\textsuperscript{105} corresponding to Leu\textsuperscript{98} of Jak3 is found to be involved in hydrophobic core interactions in the model. Thus, the published Jak3 data are also in good accordance with the β-grasp model.

Type II cytokine receptors such as IFN receptors are quite different from class I cytokine receptors, including gp130, with respect to the receptor requirements for Jak association. Interferon receptors have no clear box 1/box 2 homology. Despite this, the loop 4 mutations L80A/Y81A and Δ[Tyr\textsuperscript{81}–Ser\textsuperscript{84}] affected signal transduction in response to IFNγ and IFNα similarly to gp130-mediated signal transduction in U4C fibrosarcoma cells expressing the different Jak mutants (Fig. 5). In addition, L80A/Y81A and Δ[Tyr\textsuperscript{81}–Ser\textsuperscript{84}] showed no binding to the cytoplasmic part of the leukemia inhibitory factor receptor and the IL-5Rα, two other type I cytokine receptors, and IFNαR2, a type II cytokine receptor, as measured by coprecipitation analysis in COS-7 cells (data not shown). Thus, loop 4 seems to be crucial for binding to cytokine receptors in general. We have no evidence for an involvement of loop 4 in defining specificity for binding to different receptors.

Based on sequence similarities it has been suggested that the N-terminal region of Janus kinases might contain a divergent band 4.1 domain. These domains have also been termed “FERM domains” due to the fact that the “classical” proteins sharing such a domain are band four-point-one protein, ezrin, radixin, and moesin, or “4.1/ERF-domain” (15). The N-terminal limit of the region of Tyk2 essential for association with a GST-IFNαR1 construct corresponds exactly to the limits of the FERM domain (9). In Jak1, the putative FERM domain would comprise the region between amino acid residues 24–415 (Fig. 1C). The recently published first x-ray structure of a FERM domain, namely that of moesin (32), showed that these domains consist of three separate subdomains. Interestingly the most N-terminal subdomain, F1, has a ubiquitin-like, i.e., a β-grasp, fold. The F2 subdomain is rich in α-helices and shows structural similarity to the acyl-CoA-binding protein. The F3 subdomain is folded like a pleckstrin homology domain. This structural information and our prediction that the extreme N terminus of Jak1 contains a β-grasp fold support the hypothesis that Jak5 contains a FERM domain (15).

The three subdomains of the moesin FERM domain contact each other at defined interaction sites. Importantly the loop 4 region of the moesin F1 subdomain is not involved in domain/domain contacts. The same holds true for the corresponding regions of the radixin and 4.1R FERM domains (33, 34). Under the assumption that the subdomains of the FERM domain show an identical topology in Jak1 to those in moesin, radixin, and 4.1R, it is unlikely that the inability of our Jak1 loop 4 mutants to bind gp130 is due to abrogation of a necessary interaction between the three subdomains F1, F2, and F3. In the solved FERM domain structures the respective regions corresponding to loop 4 have not been found to be involved in interactions with any other molecules (33, 34). Taken together, in the solved FERM structures, loop 4 of the ubiquitous-like F1 domains is not buried between the subdomains but rather is exposed and should therefore be accessible for binding as one might expect for a region involved in receptor association.

Several studies with Janus kinases have underscored the importance of the N-terminal region for receptor binding, (9–12, 14, 35–38). Shortened fragments comprising only the JH6/JH7 domains of Tyk2, Jak2, and Jak3 were able to associate with appropriate receptors (9–12). For Jak1 it is known from experiments in intact cells that the N-terminal half can mediate receptor association; Jak1/Jak2 chimeras with fusion borders further N-terminal were unable to sustain an IFNγ response (14). More recently, however, similar intact cell experiments with different Jak1/Jak3 chimeras have shown that substitution of the putative Jak3 4.1/FERM domain with that from Jak1 can confer gp130 binding upon Jak3. 2 In addition, here we show that in a cell-free system an N-terminal fragment containing only the JH6 and JH7 subdomains of Jak1 is able to bind to a biotinylated, 73-amino acid box 1/box 2 gp130 peptide. It will be interesting to see whether an isolated β-grasp domain binds gp130. However, it might also be possible that an intact JH7/JH6 context is crucial for Jak binding to cytokine receptors as several studies suggest. The JH region 7 roughly corresponds to the β-grasp subdomain F1 and the JH region 6 to the helix bundle subdomain F2 of the FERM domain. Interestingly chimeric Jak3/Jak1 constructs, which contain the intact β-grasp domain of Jak3 (containing amino acids 1–109 of Jak3), are not able to bind to the IL-2Rγ (12). The same is true for chimeric Jak3/Jak2 constructs, which contain the intact β-grasp domain of Jak3 and part of the linker to the helix bundle domain (containing amino acids 1–124 of Jak3) (11). Only a Jak3/Jak1 chimera incorporating the complete Jak3 linker region between the β-grasp and the helix bundle...
region of the potential FERM domain (amino acids 1–132 of Jak3) shows a successful γ-chain association (12). It is noteworthy that the Jak kinases show differences in length and sequence of this linker region and that binding to the γ-chain can be increased by using the complete β-grasp and helix bundle region of Jak3 (i.e. the JH7-JH6 regions) in the Jak3/Jak1 chimera (12). These data suggest that the JH6 and the JH7 regions may have to be present in a very defined structural context to allow high affinity receptor binding.

The interaction of the Janus kinase N-terminal domain with cytokine receptors appears complex and implicates large portions of the kinase and the receptor, i.e. 69 amino acids of gp130 (18, 39). Our data represent a further step in the elucidation of the structural interface of the Jak/receptor interaction before full structural data are available. Knowledge of the Jak/receptor binding interface could be helpful in the design of low molecular weight inhibitors of cytokine signaling of potential therapeutic value.

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Mapping of a Region within the N Terminus of Jak1 Involved in Cytokine Receptor Interaction
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