Janus Kinase (Jak) Subcellular Localization Revisited

THE EXCLUSIVE MEMBRANE LOCALIZATION OF ENDOGENOUS JANUS KINASE 1 BY CYTOKINE RECEPTOR INTERACTION UNCOVERS THE Jak-RECEPTOR COMPLEX TO BE EQUIVALENT TO A RECEPTOR TYROSINE KINASE*

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The Janus kinases are considered to be cytoplasmic kinases that constitutively associate with the cytoplasmic region of cytokine receptors, and the Janus kinases (Jaks) are crucial for cytokine signal transduction. We investigated Jak1 localization using subcellular fractionation techniques and fluorescence microscopy (immunofluorescence and yellow fluorescent protein-tagged Jaks). In the different experimental approaches we found Jak1 (as well as Jak2 and Tyk2) predominantly located at membranes. In contrast to previous reports we did not observe Jak proteins in significant amounts within the nucleus or in the cytoplasm. The cytoplasmic localization observed for the Jak1 mutant L80A/Y81A, which is unable to associate with cytokine receptors, indicates that Jak1 does not have a strong intrinsic membrane binding potential and that only receptor binding is crucial for the membrane recruitment. Finally we show that Jak1 remains a membrane-localized protein after cytokine stimulation. These data strongly support the hypothesis that cytokine receptor-Janus kinase complexes can be regarded as receptor tyrosine kinases.

The Janus kinase family of protein-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. Janus kinases non-covalently associate with the cytoplasmic parts of cytokine receptors and play a crucial role in the initial steps of cytokine signaling (1). Upon cytokine-induced receptor dimerization the Jaks are thought to transphosphorylate and thereby autoactivate themselves. The kinases subsequently phosphorylate tyrosine residues on the receptor cytoplasmic tail providing docking sites for Src homology 2 domain-containing signaling proteins. These include transcription factors of the STAT (signal transducers and activators of transcription) family. Upon phosphorylation, STATs translocate to the nucleus where they bind to specific DNA sequences in the promoter regions of their target genes.

Based on sequence similarities between the Jaks (molecular masses of 120–140 kDa) seven Jak homology (JH) regions have been defined (2). However, the JH domains match the functional domains only partially. The JH1 domain, at the C terminus, a classical tyrosine kinase domain, is flanked by a pseudokinase domain (JH2) that may play a regulatory role (3, 4). The N-terminal half of the Jaks, domains JH3–JH7, comprises a potential Src homology 2 domain (5–7) and a postulated FERM domain (four-point-one, ezrin, radixin, moesin) that is involved in cytokine receptor binding (8–11). FERM domains are composed of three subdomains: F1 with a ubiquitin-like β-grasp fold, F2 with an acyl-coenzyme A-binding protein-like fold, and F3, which shares the fold of phosphotyrosine binding or pleckstrin homology domains. F1, F2, and F3 together form a compact clover-shaped structure (12–14). A recent mutagenesis study has highlighted the importance of the F1 subdomain of Jak1 for the interaction with gp130, the signal-transducing chain of interleukin-6-type cytokines (10).

Functionally Jaks are involved in signal transduction of cytokines as has been reviewed extensively (15, 16). The phenotypes of the respective Jak knock-out mice (Jak1 (17), Jak2 (18, 19), Jak3 (20), and Tyk2 (21, 22)) and of patients with Jak3 mutations (23–25) also reflect the importance of the Jaks in cytokine signal transduction. Jak binding to at least some cytokine receptors is crucial for receptor surface expression (26–30). However, Jak functions may not be restricted to cell surface receptors. In a recent study Jak2 has been identified as the kinase that associates with p97, a member of the AAA family (ATPases associated with different cellular activities), which is involved in membrane fusion and assembly of the transitional endoplasmic reticulum (31). Thus, the functional data imply a localization of Jaks at cellular membranes, at the cell surface, and at the endoplasmic reticulum. However, previous reports investigating Jak localization describe a predominant nuclear localization of Jak1, Jak2, and Tyk2 (32–35). The significance of nuclear Janus kinases is not clear given that all functional features of Jak proteins point to their involvement in events occurring at the cell surface or at intracellular membranes.

Therefore we reinvestigated the localization of Jak1 using cell fractionation techniques and fluorescence microscopy and always using Jak-deficient cells as controls. The subcellular
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distribution of fluorescent Jak1 fusion proteins was monitored in living cells. We found the Janus kinases were located mainly at membranes. Virtually no Jak1 seems to be present in the cytoplasm and the nucleus. Cytokine stimulation did not change the localization of Jak1. However, a Jak1 mutant unable to associate with cytokine receptors due to point mutations in the FERM subdomain F1 was distributed evenly in the cytoplasm indicating that receptor binding is crucial for the observed membrane localization.

MATERIALS AND METHODS

Cell Culture and Transfection—HEK293T cells (human embryonic kidney cells obtained from DSMZ, Braunschweig, Germany) 2C4, U4C, γ2A, 2TGH, and U1A cells (human fibrosarcoma cells kindly provided by Dr. I. Kerr, Cancer Research UK, London); HeLa cells (cervix carcinoma cells obtained from DSMZ), and Chinese hamster ovary cells obtained from DSBM were maintained in Dulbecco's modified Eagle's medium (Invitrogen). A575 cells (human melanoma cells obtained from ATCC) were maintained in RPMI 1640 medium (Invitrogen). Hep2C cells (human hepatoma cells obtained from DSMZ) were maintained in Dulbecco's modified Eagle's medium/Nutrient Mix F-12 medium with Glutamax (Invitrogen). Human umbilical vein endothelial cells (HUVECs) were maintained in C-22010 endothelial cell growth medium with the supplement mixture C-39215 (Promo cell). All media were supplemented with 10% fetal calf serum, 100 mg/liter streptomycin, and 60 mg/liter penicillin. G418 (400 μg/ml) was added to the medium of 2C4, U4C, and γ2A cells, while 2TGH and U1A cells were supplemented with 250 μg/ml hygromycin. The U4C-Jak1, U4C-Jak1-YFP, U4C-Jak1-L80A/Y81A, U4C-Jak1-L80A/Y81A-YFP were cultured with 500 μg/ml leupeptin, 1 mM EDTA, and 1 mM dithiothreitol). Cells were also tested. The monoclonal antibody against lamin A/C (636, Amersham Biosciences) was used for detection. The horseradish peroxidase-conjugated secondary antibodies were purchased from Dako. Signals were detected using the ECL system (Amersham Biosciences).

Cell Lysis, Immunoprecipitation, and Western Blot Analysis—Cells were lysed with 500 μl of lysis buffer containing 1% Brij 96 or 1% Triton X-100, 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 1 mM EDTA, and 1 mM dithiothreitol) and incubated on ice for 5 min to solubilize the membrane contaminations, and the nuclei were pelleted by centrifugation at 500 rpm. This procedure was repeated once. The purified nuclei were lysed using 1 ml of 2% Triton-containing nuclear lysis buffer (2% Triton X-100, 20 mM Tris/HCl, pH 7.5, 280 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 5 μg/ml aprotinin, 3 μg/ml pepstatin, 5 μg/ml leupeptin, 1 mM EDTA, and 1 mA dithiothreitol), and the lysate was cleared by centrifugation at 12,500 × g for 10 min. The insoluble membranes were solubilized in 1 ml of Laemmli buffer by heating and vigorous mixing for 10 min at 85 °C.

Cell Lysis, Immunoprecipitation, and Western Blot Analysis—Cells were lysed on the dish with 500 μl of lysis buffer containing 1% Brij 96 or 1% Triton X-100, 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 10 mM phenylmethylsulfonly fluoride, 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 antibodies, the immunoprecipitates were collected with protein A-Sepharose (1 h at 4 °C), washed three times with lysis buffer, and analyzed further by SDS-PAGE. The proteins were transferred to a polyvinylidene difluoro-urea membrane (Amersham Biosciences) and probed with the following antibodies. Anti-Jak1 (J24320, Transduction Laboratories), anti-Tyk2 (T20220, Transduction Laboratories), anti-lamin A/C (636, Santa Cruz Biotechnology, Inc.), anti-calcinein (610523, Transduction Laboratories), anti-flotillin (610820, Transduction Laboratories), anti- phosphotyrosine (PT99, Santa Cruz Biotechnology, Inc.), anti-STAT1 (610159, Transduction Laboratories), anti-phospho-STAT3 (Ty-705) (9131, Cell Signaling Technology Inc.), anti-STAT1 (S21120, Transduction Laboratories), and anti-phospho-STAT1 (Ty-701) (9171, Cell Signaling Technology Inc.) were used for detection. The horseradish peroxidase-conjugated secondary antibodies were purchased from Dako. Signals were detected using the ECL system (Amersham Biosciences).

Immunofluorescence Studies—The different cell types were seeded on 18-mm coverslips coated with collagen and cultivated in 12-well plates. Cells were fixed using 4% formaldehyde in PBS (1 mM MgCl2, 0.1 mM CaCl2 in PBS) for 10 min, 2% formaldehyde + 0.2% glutaraldehyde in PBS for 10 min, or acetone/methanol (1:1) or methanol for 15 min at −20 °C. After fixation the cells were washed twice with PBS 2×, permeabilized, and blocked with PBS 2× containing 0.5% saponin and 3% BSA for 10 min. The cells were then incubated with primary antibody at a dilution of 1:200–1:1000 in PBS 2× for 60 min. After washing four times with PBS 2× containing 0.05% saponin and 3% BSA, the fixed cells were incubated with the appropriate fluorescently labeled secondary antibody at a 1:100 dilution in PBS 2× containing 0.5% saponin and 3% BSA for 30 min. After washing four times with PBS 2× containing 0.5% saponin and 3% BSA the slides were mounted using a Mowiol-containing mounting medium (10% (w/v) Mowiol (Calbiochem), 25% (v/v) glycerin, 25% (v/v) water, 45% (v/v) 0.1 M Tris/HCl, pH 8.5). Substituting saponin for 0.1% Triton or leaving out BSA from the incubation and washing buffers led to no improvement of the staining pattern that was observed with the protocol above. In addition the protocols recommended by the providers of the respective antibodies were also tested. The monoclonal antibody against lamin A/C (636, Santa Cruz Biotechnology, Inc.) was used in all experiments as positive control. Another positive control was the FITC-conjugated β-tubulin antibody TUB2.1 (F2043, Sigma). For the detection of Jak1 proteins the following antibodies were tested: a polyclonal serum (kindly provided by A. Ziemiecki), the polyclonal antibody HRT875 (Santa Cruz Biotechnology, Inc.), the polyclonal antibody 06-272 (Upstate Biotechnology), the monoclonal antibody MAB3700 (Chemicon), and the monoclonal antibody J24320 (Transduction Laboratories). The Jak2 antibodies were a polyclonal serum (kindly provided by A. Ziemiecki) and the polyclonal antibody J24320 (Upstate Biotechnology). For the detection of Jak1 we used the monoclonal anti-Tyk2 antibody T02220 (Transduction Laboratories). The Cy3-labeled donkey anti-mouse IgG (715-165-150) or donkey anti-rabbit IgG (711-165-152) secondary antibodies were from Dianova (Hamburg, Germany). Imaging of the cells was carried out on the Axiosvert 100M fluorescence microscope from Zeiss (Germany) equipped with an AxioCam camera using a water-corrected Plan-Apochromat (63×) objective. FITC fluorescence was detected using a 50-watt mercury lamp and filter set 09 from Zeiss (BP 450–490 nm for excitation; LP > 551 for detection). Cy3 fluorescence was detected using a 50-watt mercury lamp and filter set described by Buckley (37). The crude nuclear pellet obtained as described above (centrifugation of Dounce-homogenized cell homogenate at 500 rpm) was resuspended in 1 ml of nuclear isolation buffer (0.5% Nonidet P-40 (IGEPAL CA630 from ICN Biomedicals Inc.), 10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl2, 1 mM sodium vanadate, 5 μg/ml aprotinin, 3 μg/ml pepstatin, 5 μg/ml leupeptin, 1 mM EDTA, and 1 mM dithiothreitol) and incubated on ice for 5 min to solubilize the membrane contaminations, and the nuclei were pelleted by centrifugation at 500 rpm. This procedure was repeated once. The purified nuclei were lysed using 1 ml of 2% Triton-containing nuclear lysis buffer (2% Triton X-100, 20 mM Tris/HCl, pH 7.5, 280 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 5 μg/ml aprotinin, 3 μg/ml pepstatin, 5 μg/ml leupeptin, 1 mM EDTA, and 1 mM dithiothreitol), and the lysate was cleared by centrifugation at 12,500 × g for 10 min. The insoluble membranes were solubilized in 1 ml of Laemmli buffer by heating and vigorous mixing for 10 min at 85 °C.
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RESULTS

In Cell Fractionation Experiments Jak1 Is Exclusively Found in Membrane Fractions but Not in the Cytoplasm or the Nucleus—We investigated the localization of endogenous Jak1 and Tyk2 in a number of cell lines and primary HUVECs by using cell fractionation techniques. The cells were fractionated as described under “Materials and Methods,” and Jak1 and Tyk2 were detected by Western blot analysis. The blots were counterstained with antibodies against marker proteins of the different subcellular fractions. Calnexin or flotillin were used as membrane fraction markers, and lamin A and C were used as nuclear marker proteins. Cytoplasmic proteins have been defined as those soluble after 1-h ultracentrifugation at 100,000 × g. A predominant membrane localization of Jak1 and Tyk2 was found in the hepatoma cell line HepG2, in the cervix carcinoma cell line HeLa, in the melanoma cell line A375, in HUVECs, in 2C4 and 2fTGH fibrosarcoma cells (Fig. 1A, lanes 2, 5, 8, 11, 17, and 23), and in Chinese hamster ovary cells (data not shown). Jak1 and Tyk2 were, however, not found in significant amounts in the cytoplasmic or nuclear fractions (Fig. 1A). In all experiments Jak1 detection in cytoplasmic or nuclear fractions could be related to membrane contamination. In the case of HepG2 and HeLa cells, a weak Jak1 and Tyk2 band in the nucleus correlates with a weak flotillin band in the nuclear fraction (Fig. 1A, lanes 3 and 6). For HUVECs, membrane contamination of the cytoplasmic fraction is probably the cause for the weak Jak1 signal (Fig. 1A, lane 10). To prove the identity of the Western blot bands of Jak1 and Tyk2 we fractionated human fibrosarcoma cells (2C4 and 2fTGH) and cells derived from those that are deficient in Jak1 (U4C) or Tyk2 (U1A) (38, 39). For an overview of the Jak-deficient cell lines see Table I. 2C4 and 2fTGH cells showed the same membrane distribution of Jak1 and Tyk2 already observed in the other cell types. In U4C cells no Jak1 (Fig. 1A, lanes 13–15) and in U1A cells virtually no Tyk2 (Fig. 1A, lanes 19–21) could be detected, thus the respective antibodies we used are specific for Jak1 or Tyk2 and can be used to detect these proteins in this assay. However, the results with the Tyk2 antibody are not entirely satisfactory since it detected a slight unspecific band in the cytoplasmic fraction that has a mobility similar to Tyk2. (In fact Jak2 was excluded from the fractionation study because strong unspecific bands in the cytoplasmic and nuclear fractions interfered with an unambiguous detection of the endogenous Jak2.)

Interestingly the membrane fraction contains only approximately half of the total Jak1 (Fig. 1B, compare lanes 2 and 4). This is due to the fact that a significant amount of plasma...
membrane and internal membranes are pelleted with the nuclei during the fractionation procedure. To solubilize these membranes the nuclei were washed twice with Nonidet P40-containing buffer. The first Nonidet P40 wash fraction of the nuclei contains the other half of the membranes as shown by the presence of calnexin and Jaks in this fraction (Fig. 1B, lane 4, labeled 1. wash). In the second wash fraction almost no membrane markers and Jaks were found (data not shown). Thus, the amount of membrane-associated Jaks is even higher in comparison with the cytoplasmic and nuclear fractions as it appears from the Western blots of membrane fractions. Importantly no lysis of the nuclei occurs upon incubation with Nonidet P-40-containing buffer as shown by the lack of lamin A/C in this fraction (Fig. 1B, lane 4). The small amount of cytoplasmic Jak1 separated on an SDS-polyacrylamide gel consisted of two bands of different mobility (Fig. 1B, lane 1 (long exp.)). The lower band of increased mobility might represent a Jak1 lacking posttranslational modifications or an intermediate degradation product.

All Tested Jak Antibodies Were Not Suited for Immunofluorescence—We intended to use immunofluorescence as a second independent method to investigate Jak subcellular localization. To identify Jak1, Jak2, and Tyk2 antibodies suited for immunofluorescence we tested a number of Jak antibodies in human fibrosarcoma cells (2C4 and 2fTGH) and cells derived from those that are deficient in Jak1 (U4C), Jak2 (γ2A), or Tyk2 (U1A) (see Table I). In the case of Jak1, we also tested the polyclonal antibodies in mouse embryonal fibroblasts from Jak1 knock-out mice or normal mice (data not shown). The cells were grown on collagen-coated coverslips, fixed, and stained using different protocols (see "Materials and Methods"). The antibodies recommended for immunofluorescence were also tested using the protocols provided by the manufacturers.

Surprisingly a specific Jak1, Jak2, or Tyk2 staining could not be achieved with any of the tested antibodies. The polyclonal sera against Jak1 and Jak2 as well as the α-Jak1–06-272 and the α-Jak2–06-255 antibodies showed an unspecific cytoplasmic staining with an occasional unspecific nuclear staining (Fig. 2, A and B). The α-Jak1-HR785 and the α-Jak1-J24320 (clone identical to α-Jak1-MAB3700, data not shown) yielded a predominantly nuclear unspecific staining. The α-Tyk2-T20220 showed an unspecific cytoplasmic staining (data not shown). As controls we also performed immunofluorescence experiments with antibodies against other proteins that are well known to generate specific stainings. Fibrosarcoma cells stained with anti-lamin A/C or anti-tubulin-FITC showed a specific staining as documented in the literature (Fig. 2C) (47, 48).

Functionally Intact Fluorescent Jak1 and Jak2 Fusion Proteins Are Localized at Membranes but Excluded from the Nucleus—Since it was not possible to detect endogenous Jak1 using immunofluorescence, a fusion protein of Jak1 with the fluorescent YFP protein was generated to determine the subcellular distribution of the kinase in living cells. We want to emphasize that in this work we studied endogenous levels of Jaks also when expressing mutants (e.g. Jak1-L80A/Y81A) or YFP fusion proteins. This has not been done before with YFP fusion proteins of Jak1. It is crucial not to use overexpressed proteins when the subcellular localization is investigated since posttranslational protein modification processes and endogenous protein/protein interactions may become limited upon overexpression. This can lead to severe artifacts in subcellular localization. In addition it is well known that overexpressed Janus kinases activate themselves and aggregate.

First we checked whether Jak1-YFP was still a functional protein that could promote cytokine signal transduction and gene transcription events. U4C cells lacking Jak1 were stably transfected with cDNAs encoding Jak1-WT and Jak1-YFP. To examine the signal transducing capacity of the U4C-Jak1 and
the U4C-Jak1-YFP cells, they were stimulated with OSM or IFN-γ, and total cellular lysates were prepared. These were subjected to SDSPAGE and Western blotting. As can be seen in Fig. 3, Jak1-YFP did not show any differences in signal transduction compared with Jak1-WT. Jak1 phosphorylation (Fig. 3A) as well as the kinetics of tyrosine phosphorylation of STAT1 and STAT3 after cytokine stimulation (Fig. 3, B and C) were identical in U4C-Jak1 and U4C-Jak1-YFP cells. U4C cells did not show STAT activation after OSM or IFN-γ stimulation (data not shown). The up-regulation of MHC-I surface expression in U4C-Jak1-YFP in response to IFN-γ stimulation was identical to the one found in U4C-Jak1 cells (Fig. 3D). Upon transient expression of Jak1, Jak1-YFP, Jak2, and Jak2-YFP in COS-7 and HEK293T cells, all tested signal transduction events were also comparable (data not shown).

Cells were seeded onto coverslips, and living cells were analyzed with a confocal laser scanning microscope. Microscopic images of living U4C-Jak1-YFP cells, expressing endogenous levels of Jak1-YFP protein, are shown in Fig. 4A. Fluorescence can be observed at the cellular margins, but no localization in the nucleus could be observed. To investigate whether overexpression of Jak1 can lead to a nuclear accumulation HEK293T cells were transiently transfected with expression vectors encoding Jak1-YFP, Jak2-YFP, or YFP alone. γ2A cells were also transiently transfected with expression vectors encoding Jak2-YFP or YFP alone. Generally a different distribution of Jak1-YFP and Jak2-YFP in comparison with YFP was observed (Fig. 4B). Whereas YFP was expressed throughout the cell, Jak1-YFP and Jak2-YFP were predominantly localized at the plasma membrane but not in the nucleus. So even overexpressed Jak1-YFP and Jak2-YFP are excluded from the nucleus.

Jak Localization Does Not Change upon Cytokine Stimulation—Using cell fractionation techniques and fluorescence microscopy in living cells, we investigated whether there is a stimulation-dependent location change in fibrosarcoma cells stimulated with OSM.

After stimulation of 2C4 cells with OSM or IFN-γ no change in Jak1 localization could be observed in fractionation experiments (Fig. 5, A and B). Jak1 remained membrane-associated upon stimulation in all cell types investigated (HepG2, A375, and HeLa) (data not shown). As a second approach U4C-Jak1-YFP cells were stimulated with IL-6 soluble IL-6 receptor complexes, and the YFP fluorescence was monitored in living cells for up to 40 min. As shown in Fig. 5C Jak1-YFP remained exclusively at the plasma membrane after stimulation.

Jak1 Does Not Seem to Have a Strong Intrinsic Membrane Binding Potential—The fact that Jak1 is recruited to transmembrane receptors provides a plausible explanation for the observed membrane staining. Nevertheless Jak1 could also

![Fig. 3. Jak1-YFP is biochemically equivalent to Jak1. A. U4C cells stably expressing Jak1 or Jak1-YFP were stimulated with OSM. Lysates were prepared and were subjected to immunoprecipitation using a Jak1 antibody (HR785). The immunoprecipitates were analyzed by SDS-PAGE. Western blots were probed with an anti-phosphotyrosine monoclonal antibody (PY-99, Santa Cruz Biotechnology, Inc.). The blots were reprobed with an anti-Jak1 antibody. B and C, U4C cells stably expressing Jak1 or Jak1-YFP were stimulated with OSM (B) or IFN-γ (C). STAT3 (Tyr-705) and STAT1 (Tyr-701) phosphorylation was investigated using phosphotyrosine-specific antibodies for detection of the Western blots of lysates. The blots were reprobed using STAT3- and STAT1-specific antibodies. D, U4C cells stably expressing Jak1 or Jak1-YFP were stimulated with IFN-γ for 3 days, and MHC-I expression was assayed by fluorescence-activated cell sorting analysis. Histograms resulting from unstimulated cells stained with the MHC-I-specific antibody are shown in gray; those from stimulated cells are shown as a black line. PY, phosphotyrosine; IP, immunoprecipitation.](image1)

![Fig. 4. Jak1-YFP and Jak2-YFP are membrane-localized. A, living U4C cells stably expressing Jak1-YFP were monitored by confocal microscopy. B, HEK293T cells were transiently transfected with Jak1-YFP, Jak2-YFP, or YFP alone. γ2A cells were also transiently transfected with Jak2-YFP or YFP. The living cells were monitored using confocal microscopy.](image2)
have an intrinsic membrane binding potential. To test this we used a non-receptor-binding Jak1 point mutant with the amino acid exchanges L80A and Y81A in the loop 4 region of the FERM subdomain F1 (10). We generated U4C clones stably expressing endogenous amounts of Jak1-L80A/Y81A and Jak1-L80A/Y81A-YFP. U4C-Jak1-L80A/Y81A clones did not show Jak1 phosphorylation (Fig. 6 A) or STAT activation (Fig. 6 B) after OSM and IFN-γ stimulation, nor did they induce an up-regulation of MHC-I expression after IFN-γ stimulation (Fig. 6 C). This is due to the fact that Jak1-L80A/Y81A does not bind to cytokine receptors and thus cannot be activated.

Fractionation experiments in U4C-Jak1-L80A/Y81A cells showed that the non-receptor-binding mutant is located mainly in the cytoplasm in contrast to Jak1-WT (Fig. 7 A). In living cells, Jak1-L80A/Y81A-YFP also exhibited a pronounced cytoplasmic localization in stable U4C-transfectants (Fig. 7 B).

Thus, the mutations that abrogate receptor association concomitantly prevent membrane association of the corresponding Jak1 mutants. This indicates that Jak1 has no strong intrinsic membrane binding potential and that membrane recruitment

Figure 5. Jak1 localization does not change upon cytokine stimulation. A, 2C4 fibrosarcoma cells were stimulated with OSM for different periods of time, fractionated, and analyzed by SDS-PAGE. Western blots were detected using antibodies against Jak1, Tyk2, and marker proteins for the different fractions. B, 2C4 fibrosarcoma cells were stimulated with OSM and IFN-γ and analyzed as described in A. C, U4C cells stably expressing Jak1-YFP were stimulated with IL-6 (100 ng/ml) and soluble IL-6 receptor (sIL6R, 1 μg/ml). Living cells were monitored by confocal microscopy. Cyt.-UC, cytoplasmic ultracentrifuged; Nuc., nuclear; Mem., membrane; 1. wash, first Nonidet P40 wash.

Figure 6. The non-receptor-binding mutant Jak1-L80A/Y81A does not induce signal transduction events upon cytokine stimulation. A, three independent clones of U4C-Jak1-L80A/Y81A (clones 1, 7, and 10) were stimulated with OSM or IFN-γ. Lysates were prepared and subjected to immunoprecipitation using a Jak1 antibody (HR785). The immunoprecipitates were analyzed by SDS-PAGE. Western blots were probed with an anti-phosphotyrosine monoclonal antibody (PY-99, Santa Cruz Biotechnology, Inc.). The blots were reprobed with an anti-Jak1 antibody. B, STAT3 (Tyr-705) and STAT1 (Tyr-701) phosphorylation was detected using phosphotyrosine-specific antibodies. The blots were reprobed using STAT3- and STAT1-specific antibodies. C, U4C cells stably expressing Jak1 or Jak1-YFP were stimulated with IFN-γ for 3 days, and MHC-I expression was monitored by fluo-

rescence-activated cell sorting analysis. Histograms resulting from unstimulated cells stained with the MHC-I-specific antibody are shown in gray; those from stimulated cells are shown as a black line. PY, phosphotyrosine; IP, immunoprecipitation.
of Jak1 crucially depends on its ability to associate with cytokine receptors.

**DISCUSSION**

The first intracellular step upon cytokine binding to their specific transmembrane receptors is the activation of Janus kinases. IL-6 type cytokines have been shown to activate Jak1, Jak2, and Tyk2. Among the Jaks, Jak1 seems to play a crucial role (40, 41). IFN-γ signaling also relies on the activation of both Jak1 and Jak2 for an efficient signal transduction. In this study we characterized the subcellular distribution of Janus kinases focusing on Jak1.

In subcellular fractionation studies Jak1 and Tyk2 were found to be localized in membrane fractions of 2C4, 2TGH, HepG2, HeLa, and A375 cells and HUVECs. Jak1 and Tyk2 were, however, not found in significant amounts in the cytoplasmic or nuclear fractions. In all experiments Jak1 detection in cytoplasmic or nuclear fractions could be related to membrane contamination.

To corroborate the results from the subcellular fractionation experiments we tested a number of Jak1 and Jak2 antibodies in immunofluorescence using antibodies against Jak1, Tyk2, and marker proteins for the different fractions. Cyt.-UC, cytoplasmic ultracentrifuged; Nuc., nuclear; Mem., membrane.

**FIG. 7.** The non-receptor-binding mutant Jak1-L80A/Y81A-YFP shows a cytoplasmic localization in living cells. A. U4C cells stably expressing Jak1-WT and Jak1-L80A/Y81A were fractionated and analyzed by SDS-PAGE. Western blots were detected using antibodies against Jak1, Tyk2, and marker proteins for the different fractions. B, living U4C-Jak1-YFP or U4C-Jak1-L80A/Y81A-YFP cells were visualized using confocal microscopy. Cyt.-UC, cytoplasmic ultracentrifuged; Nuc., nuclear; Mem., membrane.

To circumvent fixation artifacts and unspecific antibody binding in indirect immunofluorescence we generated fusion proteins of Jak1 and Jak2 with the fluorescent protein YFP to determine the subcellular distribution of both kinases within living cells. Fusion of the YFP moiety either to the C terminus (in the case of Jak1), or to the N terminus (in the case of Jak2), did not alter any tested biochemical characteristics of these molecules. Jak phosphorylation, STAT activation and MHC-I expression were comparable in cells stably expressing either Jak1 or Jak1-YFP. Jak1-YFP and Jak2-YFP were both located at the plasma membrane in stable and transient U4C transfectants (Jak1-YFP), in transient γ2A transfectants (Jak2-YFP), and in transient HEK transfectants (Jak1-YFP and Jak2-YFP). Thus, our results from confocal microscopy in living cells and from the fractionation experiments coincide.

A membrane-bound protein, like Jak1, without a transmembrane domain can be directly bound to the membrane by lipid modifications (e.g. myristoylation, palmitoylation, or farnesylation), lipid binding domains (e.g. FERM, pleckstrin homology, and FYFE domains), membrane-penetrating structures, electrostatic forces, binding to another membrane-associated protein, or a combination of some of these mechanisms. Here we provide evidence that endogenous Jaks are recruited to membranes by binding to cytokine receptors. To show this we used the Jak1-L80A/Y81A double mutant that does not bind cytokine receptors any more (10). In a model of the F1 lobe of the Jak1 FERM domain the amino acids Leu-80 and Tyr-81 are located in the exposed loop 4 region (10). Loop 4 of the F1 domain is exposed on the surface of the FERM domain in solved structures (12–14). Mutation of Leu-80 and Tyr-81 to alanine is therefore thought to disrupt receptor binding only and not to affect the structural integrity of the FERM domain. In living cells Jak1-YFP is localized at the plasma membrane, while Jak1-L80A/Y81A-YFP is evenly distributed throughout the cytoplasm. Fractionation experiments with cells stably expressing Jak1 and Jak1-L80A/Y81A also showed the cytoplasmic localization of Jak1-L80A/Y81A. Thus disruption of cytokine receptor binding is sufficient to make Jak1 a cytoplasmic protein. Interestingly the FERM domain of radixin has been described to bind phospholipids (13), and the positive residues promoting this interaction are conserved in the FERM domains of ezrin, radixin, and moesin. However, these crucial residues are not conserved in the Jaks. The Jak1 FERM domain could bind phospholipids in membranes by a mechanism different from that of ERMs (ezrin, radixin, moesin) proteins, but phospholipid binding is generally a transient phenomenon and can probably not account for the permanent membrane localization of Jak1. It is conceivable that the recruitment to different membrane compartments (raft versus non-raft localization or plasma membrane versus endoplasmic reticulum localization) could be regulated by such a mechanism.

Our data from this and our previous studies suggest that Jak1 is recruited to membranes by tight association with cytokine receptors, virtually forming a receptor tyrosine kinase. The crucial regions for gp130/Jak1 interaction comprise about 70 amino acids from gp130 and the FERM domain (418 amino acids) of Jak1 (10, 11, 42, 43). In the receptor cytoplasmic tail routinely used in cell biology (anti-lamin A/C and anti-β-tubulin—FITC). Thus our incapability to obtain specific immunofluorescence stainings with Jak antibodies was due to the fact that these antibodies are not specific enough to be used for this purpose. In fact the previously published data describing nuclear Jak1 and Jak2 (32–34) rely on the use of the polyclonal Jak1 and Jak2 antisera and the α-Jak1–α-Jak2–α-Jak2 antibodies (which we found to be unspecific in immunofluorescence) for immunofluorescence or immunohistochemistry.
and in the Jak1 FERM domain essential residues for binding have been identified (10, 42). Interestingly Jak/receptor interaction seems to be mediated by multiple contact sites that dictate the Jak position in a defined orientation critical for activation (43). The identical half-lives of gp130 and Jak1 also argue in favor of a “common fate” of the two proteins (44). Using fluorescence recovery after photobleaching analysis our group has recently shown that overexpressed gp130 and Jak1 have identical diffusion velocities characteristic for plasma membrane proteins (45). There is increasing evidence that Jaks also bind to cytokine receptors that are not presented on the cell surface (26–30). Furthermore evidence has been presented that Jaks may be present only in limited amounts and that competition exists between different cytokine receptors for the recruitment of the available kinases (46).

The fact that Jak1 has now been shown to be membrane-localized only by receptor interaction can be of practical value. We and others have described previously that the binding interfaces of cytokine receptors (e.g., gp130) and Jak1 involve large portions of both proteins. Interestingly the intracellular regions of different cytokine receptors do not show much similarity concerning the nature and the position of the residues involved in Jak binding, although they bind the same Janus kinase. Thus interfering with the receptor/Jak interaction might be a specific way to inhibit cytokine signal transduction. This approach might be superior to the use of kinase inhibitors for Janus kinases because the ubiquitously expressed Janus kinases (Jak1, Jak2, and Tyk2) are often used in different cytokine signaling pathways. In disease treatment this approach might represent an alternative to cytokine antagonists, neutralizing antibodies, or kinase inhibitors. In addition, the fact that we have shown that endogenous Jak1 has no strong membrane binding potential and that Jak1-YFP expressed at endogenous levels is biochemically equivalent to Jak1-WT presents a chance of establishing a Jak1-YFP screening assay. A receptor and a Janus kinase of interest can be expressed as fluorescently labeled proteins in cells and screened for membrane localization with or without possible interaction inhibitors.

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Note Added in Proof—As opposed to human Tyk2, the murine orthologue is excluded from the nucleus, as seen by immunofluorescence (S. Pellegrini, personal communication).

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