The Human Homologue of the Yeast Proteins Skb1 and Hsl7p Interacts with Jak Kinases and Contains Protein Methyltransferase Activity*

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To expand our understanding of the role of Jak2 in cellular signaling, we used the yeast two-hybrid system to identify Jak2-interacting proteins. One of the clones identified represents a human homologue of the Schizosaccharomyces pombe Skb1 kinase-binding protein 1, Skb1, and the protein encoded by the Saccharomyces cerevisiae HSL7 ('histone synthetic lethal 7') gene. Since no functional motifs or biochemical activities for this protein or its homologues had been reported, we sought to determine a biochemical function for this human protein. We demonstrate that this protein is a protein methyltransferase. This protein, designated JBP1 (Jak-binding protein 1), and its homologues contain motifs conserved among protein methyltransferases. JBP1 can be cross-linked to radiolabeled S-adenosylmethionine (AdoMet) and methylates histones (H2A and H4) and myelin basic protein. Mutants containing substitutions within a conserved region likely to be involved in AdoMet binding exhibit little or no activity. We mapped the JBP1 gene to chromosome 14q11.2–21. In addition, JBP1 co-immunoprecipitates with several other proteins, which serve as methyl group acceptors and which may represent physiological targets of this methyltransferase. Messenger RNA for JBP1 is widely expressed in human tissues. We have also identified and sequenced a homologue of JBP1 in Drosophila melanogaster. This report provides a clue to the biochemical function for this conserved protein and suggests that protein methyltransferases may have a role in cellular signaling.

The Jak1-Stat pathway plays a crucial role in the signal transduction of many cytokines, growth factors and hormones. Central to this pathway are the Jak family of protein tyrosine kinases. This family includes the mammalian kinases Jak1, Jak2, Jak3, and Tyk2 and the Drosophila melanogaster kinase encoded by the hopscotch (hop) locus (1–14). The Jaks are essential for the biological activities mediated by these ligands and defects in this family of kinases have been shown to lead to a number of disease states in both mammals and D. melanogaster (15–23).

The role of the Jak kinases in cytokine signal transduction was first shown for the interferons (IFNs) (24, 25). Subsequently, many reports have demonstrated that Jak activation occurs rapidly after ligand stimulation (1, 8, 9, 26). This activation initiates a cascade of events, which includes receptor phosphorylation and recruitment, subsequent phosphorylation and nuclear translocation of members of the Stat (signal transducers and activators of transcription) family of proteins, which then activate cytokine-inducible genes (27). In addition to their enzymatic role, several reports have demonstrated that the Jaks play a structural role in the receptor complex and that the Jaks may have functions in addition to their kinase activity that are important for signaling. For example, introduction of a kinase-inactive mutant of Jak1 into cells that lack this kinase (and are unresponsive to interferon-γ (IFN-γ)) restores partial IFN-γ-induced gene expression (28, 29). Furthermore, the amino terminus of Tyk2 stabilizes the IFNAR1 chain of the IFN-α receptor complex (30).

In addition to their interactions with cytokine receptor chains, a large body of evidence has accumulated demonstrating that the Jak kinases interact with other signaling proteins. In particular, Jak2 was reported to interact with SHPTP1, SHPTP2, PP2A, PI3K, Yes, Fyn, She, Syp, Grb2, the angiotensin II AT1 receptor, and the serotonin 5-HT2A receptor (31–44). The ability to interact with such diverse proteins underscores the complex role of Jak2, which is activated by the majority of cytokine receptor ligands that utilize the Jak-Stat pathway (45). While the physiological roles for these interactions have not been characterized, they suggest that the Jaks play a role in other pathways and/or facilitate cross-talk between signaling pathways.

In identifying Jak2-interacting proteins with the yeast two-hybrid system, we cloned a human homologue of the Schizo-
saccharomyces pombe Skb1 protein and the Saccharomyces cerevisiae protein encoded by the HSL7 gene (46–48). The skb1 gene was initially identified during a two-hybrid screen for proteins interacting with the skb1 kinase which represents a member of the p21^{cdcl121}/Roc1-activated kinase (PAK) family of protein kinases (47). Recent data suggest that removal of this protein results in cell cycle abnormalities and that the human homologue of this protein can functionally substitute for Skb1 (49). The HSL7 (histone synthetic lethal 7) gene was initially identified as a gene whose mutation is lethal in combination with a mutation in the histone H3 and was described to be a negative regulator of Sve1 function (48). Disruption of HSL7 also results in cell cycle abnormalities of S. cerevisiae (48). Taken together these data suggest that this family of proteins is involved in coordinating cellular events such as the cell cycle or cellular signaling. Since no functional motifs or biochemical activities had been identified for Skb1 or Hsl7p, we focused on identifying a biochemical activity for JBP1. This report shows that JBP1 is a protein methyltransferase.

MATERIALS AND METHODS

Creation of Plasmid Encoding GAL4 DBD-Jak2—Expression vectors for Jak1, Jak2, Jak3, and Tyk2 were gifts from James Ihle and John Krown. The yeast two-hybrid system vector pAS2 which contains the yeast two-hybrid (TRP1)-selectable marker and a hemagglutinin (HA) tag, was a gift from Stephen Elledge (50). To create pAS2-Jak2, the murine Jak2 cDNA was modified in the following manner. First, the 4Trg1 site in the 3’ untranslated region of the plasmid Bluescript SK-muJak2 (a gift from James Ihle) was removed by digesting with NheI, blunt ending with the large fragment of DNA polymerase I (Klenow fragment), digesting with EcoRV, and recircularizing with T4 DNA ligase. This created the plasmid muJak2-4Trg1. A linker containing a SfiI site was placed into the remaining 4Trg1 site located 53 base pairs downstream from the translational start codon. This linker was created by annealing two oligonucleotides (5'-GTACGGCATGGAGGCC-3' and 5'-GTACGGCTCATTGGAGGCC-3'), then heating equimolar amounts to 100 °C, and cooling slowly to 4 °C in 10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl2 (pH 8.3 at 20 °C) in a total volume of 0.05 ml. Thirty cycles at 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 45 s were performed. The product from this reaction was digested sequentially with NotI and KpnI restriction endonucleases and cloned into yeast plasmid p41Δ6-Bluescript digested with the same enzymes (56). The PCR product was created with NotI and KpnI restriction endonucleases. To create plasmid pEF2-HA, plasmid pCDNA3-HA was digested with KpnI and SfiI restriction endonucleases and the fragment containing the HA tag and multiple cloning site was cloned into vector pCDF3 digested with the same enzymes (56).

The plasmid to express HA-tagged JBP1 was constructed in three steps. First, PCR was used to amplify the insert with a 5’ NorI site whose frame was compatible with the NorI site of vector pCDNA3-HA. This was accomplished by PCR with the plasmid p41Δ6-Bluescript as the template and the following 3’ and 5’ oligonucleotide primers, respectively: U2–5’ (5’-TTTGCCACACATCACTGCTG3’) and HA–41’ (5’-CGGAATTCGGCCTCGCCGGGTCGCTGTTG3’). The PCR product was digested sequentially with NotI and Kpn1 restriction endonucleases and cloned into plasmid pCDNA3 (Invitrogen), which was digested with NotI and Kpn1 restriction endonucleases. The vector pEF2-ATG was digested with HindIII and NotI restriction endonucleases and cloned into yeast plasmid pEF2Δ6-Bluescript digested with the same enzymes. The PCR product was created with HindIII and NotI restriction endonucleases and cloned into yeast plasmid pEF2Δ6-Bluescript digested with the same enzymes. The fragment containing the HA tag and multiple cloning site was cloned into vector pCDF3 digested with the same enzymes (56).

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GAGTC-3'. The conditions for PCR were the same as above. The amplified fragment was digested with the BamHI and KpnI restriction endonucleases and cloned into vector pDE3P3 digested with the same enzymes (56). The plasmid pEF2-ATG-Myc-N vector was created by digesting the plasmid pEF2-ATG-Myc with the BamHI restriction endonuclease, purifying the insert from agarose, digesting with the NotI restriction endonuclease, and cloned into the plasmid vector pCR2.1 (Stratagene) and then subcloned as a NotI/SpeI fragment into vector pEF2HA digested with NotI and XhoI restriction endonucleases.

Creation of JBP1 Point Mutants—The PCR was used to create two point mutants of JBP1 called JBP1R368A and JBP1G367A. To create JBP1R368A, a fragment of JBP1 was amplified with the oligonucleotides 41del-1 (5'-GGCGGCGCCCTTGGTGGACACAG-GAG-3') and MUT1 (5'-GGTTCACCGGCGTCCCTGCTCTGCCC-3'; changes bases shown in boldface) and the plasmid pEF2-Flag-JBP1 as the template. Thirty cycles at 94 °C for 40 s, 42 °C for 40 s, and 72 °C for 40 s were performed in a PCR machine in a 10-ml reaction volume. Afterward, the beads were washed twice in 1 ml of Buffer B (25 mM HCl, 136 mM NaCl, and 2.7 mM KCl, pH 7.4) and then addropwise to the 800-ml DMEM/DEAE-dextran/chloroquine suspension containing 10% FBS. Medium was aspirated from a dish of cells, then washed twice for 2 min with rocking and then incubated for 1 h with the indicated secondary antibody conjugated to horseradish peroxidase followed by washing as described above for immunoprecipitation. Cell lysate was passed and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) with the Trans-Blot apparatus. After incubation, the membrane was removed by aspiration and the cells were washed with the addition of 3 ml of PBS containing 10% Me2SO. After 2 min the Me2SO was aspirated, the cells rinsed with 3 ml of PBS, and then returned to the 37 °C incubator with 10 ml of complete DMEM. Cells were harvested for assays after 48–72 h.

Isolation of HeLa Cell Lysate—Dishes of adherent COS cells were washed with ice-cold PBS and scraped with a silicone policeman in the DEAE-dextran/Me2SO shock protocol (59–61). Tissue culture dishes were washed twice with ice-cold PBS and then scraped with a silicone policeman in 1 ml of DMEM. Cells were harvested for assays after 48–72 h.

In Vitro Binding Assay—To express the recombinant protein fused to glutathione S-transferase (GST), the insert from plasmid p31–2B was cloned into vector pGEX1 (Amersham Pharmacia Biotech) as a BamHI/XhoI fragment creating plasmid pGST-JBP1. This fused amino acids sequence was amplified by PCR with the plasmid pEF2-ATG-Myc as a template and the T7 oligonucleotide and the oligonucleotide 41del-1. The fragments containing the mutations were purified from agarose and ligated into the plasmid vector pEF2-ATG-Myc with the NotI restriction endonuclease. The inserts from the Flag constructs were cloned into the pEF2-ATG-Myc expression vector. The fragments fused to the Myc epitope, except that the oligonucleotide MUT2 (5'-GGTCCACCAGGGGTCCC-3') was used instead of MUT1. After amplification, these fragments were extracted with phenol/chloroform/isonamyl alcohol, precipitated with ethanol, and digested with the restriction endonucleases SaclI and NdeI. The fragments containing the mutations were purified from agarose and ligated into the plasmid pEF2-Flag-JBP1 digested with the same restriction endonucleases. To express the JBP1R368A and JBP1G367A proteins fused to the Myc epitope, the inserts from the Flag constructs were cloned into the pEF2-ATG-Myc-N vector as NotI/XbaI fragments.

Cell Culture and transient DNA Transfections—COS-1 cells (58), derived from a simian kidney line, were transfected with plasmids by the DEAE-dextran/Me2SO shock protocol (59–61). Tissue culture dishes (10-cm dish, Falcon) containing 10 ml of 10% calf serum-supplemented Dulbecco’s modified Eagle’s medium (DMEM) were seeded with 1.75–2.0 x 10⁶ cells trypsinized at confluence. Cells were incubated overnight at 37 °C before transfection. For each dish, 636 ml of DEAE-dextran (10 mg/ml, filter-sterilized), and 4 µl of the DNA (approximately 10 µg) were added dropwise to the 800-ml DMEM/DEAE-dextran/chloroquine suspension containing 10% FBS. Medium was aspirated from a dish of cells, then washed twice for 2 min with rocking and then incubated for 1 h with the indicated secondary antibody conjugated to horseradish peroxidase followed by washing as described above for immunoprecipitation. Cell lysate was passed and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) with the Trans-Blot apparatus. After incubation, the membrane was removed by aspiration and the cells were washed with the addition of 3 ml of PBS containing 10% Me2SO. After 2 min the Me2SO was aspirated, the cells rinsed with 3 ml of PBS, and then returned to the 37 °C incubator with 10 ml of complete DMEM. Cells were harvested for assays after 48–72 h. Isolation of HeLa Cell Lysate—Dishes of adherent COS cells were washed with ice-cold PBS and scraped with a silicone policeman in the DEAE-dextran/Me2SO shock protocol (59–61). Tissue culture dishes (10-cm dish, Falcon) containing 10 ml of 10% calf serum-supplemented Dulbecco’s modified Eagle’s medium (DMEM) were seeded with 1.75–2.0 x 10⁶ cells trypsinized at confluence. Cells were incubated overnight at 37 °C before transfection. For each dish, 636 µl of DMEM, 160 µl of DEAE-dextran (10 mg/ml, filter-sterilized), and 4 µl of chloroquine (200 µg/ml, filter-sterilized) was added. After 2 min, 5 µg of plasmid DNA was diluted in 200 µl of TBS (25 mM HCl, 136 mM NaCl, and 2.7 mM KCl, pH 7.4) and then added dropwise to the 800-ml DMEM/DEAE-dextran/chloroquine mixture. Medium was aspirated from a dish of cells, then this DNA/DMEM/DEAE-dextran/chloroquine mixture was then added to the dish, swirled gently for a few seconds, topped with 3 ml of DMEM/DEAE-dextran/chloroquine, and then incubated with rocking at 37 °C. After the incubation, the medium was removed by aspiration and the cells were shocked by the addition of 3 ml of PBS containing 10% Me2SO. After 2 min the Me2SO was aspirated, the cells rinsed with 3 ml of PBS, and then returned to the 37 °C incubator with 10 ml of complete DMEM. Cells were harvested for assays after 48–72 h.

Isolation of HeLa Cell Lysate—Dishes of adherent COS cells were washed with ice-cold PBS and scraped with a silicone policeman in 0.5–1.0 ml of ice-cold Lysis Buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2.5 mM MgCl2, 10.3 mM NaF, 1.05 mM Na3VO4, 5 mM sodium pyrophosphate, 1% IGEPAI (Sigma catalog no. I-3201), 0.8% phenylmethylsulfonyl fluoride (PMSF), 1 µl/ml leupeptin, 2 µl/ml antipain, 10 µg/ml benzamidine, 10 µl/milliliter inactivating units/ml aprotinin, 1 µg/ml chymostatin, 1 µg/ml pepstatin). Cells were incubated on ice for 20 min to 12 h and spun down at 16,000 x g for 10 min at 4 °C. Supernatants were transferred to a new 1.5-ml microcentrifuge tube, and lysates were stored at -70 °C or in a nitrogen freezer until used.

Isolation of HeLa Cell Lysate—Dishes of adherent COS cells were washed with ice-cold PBS and scraped with a silicone policeman in 0.5 ml of Lysis Buffer. Cells were incubated on ice for 20 min to 12 h and spun down at 16,000 x g for 10 min at 4 °C. Supernatants were transferred to a new 1.5-ml microcentrifuge tube. To immunoprecipitate epitope-tagged JBP1, 0.2 ml of the above lysate was incubated with 0.5–2.0 µg of the appropriate antibody for 1 to 12 h with rocking. Protein A/G plus beads (Santa Cruz sc-2003) were added and incubated for 1 h with rocking. The beads were pelleted and then washed three times in 1 ml of ice-cold Lysis Buffer for 1 to 10 min per wash. The beads were then resuspended with SDS-PAGE buffer and resolved with SDS-PAGE. Proteins were transferred and blotted for Western analysis.

Affinity Purification of Flag-JBP1 and His6-JBP1—Flag-JBP1 was transiently expressed in COS cells, and cell lysates were prepared as described above for immunoprecipitation. Cell lysate was passed over an anti-Flag M2 affinity column created by packing 1 ml of anti-Flag M2 affinity gel (Eastman Kodak Co. catalog no. IB13020) into a 2.0-ml column.
Human Homologue of Skb1 and Hsl7p Interacts with Jak

Econo-Pac disposable chromatography column (Bio-Rad catalog no. 732—1010). The column was first washed in 15 ml of ice-cold glycine buffer containing 0.1 M glycine-HCl, pH 3.5, followed by washing with 15 ml of TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5). Cell lysate was then passed over the column by gravity flow three to four times, and the column was washed with 50 ml of ice-cold TBS. Flag-JBP1 was eluted in 1-ml fractions in TBS containing 250 μg/ml Flag peptide (Kodak catalog no. IB13070), and fractions were analyzed by SDS-PAGE and Coomassie Blue staining.

To purify recombinant protein, the insert from plasmid pEF2HAJB1P1D4 was cloned into the BamHI site of the pRSBII six-histidine vector of the appropriate frame. A Flag epitope and a histidine epitope tag to the NH2 terminus of proteins encoded by the JBP1D4 DNA insert. E. coli carrying the appropriate plasmid were grown with 1 ml IPTG for 3–12 h at 37 °C. Cells were pelleted at 4651 × g for 4000 rpm in a RC3C Sorvall centrifuge with a H600A rotor. Cells were resuspended in denaturing Lysis Buffer (final pH of 8.0, 50 mM Na2PO4, 10 mM Tris-HCl, 100 mM NaCl) at 0.5 ml2 of liquid culture. The lysate was centrifuged at 14,000 rpm (23, 420 × g) in a Sorvall RC5B centrifuge with an SS-34 rotor. Supernatant was loaded onto a column containing 2 ml of Talon resin (CLONTECH) prewashed with denaturing lysis buffer. After loading, the column was washed with at least 20 rinses of Wash Buffer (pH 7.0, 8 M urea, 50 mM NaH2PO4). Protein was eluted with either Elution Buffer A (pH 5.0, 8 M urea, 20 mM Pipes, 100 mM NaCl) or Elution Buffer B (pH 7.0, 8 M urea, 50 mM NaH2PO4, 100 mM EDTA). Fractions were analyzed by Bio-Rad protein assay and Coomassie Blue staining after SDS-PAGE.

UV Cross-linking of [3H]Adenosylmethionine to Flag-JBP1—One half μg to 2 μg of BSA, CheR (15), Flag-JBP1, GST, or GST-JBP1-N268 were each incubated with 5.5 μCi of [3H]Adenosylmethionine (NEN catalog no. NET1553) in cross-linking buffer (50 mM Tris, pH 7.5, 0.1 mM NaCl, 2 mM EDTA, 1 mM DTT) in a total volume of 0.065 ml. Samples were added to 96 well plates and incubated on ice at a distance of 3.5–5.0 cm from the UV source (Stratalinker 2400). Samples were exposed to two 0.96 joules of UV irradiation and the reaction stopped by the addition of 30 μl of 3 × SDS-PAGE sample buffer. Samples were stored at −20 °C overnight, and then proteins were separated by SDS-PAGE and stained with Coomassie Blue. This was followed by incubation with Enzyme reagent (NEF992) and radiography with Kodak Biomax MR film at −70 °C for 7–14 days.

Methylation of Proteins—HA-JBP1 was immunoprecipitated with anti-HA antibody from the HeLa-HA-SMT cell line as described above and washed three times for 10 min in 0.5 ml of ice-cold Lysis Buffer. As a control for immunoprecipitation, HeLa cells transfected with the parent vector pEF2HA containing no insert were used. This was followed by rinsing the immunoprecipitates two times with Methylation Buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM EGTA) at room temperature. Protein A/G beads with bound HA-JBP1 were resuspended in 0.1 ml Lysis Buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA) at a distance of 10 cm from the UV source. The protein A/G beads with bound HA-JBP1 were incubated with the aid of avidin antibody (Oncor), washed in 2 × SDS, dehydrated, and then denatured at 70 °C in 10% formamide, 2 × SSC immediately prior to hybridization. The hybridization mixture (15 μl/slide) contained the biotinylated probe (300 ng/slide) and excess repetitive human DNA (Blockit, Oncor) in Hybridization Buffer (pH 6.5). After overnight hybridization at 37 °C, slides were washed at 45 °C in 0.5 M formamide/1× SSC (2 × 10 min), 1× SSC (2 × 5 min), 0.1× SSC (1 × 5 min), and 0.1× SSC (1 × 5 min) at room temperature. Probe detection was performed by incubation of the slides with fluorescein isothiocyanate-avidin (Oncor) for 20 min at 37 °C. Signal amplification was performed by subsequent incubation with an anti-avidin antibody (Oncor), followed by incubation with fluorescein isothiocyanate-avidin. The slides were mounted in the antifade medium (Oncor) containing diammonium fluorescein-2-(p-trifluoroacetoxyethyl)amine and 200 mM EM60 Olympus fluorescent tissue microscope. Color prints of the metaphase spreads showing hybridization with JBP1 were obtained with Cytovision (Applied Imaging). The slides were subsequently destained and Giemsa-banded (63).

RESULTS
Identification and Cloning of JBP1 with the Yeast Two-hybrid System—To screen for Jak2 interacting proteins with the two-hybrid system, we cloned the murine Jak2 cDNA into vector pAS2 (50) as described under “Materials and Methods.” This construct fused the GAL4 DNA binding domain (GAL4DBD) to amino acids 19–1129 of the murine Jak2 cDNA.

The yeast strain Y190 was cotransformed with the plasmid pAS2-Jak2 and a HeLa cell library created for use in the two-hybrid system (51). Yeast strain Y190 contains two reporter genes whose transcription indicates an interaction between the two GAL4 fusion proteins. We first selected transformants for their ability to activate the HIS3 reporter gene by plating onto media lacking histidine. Histidine prototrophs were selected and subsequently tested for activation of the lacZ reporter gene. After screening 1.6 × 109 transformants, 143 histidine prototrophs were selected to assay for β-galactosidase activity. Twenty-eight of those assayed showed evidence of β-galactosidase activity. To determine whether the interactions were dependent on expression of the GAL4–Jak2 fusion protein, we performed a mating assay as described previously (50). Transformants activating both reporter genes were cured of the plasmid pAS2-Jak2 by selecting on media containing cycloheximide. The plasmid pAS2 contains a marker that confers cycloheximide resistance (50). We confirmed loss of the pAS2-Jak2 plasmid by selecting the cycloheximide-resistant colony for tryptophan auxotrophy. Colonies, which were checked only on the library plasmid, were grown to early log phase in Y187 expressing “decoy” GAL4 fusion proteins including lamin, CDK2, or SNF1. We also re-tested for the original Jak2 interaction by including yeast expressing the GAL4-Jak2 fusion protein in this mating assay. Of the 28 clones that exhibited β-galactosidase activity, 10 activated the lacZ reporter gene in the presence of the GAL4-Jak2 fusion protein, but not in the pres-
ence of the “decoys.” Results from one of these mating assays can be seen in Fig. 1. Library plasmids from these 10 clones were rescued, characterized by restriction endonuclease digestion, and sequenced.

The 10 clones which activated the lacZ reporter gene only in the presence of the pAS2-Jak construct represented four different cDNAs (46). Three of these cDNAs will be described elsewhere. Two of the 10 clones, called 31–2B and 41–3A, represented independent isolates of the same cDNA. The inserts from clones 31–2B and 41–3A were each roughly 1.5 kb. To isolate a longer clone, we used the 41–3A insert as a probe to screen a human M426 cDNA library (54). This led to the isolation of a 2.4-kb cDNA clone, which codes for a protein of 637 amino acids called JBP1 (for Jak-binding protein 1), with a predicted molecular mass of 72.4 kDa. The putative full-length sequence (GenBank accession no. AF167572) was compiled from our sequence with an additional 97 nucleotides from a sequence tag recently entered into the GenBank (accession no. AA417623). This added an additional two amino acids plus some 5'-untranslated region nucleotides to our cDNA. The open reading frame of the JBP1 cDNA encoded the amino acid sequence shown in Fig. 2A.

In Vitro Interaction between GST-JBP1 and the Jak Kinases—To determine whether JBP1 could interact with Jak2 outside of the two-hybrid system, we created a GST fusion protein by cloning the insert from the plasmid p31–2B into the pGEX1 expression vector through a compatible BamHI site (see “Materials and Methods”). This fused amino acids 268–637 of JBP1 to GST and is referred to as GST-JBP1-N268. We then purified and immobilized recombinant GST-JBP1-N268 onto glutathione-agarose beads. To provide a source of the Jak proteins, COS-1 cells were transiently transfected with different Jak kinase expression plasmids. Lysates from these cells were prepared and incubated with immobilized GST or GST-JBP1-N268. Proteins that bound to the beads were eluted by boiling in sample buffer, separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with the respective anti-Jak antibodies. GST-JBP1-N268, but not GST alone, was able to bind murine Jak1, murine Jak2, murine Jak3, and human Tyk2 from COS-1 cells (Fig. 3). The interaction (with Jak2) did not appear to require an active kinase domain, as GST-JBP1-N268 was also able to interact with a kinase inactive mutant of Jak2.

Identification of Sequence Homology between JBP1 and Protein Methyltransferases—Initial searches of the GenBank data bases with our 41–3A sequence revealed homology to sequence tags from a variety of different libraries. Subsequent entries led to the identification of JBP1 as a homologue of the protein encoded by the S. pombe gene skb1 and the S. cerevisiae gene HSL7 (47, 48). In these reports, homologous open reading frames from an uncharacterized human cDNA sequence were identified (47, 48). Since our original report (46), additional sequence data for this human cDNA have been entered into the GenBank data base (accession no. AF015913). This sequence is identical to our JBP1 sequence with the exception of a substitution of valine for glycine at position 553 and phenylalanine for serine at position 247 (Fig. 2). In addition, when we searched the data bases with our sequence, it matched highly to the complement of the 3'-untranslated region of a murine cDNA encoding basic protein 2 as well as other murine sequence tags. When these murine sequences were compiled and translated (GenBank accession no. AF167573), they encoded a protein with extremely high homology to our human cDNA as shown in Fig. 2A. We have also obtained a D. melanogaster homologue of JBP1. By searching available data bases, we identified Drosophila expressed sequence tags with homology to JBP1. After obtaining these clones (LD07634 and LD08768) from Genome Systems Inc., they were sequenced with vector and internal primers (GenBank accession no. AF167574). Initial sequencing revealed that clone LD08768 had a deletion of amino acids 126–130. Whether this represents a physiological splice variant of this protein remains to be seen. Fig. 2A shows the homology between members of the JBP1 family which currently includes sequences or cDNA clones from S. pombe, S. cerevisiae, Caenorhabditis elegans, D. melanogaster, Mus musculus, and Homo sapiens. Since we used the murine Jak2 cDNA as the bait to screen a human library, we anticipated that any proteins identified would be highly conserved between mice and humans as is the case with these proteins.

Other than the open reading frames previously reported (47, 48) and the Drosophila and murine homologues of JBP1 reported here, JBP1 shares little similarity to other known proteins and contains no easily recognizable domains. However, continued analysis revealed that JBP1 shared some homology to protein arginine methyltransferases (64–67). Regions that had been described to be conserved in methyltransferases appeared to be present in JBP1 and its homologues (68, 69). These three regions are shown in Fig. 2B. Because of this homology and the fact that methyltransferases have been shown to vary widely in their primary sequence, we determined whether JBP1 exhibited protein methyltransferase activity (70).

Cross-linking of JBP1 to [3H]AdoMet—To determine whether JBP1 represented a new methyltransferase, we first measured whether this protein could bind to the universal methyl donor AdoMet. One method that has been frequently employed to determine AdoMet binding is UV cross-linking (71, 72). To express JBP1 in mammalian cells, we cloned this sequence into mammalian expression vectors containing the Flag epitope. This construct fused amino acids 5–637 of JBP1 with the Flag epitope. We purified FLAG-JBP1 from transiently transfected COS cells by affinity chromatography, incubated this protein with [3H]AdoMet, and exposed the reaction mix-
Human Homologue of Skb1 and Hsl7p Interacts with Jaks

| A          | B          | C          | D          | E          | F          | G          | H          | I          | J          |
|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| JB1        | M. musculus| D. melanogaster| Skb1       | C. elegans| Hal7p      | consensus  | JB1        | M. musculus| D. melanogaster| Skb1       | C. elegans| Hal7p      | consensus  |
| 1          | 1          | 1          | 1          | 1          | 1          | 1          | 57         | 52         | 42         | 64         | 61         | 76         | 1          |
| M. musculus| 1          | 1          | 1          | 1          | 1          | 1          | 136        | 131        | 116        | 143        | 159        | 156        | 161        |
| D. melanogaster| 1        | 1          | 1          | 1          | 1          | 1          | 215        | 210        | 197        | 207        | 234        | 222        | 241        |
| Skb1       | 1          | 1          | 1          | 1          | 1          | 1          | 291        | 286        | 271        | 284        | 314        | 303        | 321        |
| C. elegans| 1          | 1          | 1          | 1          | 1          | 1          | 360        | 355        | 336        | 348        | 391        | 380        | 403        |
| Hal7p      | 1          | 1          | 1          | 1          | 1          | 1          | 427        | 422        | 404        | 415        | 470        | 452        | 481        |
| consensus  | 1          | 1          | 1          | 1          | 1          | 1          | 489        | 484        | 464        | 477        | 549        | 532        | 561        |
| JB1        | M. musculus| D. melanogaster| Skb1       | C. elegans| Hal7p      | consensus  | JB1        | M. musculus| D. melanogaster| Skb1       | C. elegans| Hal7p      | consensus  |
| 1          | 1          | 1          | 1          | 1          | 1          | 1          | 555        | 550        | 529        | 549        | 547        | 538        | 561        |
| M. musculus| 1          | 1          | 1          | 1          | 1          | 1          | 624        | 619        | 616        | 622        | 621        | 628        | 668        |
| D. melanogaster| 1        | 1          | 1          | 1          | 1          | 1          | 651        | 646        | 645        | 650        | 648        | 653        | 721        |
| Skb1       | 1          | 1          | 1          | 1          | 1          | 1          | 624        | 619        | 616        | 622        | 621        | 628        | 668        |
| C. elegans| 1          | 1          | 1          | 1          | 1          | 1          | 651        | 646        | 645        | 650        | 648        | 653        | 721        |
| Hal7p      | 1          | 1          | 1          | 1          | 1          | 1          | 624        | 619        | 616        | 622        | 621        | 628        | 668        |
| consensus  | 1          | 1          | 1          | 1          | 1          | 1          | 651        | 646        | 645        | 650        | 648        | 653        | 721        |
Fig. 2. Predicted amino acid sequence of JBP1 and its homologues. A, The open reading frame of JBP1 and its homologues are shown. The sequences for the S. pombe protein Skb1, C. elegans, S. cerevisiae Hsl7p homologues have been reported previously (47, 48). The murine homologue of JBP1 called JBP1MM was obtained by translating the murine bach 2 cDNA sequence (accession no. D86604) and available murine expressed sequence tag sequences. The D. melanogaster homologue of JBP1 was obtained from a clone identified and sequenced as detailed under “Results.” The consensus sequence is shown on the bottom of each panel. Identical amino acids corresponding to the consensus sequence are shown in black outline with white lettering. Similar amino acids are shown in gray outline with white lettering. Dots represent spaces inserted to maintain alignment. The sequences for Hsl7p (GenBank accession no. P38274) and the JBP1 homologue in C. elegans (GenBank accession no. P46580) are not shown in their entirety. These sequences can be accessed from the GenBank. Alignment was generated with the Genetics Computer Group (GCG) (93) pileup software and shading generated with Boxshade software available via the World Wide Web.

B, comparison of JBP1 and several protein methyltransferases (or putative methyltransferases). Asterisks and Roman numerals indicate methyltransferase regions I, II, and III as described (68, 69). Alignment was generated as above and the accession numbers are as follows: PRMT1 (rat) Q63009, PRMT1 (human) Q99873, RMT1 (yeast) P38074, and PRMT2 (human) P55345. Human and rat PRMT1 and yeast RMT1 have been shown to have methyltransferase activity. The gene for PRMT2 has been localized, but no biochemical activity for its encoded protein has been reported.
ture to UV light as described under “Materials and Methods.” As a positive control, we used the CheR methyltransferase, a well studied bacterial methyltransferase whose crystal structure was determined (70). We included BSA, GST, and GST-JBP1-N268 as additional controls. Fig. 4A shows that of the four proteins tested only CheR and Flag-JBP1 were able to be cross-connected to [3H]AdoMet.

**JBP1 Immunoprecipitates Contain Protein Methyltransferase Activity**—Given that JBP1 had homology to a protein methyltransferase and was able to be cross-connected to [3H]AdoMet, we determined whether JBP1 could transfer labeled methyl groups from [3H]AdoMet to proteins. HeLa cells were transfected with the plasmid pEF2HA-JBP1, and stable cell lines expressing HA-JBP1 were isolated. We immunoprecipitated HA-JBP1 from HeLa cells and incubated this protein with histones and [3H]AdoMet. Histones were selected because they have previously been shown to function as a methyl acceptor for some protein methyltransferases (73–75) and because of the genetic link between HSL7 and histones in yeast (48, 75). As a control we used immunoprecipitates from HeLa cells transfected with the pEF2-HA vector. Incubations of histones with [3H]AdoMet or [3H]AdoMet plus immunoprecipitates from pEF2-HA vector-transfected HeLa cells resulted in no transfer of the radioactive methyl groups (Fig. 4B). However, incubation of histones with [3H]AdoMet plus immunoprecipitates from transfected cells expressing HA-JBP1 resulted in transfer of methyl groups from [3H]AdoMet to histones. To determine whether HA-JBP1 could transfer methyl groups to other methyl acceptors, we also included two additional known methyl acceptors, cytochrome c and myelin basic protein (74, 76). Myelin basic protein, but not cytochrome c, could serve as a methyl acceptor for HA-JBP1.

In addition to reactions containing the exogenously added methyl acceptors, we observed labeled proteins in reactions containing only HA-JBP1 immunoprecipitates. These additional substrates which co-immunoprecipitate with HA-JBP1 vary in molecular weight and may represent additional physiological substrates of JBP1.

**Analysis of JBP1 R368A and JBP1 G367A Point Mutants**—While we identified homology between JBP1 and putative protein methyltransferases and demonstrated cross linking of JBP1 to AdoMet, the possibility of a contaminating enzyme in our immunoprecipitates could not be excluded. To address this issue, we created two point mutants of JBP1 and analyzed immunoprecipitates of these mutants for protein methyltransferase activity. Because of the highly conserved nature of the GXGXR motif in JBP1 and its homologues and the similar GXGXR motif in other protein methyltransferases (69), we selected this region for mutational analysis. Both the conserved arginine in JBP1 R368A and the central glycine in JBP1 G367A were mutated to alanine residues. A similar region has been shown to be involved in AdoMet binding of the Hhal DNA methyltransferase (77). We compared the activity of the two point mutants with that of the wild type enzyme after expression in COS cells. While all three proteins were expressed in COS cells, the Myc-JBP1 protein had the ability to methylate histones whereas the mutants exhibited little or no activity as shown in Fig. 4C. Similar quantities of JBP1 and the two mutants were present in the immunoprecipitates as determined by Coomassie Blue staining.

**JBP1 Specifically Methylates Histones H2A and H4**—To determine which of the five histones could serve as a substrate for JBP1, methylation reactions were carried out with preparations of individual histones. HA-JBP1 was immunoprecipitated from HA-JBP1-producing HeLa cells as described above. Immunoprecipitates were incubated alone or with 10 μg of pooled histones, histone H1, H2A, H2B, H3, H4, myelin basic protein, or cytochrome c (Fig. 5). After separation of the proteins by SDS-PAGE and radiography, it was seen that only histone H2A and H4 were methylated in this experiment. Bands in other lanes were due to the fact that the individual histone preparations were not homogeneous and contained histone H2A or H4 as contaminants. The protein bands corresponding to histones H1, H2B, and H3 were not radiolabeled. The radiographic signals present in the lanes containing histones H1, H2B, and H3 appear to be the same size as histones H2A and H4 (Fig. 5B). These labeled proteins likely represent histones H2A and H4, which were present within these preparations. In addition, labeled proteins of larger molecular weight can be seen in the lanes for histones H2A and H3. These represent other proteins within these preparations which can serve as substrates for JBP1. As shown previously in Fig. 4, myelin basic protein, but not cytochrome c, could also serve as a substrate for JBP1.

**HA-JBP1 Interacts with Itself**—The homologue of JBP1 was shown to form a homodimer in the two-hybrid system (47). In addition, we observed a doublet in our HA-JBP1 immunoprecipitates after staining with Coomassie Blue even though blotting with anti-HA antibodies revealed only a single band (Figs. 4B and 6). To determine whether both of the bands of this doublet were JBP1, we blotted our HA-JBP1 immunoprecipitates with antisera generated against JBP1. Both bands of the doublet reacted with this antisera as shown in Fig. 6, suggesting that the lower band of the doublet represents the endogenous JBP1 that co-immunoprecipitated with HA-JBP1. We confirmed the ability of JBP1 to bind to itself with an *in vitro* binding assay (data not shown). In this assay, the GST-JBP1-N268 protein bound the full-length JBP1 protein produced in COS cells. These data indicate that JBP1 forms homodimeric or multimeric complexes.

**Northern Analysis of JBP1**—In our initial searches for related proteins in the GenBank data base, we noticed that there were expressed sequence tags corresponding to JBP1 from a wide variety of tissues including those from embryonic and fetal tissue. To determine the tissue expression pattern and transcript size, we probed two human multiple tissue Northern blots with the JBP1 sequence. As can be seen in Fig. 7, the JBP1 mRNA is widely expressed in human tissues as a major transcript of 2.5 kb.

**FISH**—A combination of FISH and sequential G-banding was used to determine the chromosomal localization of JBP1. *In situ* hybridization with the biotinylated cDNA probe regionally localized JBP1 to a medium-sized acrocentric chromosome.
Representative results of hybridization of JBP1 to prepared metaphase spreads are shown in Fig. 8A. Hybridization of JBP1 was specific to the proximal long arm of a group D chromosome in 10 metaphase spreads analyzed by FISH. A combination of FISH and sequential G-banding of the same metaphase spreads examined for hybridization further localized JBP1 between bands q11.2-q21 on the long arm of chromosome 14 (Fig. 8B).

**DISCUSSION**

We used the yeast two-hybrid system to identify new Jak2-interacting proteins. One of the proteins identified was a human homologue of the protein encoded by the S. pombe gene skb1 (shkl kinase-binding protein 1) and that encoded by the S. cerevisiae gene, HSL7 (histone synthetic lethal 7) (47, 48). While the defects resulting from the genetic disruption of skb1 and HSL7 implies that these proteins may have a role in regulating the cell cycle or the control of cell morphology, no functional motifs or activities for these proteins were reported (47, 48). Comparison of the human protein with homologues in S. cerevisiae (48), S. pombe (47), C. elegans (47), D. melanogaster (this study), and M. musculus (this study) demonstrate that their primary sequence has been well conserved as shown in Fig. 2A. Given the conserved nature of these proteins and their implication in the control of cellular morphology and the cell cycle, we sought to determine a biochemical activity for this newly identified human protein.

Sequence analysis of these proteins revealed homology to protein methyltransferases (Fig. 2B). While the primary sequence of protein methyltransferases is poorly conserved, three motifs have been reported to exist among this diverse family of enzymes (68–70). The homology between the human protein which we have identified, called JBP1 and protein methyltransferases included these motifs as shown in Fig. 2B. One of these motifs, called motif I, frequently contains the sequence G\(X\)XXXG (68, 69). Motif I is present in many methyltransferases and was shown by crystallography to be within the AdoMet binding pocket of the HpaI DNA methyltransferase (68, 69, 77–79). The conserved GXGRGP region of JBP1 and its homologues likely represents motif I in these proteins and is followed by regions homologous to motif II and III as shown in Fig. 2B.

We first assayed the ability of JBP1 to bind the universal methyl group donor AdoMet with a UVcross-linking assay (Fig. 4A). After demonstrating that JBP1 could bind AdoMet, we tested whether this protein could methylate proteins such as histones, cytochrome c, and myelin basic protein, which are commonly used as methyl group acceptors. HA-JBP1 immunoprecipitates could methylate histone H2A, histone H4, and myelin basic protein. In addition, we also observed the methylation of proteins that co-immunoprecipitated with HA-JBP1 from HeLa cells. We speculate that these proteins likely represent some of the endogenous substrates for JBP1. The ability of JBP1 to bind AdoMet along with the homology between JBP1 and a number of protein methyltransferases suggests that the methyltransferase activity present in our JBP1 immunopre-
arginine methyltransferases and the ability of JBP1 to label proteins known to be arginine-methylated, we hypothesize that JBP1 and its homologues are likely to represent a new group of proteins involved in arginine methylation. Based on its ability to methylate myelin basic protein, JBP1 (and its homologues) may represent the first cloned components of a type II arginine methyltransferase (80, 81). Unlike the large number of protein kinases which have been described over the past two decades, it is only recently that cDNAs encoding arginine methyltransferases have been identified and characterized (64, 65, 75, 84).

While the link between protein kinases and protein methylation may appear tenuous, protein methylation may add another mechanism by which complex cellular events are controlled. It is possible that involvement of JBP1 may be one mechanism by which complex cellular events are controlled. It is possible that involvement of JBP1 may be one mechanism through which the Jaks exert their influence on the cell cycle.

Methyltransferases represent a diverse family of enzymes which can transfer methyl groups from AdoMet to a variety of substrates including nucleic acids, small molecules and proteins (85, 86). Protein methylation reactions can vary with respect to the site of methylation and the nature of the covalent bond formed. Methylation of the α-amino group of certain NH₂-terminal amino acids as well as the N-methylations of histidine, lysine, and arginine residues are irreversible, and are likely to play a structural role (70, 85, 86). Other methylations occurring at carboxyl groups are reversible and may therefore be involved in more dynamic processes (86–89). Protein meth-

**Fig. 5. Specificity of JBP1-histone methylation.** A, immunoprecipitates of HA-JBP1 were incubated with [3H]AdoMet, plus 10 µg of mixed histones, individual histones, myelin basic protein, or cytochrome c as shown. Proteins were separated with 15% SDS-PAGE and visualized by staining with Coomassie Blue. Labeled proteins were visualized with radiography after the gel was treated with Entensify. Top panel, Coomassie Blue-stained gel. Bottom panel, radiography of top panel. B, Coomassie lanes (C) are shown adjacent to the corresponding radiography lanes (R) from panel A. All represents the pooled histone lanes, H2A, H2B, H3, and H4 represent those lanes containing the respective histones.

**Fig. 6. Homodimerization of JBP1.** HA-JBP1 was immunoprecipitated from HeLa cells stably transfected with pEF2-HA-JBP1. Immunoprecipitates were washed and eluted proteins separated by SDS-PAGE. After proteins were transferred to PVDF membranes, the membranes were Western blotted with antibodies against HA or JBP1. After transfer, the remaining proteins were visualized by staining with Coomassie Blue.

**Fig. 7. Northern analysis of JBP1 in human tissues.** Blots containing oligo(dT)-selected mRNA from multiple human tissues (CLONTECH) were hybridized with labeled DNA corresponding to the sequence encoding JBP1. After hybridization and washing, autoradiography was performed which revealed the presence of a major transcript between 2.4 and 2.6 kb. Molecular weight standards are indicated on the left of each panel. The tissues are as follows: H, heart; Br, brain; Pl, placenta; Lu, lung; Li, liver; SM, skeletal muscle; K, kidney; Pa, pancreas; Sp, spleen; Th, thymus; Pr, prostate; Te, testicles; Os, ovaries; SI, small intestine; LI, large intestine; Le, peripheral blood leukocytes.
of chromosome 14. The bracketaphase spread shown in FISH with the JBP1 probe.

protein); however, its interaction with a protein speculated toported for this 72-kDa protein (IBP72 for 72-kDa pICln-binding ride channel (92). No activity or functional homology was re-
sensitive), a protein lacking a definitive function that was

Activity has been identified for JBP1, additional experiments
can be designed to help elucidate the role of this protein and its
homologues in cellular signaling, the cell cycle and the control of
cell morphology.

Note Added in Proof—Fujita et al. (Fujita, A., Tonouchi, A., Hiroko,
T., Inose, F., Nagashima, T., Satoh, R., and Tanaka, S. (1999) Proc.
Natl. Acad. Sci. U. S. A. 96, 8522–8527) recently reported that
the homologue of JBP1 in S. cerevisiae, Hs17p, interacts with the p21Cdc42Rac-activated kinase (PAK) Ste20 and appears to function as a
negative regulator of Ste20p in the filamentous growth-signaling path-
way. Although they reported no functional homologies or enzymatic
activities for Hs17p, their results in combination with our discovery
that JBP1 exhibits methyltransferase activity suggests that methyla-
tion may be involved in this regulation.

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J.-H. Lee, J. R. Cook, B. P. Pollack, and S. Pestka, unpublished results.

Fig. 8. Localization of the JBP1 gene. Localization of JBP1 to chromosome 14 by FISH. A, metaphase chromosomes prepared from peripheral blood lymphocytes were hybridized with a biotin-labeled cDNA probe (JBP1) and visualized with fluoroscinc isothiocyanate-avidin. Hybridization signals detected with JBP1 were specific to the proximal long arm of a group D [human chromosomes 13–15] chromosome (arrows). B, composite of chromosome 14 homologues shown in A after sequential G-banding. Sequential G-banding of the same metaphase spread shown in A localized JBP1 between bands q11.2–q21 of chromosome 14. The bracket to the right of the human chromosome 14 ideogram shows the location of the hybridization signals detected by FISH with the JBP1 probe.

A multikinases have been implicated in the repair of damaged proteins, and knockout of one such protein-repair enzyme in mice results in the accumulation of altered proteins, retardation of growth, and fatal seizures (80, 86). Despite the fact that protein methylation has been known for some time, and a role for protein methylation in cellular signaling has been the subject of a recent review, the exact role of this modification in many processes is still not well understood (85, 86, 90). However, some interesting observations have been recently reported. Arginine methylation was shown to facilitate the export of certain hnRNPs (heterogeneous nuclear ribonucleoproteins) from the nucleus (91). Another report demonstrated binding of a protein arginine methyltransferase to the IFNAR1 chain of the IFN-α/β receptor complex and experiments with antisense oligonucleotides provided evidence that this methyltransferase is involved in mediating the antiproliferative effect of IFN-α/β (75). Recently, a 72-kDa protein, identical to JBP1, was shown to bind to pICln (I = current, Cl = chloride, n = nucleotide-sensitive), a protein lacking a definitive function that was proposed to be a cytosolic regulator of a swelling-induced chloride channel (92). No activity or functional homology was reported for this 72-kDa protein (IBP72 for 72-kDa pICln-binding protein); however, its interaction with a protein speculated to be involved in signaling is intriguing (92). The sequence homol-
