Identification of Tubulin as a Substrate of Jak2 Tyrosine Kinase and Its Role in Jak2-Dependent Signaling†

Xianyue Ma and Peter P. Sayeski*

Department of Physiology and Functional Genomics, University of Florida College of Medicine, P.O. Box 100274, Gainesville, Florida 32610

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ABSTRACT: Jak2 is a nonreceptor tyrosine kinase that acts in numerous cellular signal transduction systems. Here, large amounts of recombinant Jak2 protein were expressed in eukaryotic cells, and an unknown 55 kDa protein was copurified with it. Mass spectrometry and Western blot analysis identified the 55 kDa protein as the α- and β-isoforms of tubulin. Biochemical experiments determined that Jak2 and tubulin specifically coassociate with one another, and the region of Jak2 that binds tubulin is the pseudokinase domain. Immunofluoresence indicated that Jak2 and tubulin (microtubules) colocalize within intact cells. The functional consequence of the coassociation between Jak2 and tubulin is that Jak2 phosphorylates tubulin on tyrosine residues. Specifically, in response to growth hormone, tubulin was phosphorylated in a Jak2-dependent manner. Tubulin was also found to interact with signal transducers and activators of transcription 1 (STAT1) and be involved in STAT1 nuclear transport. As such, this work suggests that tubulin is a substrate of Jak2 and facilitates Jak2/STAT1-dependent signaling.

Jak2[1] is a nonreceptor tyrosine kinase belonging to the *Janus* family of tyrosine kinases. It is activated by a variety of ligands resulting in signaling cascades that facilitate the activation of various downstream target genes. Jak2 mediates gene transcription through its well-characterized downstream signaling molecules, the signal transducers and activators of transcription (STAT) proteins. Specifically, the binding of a ligand to its cognate receptor at the cell surface activates Jak2 (*1*). An activated Jak2 in turn phosphorylates the STAT proteins on tyrosine residues. Activated STATs subsequently translocate into the nucleus where they mediate gene transcription. Thus, Jak2 is regarded as an important mediator of ligand-induced gene transcription.

Tubulin is a major component of cellular microtubules (*2*). It has been found to play a role in numerous cellular events including motility, intracellular transport, chromosome segregation, mitosis, cell shape, and organelle distribution within the cell (*3*, *4*).

Here, we demonstrate for the first time that Jak2 tyrosine kinase specifically associates with both soluble tubulin heterodimers and insoluble assembled microtubule polymers. The consequence of this association is that Jak2 phosphorylates tubulin on tyrosine residues. Furthermore, in response to growth hormone, tubulin interacts with STAT1 and facilitates the nuclear transport of STAT1. Collectively, our work suggests that tubulin is a substrate of Jak2 and is a component in Jak2/STAT1-dependent signaling.

METHODS

Cell Culture. BSC-40 cells were obtained from ATCC. Creation of the γ2A (*5*), γ2A+Jak2 (*6*), γ2A-GHR-Jak2 (*7*), RASM/control (*8*), and RASM/DN (*8*) cells has been previously reported. Dulbecco's modified Eagle's medium (DMEM) cell culture media and antibiotic supplements were purchased from Invitrogen. Heat inactivated fetal bovine serum was from Hyclone.

Antibodies and Reagents. The anti-HA antibody (clone F-7) used for immunopurification of the recombinant Jak2-HA protein was purchased from Santa Cruz Biotechnology. The anti-phosphotyrosine blotting monoclonal antibodies were a mixture of PY99, PY20, and 4G10 antibodies from Santa Cruz Biotechnology, BD Transduction Laboratories, and Upstate Biotechnology, respectively. The Western blotting anti-Jak2 antibody was from Upstate Biotechnology, Inc. The α- and β-tubulin antibodies used for immunoprecipitation and Western blotting were from Sigma Chemical. Pure tubulin was purchased from Cytoskeleton, Inc. All other antibodies and reagents were from Fisher Scientific or Sigma Chemical.

Cell Lysates and HA-Tagged Jak2 Protein Preparation. Recombinant Jak2-HA protein was expressed in cultured cells using the vaccinia virus-mediated expression system, and the Jak2-HA protein was isolated via anti-HA immunoaffinity purification as described (*9*).

In Cell Phosphorylation Assay. To promote tyrosine autophosphorylation of the Jak2-HA protein prior to cell lysis, each 100-mm dish of cells was incubated in 0.9 mL

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* To whom correspondence should be addressed. Phone: 352-392-1816. Fax: 352-846-0270. E-mail: psayeski@phys.med.ufl.edu. URL: http://www.med.ufl.edu/phys/sayeski/index.shtml.

[1] Abbreviations: Jak2, Janus kinase 2; STAT, signal transducers and activators of transcription; GH, growth hormone.

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of kinase buffer (50 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 100 mM NaCl, 0.5 mM DTT, 1 mM Na_3VO_4, 10 μg/mL aprotinin, and 2 mM ATP) for 1 h at 23 °C. The cells were then lysed using the Triton X-100 method of lysis as described (*9*).

Mass Spectrometry. Protein samples were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and visualized by Coomassie blue staining. Targeted protein bands were excised and subjected to trypsin digestion. Tryptic peptides were analyzed on a matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometer (Burnham Institute, La Jolla, CA). The mass spectrum was acquired in the reflector mode and the tryptic peptide ions obtained were scanned against the ProFound databases using the MS−FIT program of Protein Prospector.

In Vitro Pull Down of Tubulin with GST/Jak2 Fusion Proteins. The creation, expression, and purification of the four GST/Jak2 fusion proteins were described (*10*). Approximately 0.1 nmol of each GST/Jak2 fusion protein was incubated with 5 μg tubulin in 100 μL of binding buffer (50 mM HEPES, pH 7.6, 20% glycerol, 200 mM KCl, 25 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM IGEPAL CA-630, 2 mM DTT, and 2 mM AEBSF) and incubated for 2 h at 4 °C with constant shaking. Next, 30 μL of a 50% slurry of glutathione-Sepharose 4B beads (Amersham) was added to each sample and incubated for an additional 15 min. The beads were then washed three times and resuspended in 70 μL of SDS-containing sample buffer. The samples were boiled, separated by SDS−PAGE, transferred onto nitrocellulose membranes, and immunoblotted as described.

In Vitro Phosphorylation of Tubulin by Jak2. In vitro kinase reactions were performed by incubating 3 μg of pure tubulin with 100 ng of pure Jak2 protein in 27 μL of kinase reaction buffer, either in the presence or in the absence of 2.5 mM ATP. The samples were incubated for 30 min at room temperature, a time within the linear reaction range. Reactions were terminated by the addition of 9 μL of 4× SDS sample buffer. The samples were then boiled, separated by SDS−PAGE, and immunoblotted with anti-phosphotyrosine antibody.

Protein Alkylation. Tubulin alkylation was carried out via the ProteoPrep reduction and alkylation kit (Sigma Chemical) following the manufacturer's instructions. Briefly, ~5 μg of tubulin was placed in 60 μL of solution (pH > 7.5) and reduced via the addition of 1.5 μL of 0.2 M tributylphosphine (TBP). The solution was then alkylated by adding 1.8 μL of 0.5 M iodoacetamide (IAA) and incubated at room temperature for 1 h. Excess IAA was quenched by the addition of another 1.5 μL of 0.2 M TBP. Reactions were terminated by adding 22 μL of 4× SDS sample buffer, and then samples were applied to SDS−PAGE for analysis.

Selective Detergent Extraction of Cellular Proteins. Adherent cells were washed with PBS and detached from the dishes using harvest buffer (40 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 150 mM NaCl) with pipeting. The cells were then collected by centrifugation (1000*g*, 10 min) and washed once with harvest buffer. The cells from one 100-mm dish were resuspended in 0.6 mL of buffer A (10 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 10 mM NaCl, 10 mM NaF, 1 mM DTT and protease inhibitors) supplemented with 0.5% Triton X-100. After a 5 min extraction at room temperature, the supernatant (soluble proteins) was collected by centrifugation at 700*g* for 5 min. The insoluble matrix was washed once with buffer A and then placed in 0.3 mL of buffer A containing 1% SDS. The samples were incubated for 60 min at 37 °C, and then 0.3 mL of RIPA buffer was added to each sample. The samples were briefly sonicated, and the supernatant (insoluble proteins) was collected by centrifugation at 10000*g* for 10 min.

Fluorescent Microscopy. Cells were seeded in 4-well Lab-Tek II Chamber Slides. The cells were subjected to either viral infection or transient cDNA transfection. For the infection, 90% confluent cells were infected for 1 h with 0.5 MOI of vJak2-WT-HA and 1.0 MOI of vTF7-3 recombinant viruses in serum-free media (*9*). For transient transfection, 60% confluent cells in each well (1.7 cm^2) were incubated for 5 h with 0.3 mL of DMEM containing 1 μg of a plasmid DNA encoding wild type murine Jak2 (*11*) or STAT1-GFP (*12*) and 2 μL of Lipofectin (Life Technologies Inc). After infection/transfection, the medium was aspirated, it was replaced with serum-containing DMEM, and the samples were incubated for ~36 h. The cells were washed with PBS and fixed for 30 min at room temperature with 4% paraformaldehyde (wt/vol) in 0.1 M Na^+ phosphate buffer (pH 7.5). The cells were then permeabilized for 10 min with 0.2% Triton X-100 in PBS. After the samples were blocked for 2 h with 5 mg/mL BSA in PBS, they were incubated with a primary antibody of anti-α-tubulin mAb (1:500 dilution) or a mixture of α-tubulin mAb and anti-HA pAb (1:125 dilution), or a primary antibody mixture of anti-α-tubulin mAb (1:500 dilution) and anti-Jak2 pAb (1:125 dilution), overnight at 4 °C in PBS containing 5 mg/mL BSA. The mouse and rabbit primary antibodies were revealed with a goat anti-mouse IgG antibody conjugated to Texas Red and a goat anti-rabbit IgG-FITC conjugated antibody, respectively. Fluorescent microscopy was performed with a Zeiss Axioplan II microscope using a 40× lens.

RESULTS

A 55 kDa Protein Specifically Interacts with Jak2 and Is Identified as Tubulin. Recently, we succeeded in expressing large amounts of recombinant, hemeagglutinin (HA)-tagged, Jak2 protein in cultured cells using a vaccinia virus-mediated expression system (*9*). Taking advantage of the HA epitope tagging, immunoaffinity purification was applied to isolate the Jak2 protein. We found that when the cells were lysed using a detergent-free method of extraction, an unknown protein of 55 kDa in mass, copurified with Jak2 (Figure 1 A). Lane 1 shows the input lysate, while lane 2 shows the purified products. The 55 kDa protein was not detected by antibodies directed against either Jak2 or the HA epitope, suggesting that the 55 kDa protein was not a Jak2 degradation product nor a protein containing the HA epitope (data not shown). To demonstrate that the 55 kDa protein specifically interacted with Jak2-HA, a control lysate was immunoaffinity purified in parallel with the Jak2-expressing lysate. The control sample was prepared from the cells that were infected with a vaccinia virus devoid of the Jak2 insert (lane 3). The mock infected lysate failed to yield both Jak2-HA and 55 kDa proteins, indicating that the isolation of the 55 kDa protein was not due to nonspecific binding to the anti-HA column. Additionally, the binding interaction between Jak2

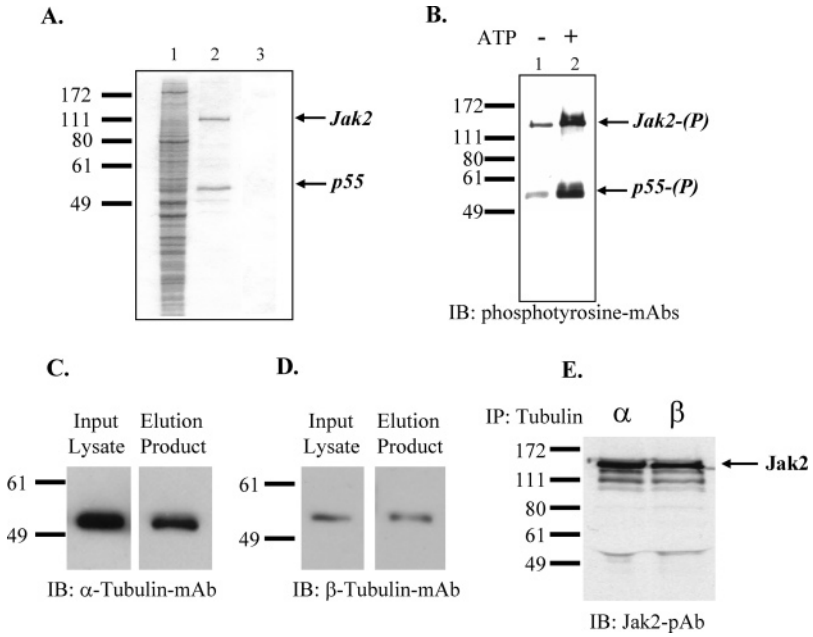

FIGURE 1: A 55 kDa protein copurifies with Jak2 and is identified as tubulin. (A) BSC-40 cells were infected with either the Jak2-HA expressing vaccinia virus (lanes 1 and 2) or a vaccinia virus devoid of the Jak2-HA insert (lane 3). The lysates were collected via the Dounce method and loaded to the anti-HA immunoaffinity column. After washing, the bound proteins were eluted with an excess of HA peptide. The original input lysate (lane 1) and the immunopurified eluates (lanes 2 and 3) were applied to SDS−PAGE and visualized by Coomassie blue staining. (B) The elution product containing Jak2 and the 55 kDa proteins were incubated with kinase buffer either lacking (−) or containing (+) 2.5 mM ATP. The proteins were separated by SDS−PAGE and Western blotted with anti-phosphotyrosine antibody. (C) and (D) Input lysates or the immunoaffinity purified Jak2-HA/55 kDa elution products were Western blotted using either anti-α-tubulin or anti-*β*-tubulin antibodies. (E) Whole cell protein lysates were immunoprecipitated with either anti-α-tubulin or anti-*β*-tubulin monoclonal antibodies and then Western blotted with anti-Jak2 antibody to measure tubulin/Jak2 coassociation. Shown is one of three independent experiments.

and the 55 kDa protein appeared to be quite strong as it persisted after extensive washing with 1.2 M NaCl (data not shown).

An in vitro kinase phosphorylation assay was applied to the elution product either in the presence or absence of ATP (Figure 1B). Addition of ATP resulted in a marked increase in the tyrosine phosphorylation levels of both Jak2-HA and the 55 kDa protein, suggesting that the 55 kDa protein either had intrinsic tyrosine kinase activity itself or was being tyrosine phosphorylated by Jak2.

To determine the identity of the 55 kDa protein, the band was excised from the gel, digested with trypsin, and analyzed via MALDI-TOF mass spectrometry (Supporting Information). Analysis of the fragment masses identified the 55 kDa protein as tubulin, including both the α- and *β*-isoforms. To confirm the identity of the 55 kDa protein as tubulin via a method other than mass spectrometry, the immunoaffinity purified protein product was Western blotted with either anti-α-tubulin (Figure 1C) or anti-*β*-tubulin (Figure 1D) antibodies. The results demonstrated that the 55 kDa protein was immunoreactive against both antibodies, when compared to the input protein lysates.

The results in Figure 1A−D indicate that Jak2 and tubulin copurify together. However, to determine whether they coassociate with one another, protein lysates were immunoprecipitated with either anti-α- or anti-*β*-tubulin antibodies and Western blotted with anti-Jak2 antibody (Figure 1E). We found that Jak2 coimmunoprecipitated equally with both the α- and the *β*-isoforms of tubulin. Furthermore, elimination of the immunoprecipitating antibodies from the protocol resulted in a specific loss of the Jak2 signal (data not shown). These results indicated that Jak2 and tubulin physically coassociate.

The Jak2 Pseudokinase Domain Mediates the Association of Jak2 with Tubulin. Jak2 contains four major structural regions: FERM, SH2-like, pseudokinase, and tyrosine kinase domains. A series of GST fusion proteins encoding each of these four regions were used in GST pull down assays so that the region of Jak2 that binds tubulin could be ascertained (Figure 2 A). Each GST/Jak2 pull down reaction was initiated by incubating purified soluble tubulin heterodimers with the different GST/Jak2 fusion proteins. The samples were subsequently precipitated via the addition of glutathione-Sepharose 4B beads. The pull downs were then Western blotted with anti-α-tubulin monoclonal antibody to detect bound tubulin (Figure 2B, top panel). We found that tubulin only bound the GST/Jak2 fusion protein encoding the pseudokinase domain. The membrane was subsequently stripped and reprobed with anti-GST antibody to show equal capture of the fusion proteins (Figure 2B, bottom panel).

In summary, the data indicate that the pseudokinase domain is the binding determinant of Jak2 for tubulin.

Jak2 Interacts with Assembled Microtubules and colocalizes with Tubulin in Cultured Cells. Tubulin exists in cells either as soluble heterodimers or as insoluble assembled microtubule polymers. In the above experiments, the conditions were set to test the interaction between Jak2 and soluble tubulin dimers. Here, the interaction between Jak2 and assembled microtubule polymers was investigated via biochemical fractionation and subsequent analysis of the distribution of tubulin and Jak2 within the fractions. As described in the methods, protein lysates were prepared and

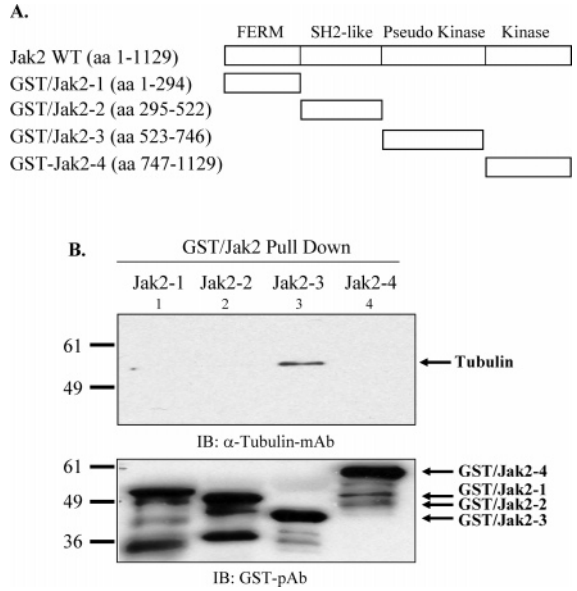

FIGURE 2: The pseudokinase domain of Jak2 binds tubulin in vitro. (A) Cartoon representing the four GST/Jak2 fusion proteins used for pull down analysis and the corresponding Jak2 domains that each encodes. (B) Pure tubulin was incubated with the indicated GST/Jak2 fusion protein and tubulin binding was measured by Western blotting the GST/Jak2 pull down products with anti-α-tubulin antibody (top). The same membrane was also probed with anti-GST antibody to demonstrate similar loading across all lanes (bottom). Shown is one of two independent experiments.

then separated into the soluble fraction that contained tubulin heterodimers (Sol) or the insoluble fraction that contained tubulin microtubules (Ins). The proteins in the insoluble fraction were subsequently dissolved via the addition of SDS. A portion of the protein preparations from both fractions was then analyzed via either direct Western blot analysis or by anti-tubulin immunoprecipitation followed by Western blot analysis. In the control cells that were not infected with the Jak2-HA vaccinia construct, endogenous Jak2 and tubulin were found in both the soluble and the insoluble fractions, although Jak2 was more abundant in the Sol fraction (Figure 3A, left panels). For cells infected with the Jak2-HA construct, Jak2 and tubulin were similarly found in both cellular fractions (Figure 3A, center panels). Furthermore, Jak2 coimmunoprecipitated with tubulin in both the soluble and the insoluble fractions as determined by the Jak2 that was detected in the anti-tubulin immunoprecipitates (Figure 3A, right panels). Collectively, the data indicate that Jak2 can associate with both soluble tubulin heterodimers and insoluble microtubule polymers.

We next used indirect immunofluorescence to investigate the distribution of Jak2 and tubulin within intact cells. BSC-40 cells were infected with the Jak2-HA expressing viral construct and then fixed with paraformaldehyde. The cellular distribution of tubulin and Jak2-HA was then determined via anti-tubulin and anti-HA immunostaining, followed by fluorescent microscopy. As seen in Figure 3B, tubulin staining was observed in organized filamentous patterns that resembled the microtubule network. HA staining was predominantly cytoplasmic in its distribution with a strong emphasis around the nucleus. Merging of the separate pictures found that the anti-HA and anti-α-tubulin antibodies showed strong colocalization between Jak2 and tubulin.

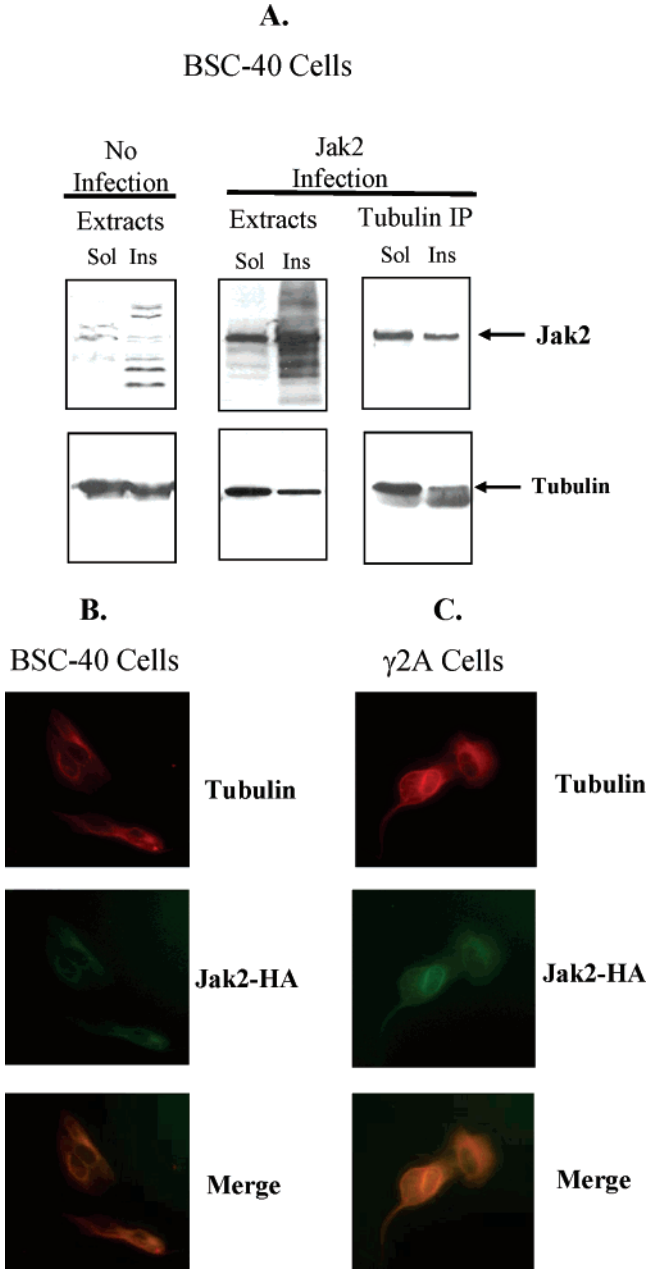

FIGURE 3: Jak2 associates with both soluble tubulin dimers and insoluble microtubule polymers. (A) BSC-40 cells that were either left uninfected (left panels) or infected with the Jak2-HA expressing vaccinia virus (center and right panels) were collected and then subjected to selective detergent extraction (see Methods). Equivalent volumes of the soluble (Sol) and insoluble (Ins) protein fractions were either directly subjected to SDS−PAGE (left and center panels) or were first immunoprecipitated with anti-α-tubulin mAb and then subjected to SDS−PAGE (right panels). Western blot analyses were conducted with anti-Jak2 antibody (upper panels) or anti-α-tubulin antibody (lower panels). BSC-40 (B) or γ2A (C) cells were infected with the Jak2-HA expressing recombinant vaccinia virus. After the cells were fixed and permeabilized, they were double-stained for α-tubulin (red) and HA (green). Analysis was performed via fluorescent microscopy, and individual images were merged digitally. Shown is one of three representative results.

To determine whether this Jak2 and tubulin colocalization pattern extended beyond that observed in BSC-40 cells, a human fibrosarcoma-derived cell line termed, γ2A, was infected with the Jak2 expressing vaccinia virus, and the same fluorescent microscopy procedure was repeated (Figure 3C). We observed a staining patttern nearly identical to that

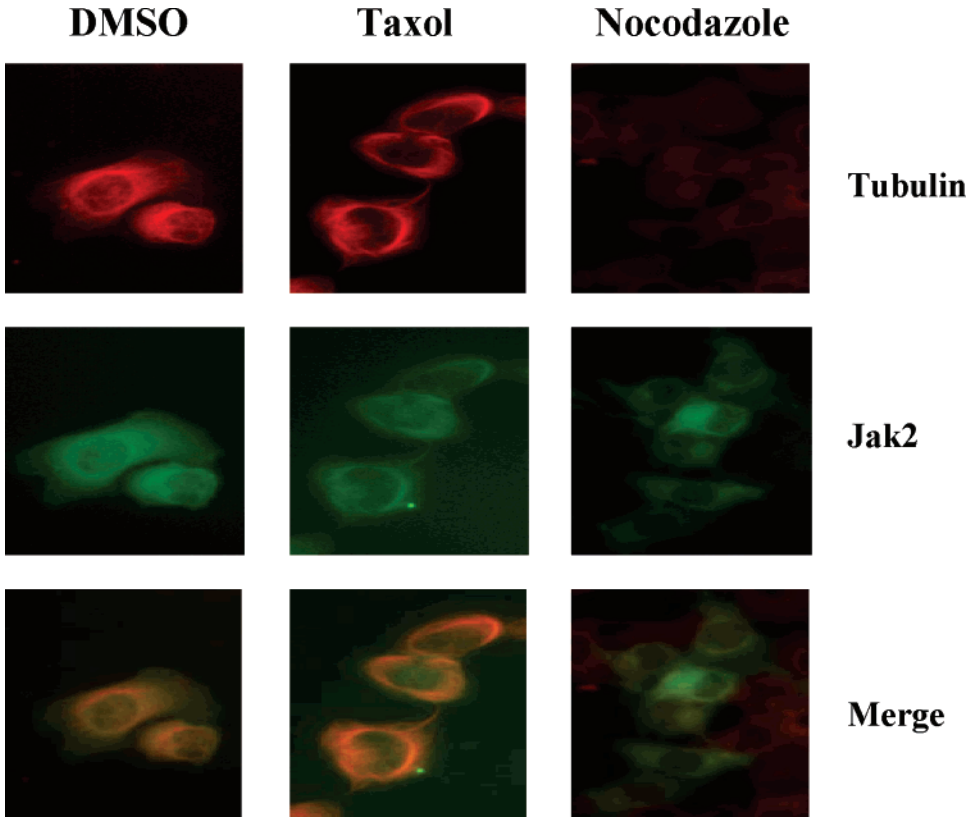

FIGURE 4: The effects of tubulin agents on the subcellular localization of Jak2 and tubulin. γ2A cells were transfected with an untagged wild type Jak2 cDNA. The cells were treated with vehicle control (DMSO) or treated with either taxol or nocodazole (10 μM for 1.5 h for both). After the cells were fixed and permeabilized, they were double-stained for α-tubulin (red) and Jak2 (green). Analysis was performed via fluorescent microscopy and individual images were merged digitally. Shown is one of three representative results.

seen in the BSC-40 cells. Collectively, the data in Figure 3 indicate that Jak2 interacts with assembled microtubules in cultured cells.

Disruption of Microtubule Polymers Attenuates Jak2/Tubulin Colocalization. We next hypothesized that if we altered microtubule polymer formation, we would similarly alter Jak2/tubulin cellular colocalization. For this set of experiments, we transiently transfected γ2A-derived cells with an untagged, wild type Jak2 cDNA expression plasmid. This level of Jak2 expression would allow us to rule out the possibility that the results observed in Figure 3 were due to an artifact induced by the vaccinia infection or by the anti-HA antibody itself. Two days after transfection, the cells were treated with either dimethyl sulfoxide (DMSO), the microtubule stabilizing agent taxol, or the microtubule destabilizing agent, nocodazole. Cells treated with DMSO displayed a Jak2 and tubulin distribution pattern similar to that seen in Figure 3; both Jak2 and tubulin colocalized with particular emphasis around the nucleus (Figure 4, left panels). Treatment of the cells with the microtubule stabilizing agent taxol led to an enhanced staining pattern of the microtubule polymers and to concomitant changes in the distribution of Jak2 and an even stronger colocalization between Jak2 and tubulin (Figure 4, center panels). Conversely, destabilization of the microtubule polymers with nocodazole disrupted both Jak2 and tubulin filamentous staining patterns (Figure 4, right panels).

Together, the data demonstrate that Jak2 colocalizes to microtubule polymers, and disruption of these polymers reduces Jak2/tubulin cellular colocalization. Additionally, the data suggest that this colocalization is not due to a vaccinia artifact nor to nonspecific anti-HA immunostaining.

Both α- and β-Tubulin Are Tyrosine Phosphorylated by Jak2. Given the strong colocalization of Jak2 and tubulin, we hypothesized that Jak2 was phosphorylating tubulin. To test this, we measured the ability of Jak2 to phosphorylate tubulin by incubating various combinations of Jak2, tubulin, and ATP in vitro. Jak2 and tubulin tyrosine phosphorylation levels were then determined by immunoblotting the samples with anti-phosphotyrosine antibodies (Figure 5A). The result suggests that tubulin is specifically phosphorylated by Jak2 since omission of either Jak2 or ATP from the kinase reaction resulted in a complete loss of tubulin tyrosine phosphorylation.

To demonstrate this another way, we expressed and isolated either a kinase active or a kinase inactive form of Jak2 from cultured cells as described (*9*). The two forms of Jak2 were then incubated with recombinant tubulin in the presence of ATP. Tyrosine phosphorylation levels were again measured by subjecting the samples to Western blot analysis with anti-phosphotyrosine antibodies (Figure 5B). The results indicate that only the kinase active form of Jak2 was able to tyrosine phosphorylate tubulin.

Tubulin exists as a heterodimer of its α- and β-isoforms. These two isoforms have the same molecular weight but are differentially susceptible to carboxymethylation, thus allowing for an electrophoretic separation of the two isoforms (*13*). Here, we sought to determine which isoform(s) of tubulin Jak2 phosphorylates. Recombinant tubulin was either left untreated or alkylated via iodoacetamide treatment. The samples were then separated via SDS−PAGE and Coomassie stained to differentiate the two isoforms. The results dem-

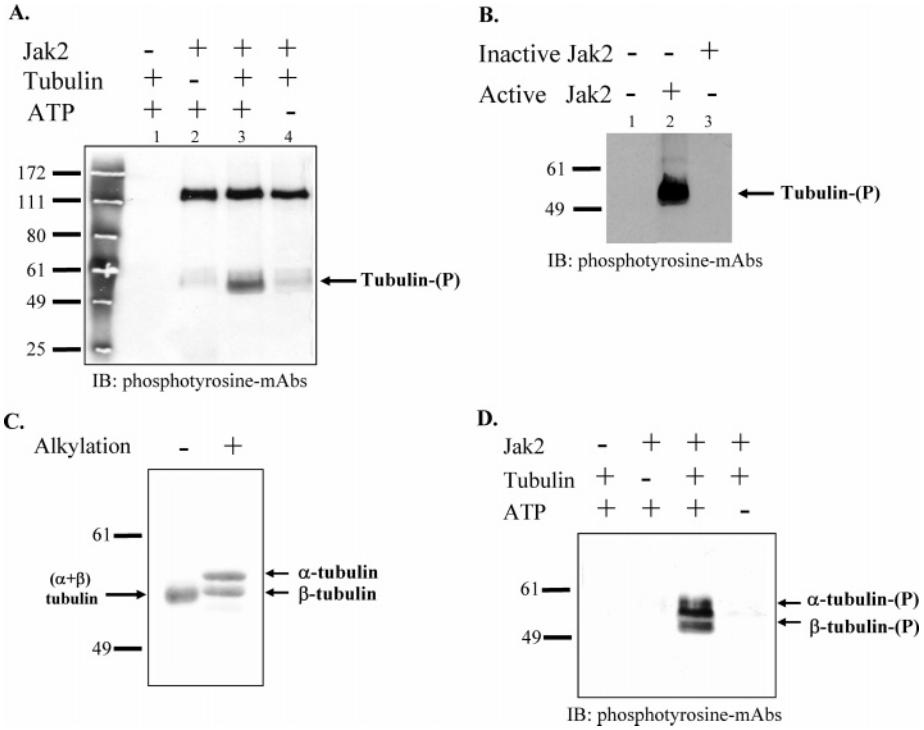

FIGURE 5: Jak2 phosphorylates tubulin in vitro. (A) Pure tubulin alone, pure Jak2 alone, or tubulin plus Jak2 were incubated in kinase reaction buffer either with or without ATP. The samples were subsequently separated by SDS−PAGE, and protein tyrosine phosphorylation levels of tubulin and Jak2 were determined by Western blotting the samples with anti-phosphotyrosine antibodies. (B) Pure tubulin was incubated with either kinase active or kinase inactive Jak2 in the presence of ATP and then Western blotted with anti-phosphotyrosine antibodies to measure the relative tyrosine phosphorylation levels of tubulin. (C) Pure tubulin was either left untreated (−) or alkylated (+) and then resolved on SDS−PAGE. The samples were Coomassie stained to distinguish between the α- and *β*-isoforms of tubulin. (D) Pure tubulin was incubated with Jak2 and ATP as indicated. The samples were further treated with the alkylation reaction and then applied to SDS−PAGE. The relative tyrosine phosphorylation levels of α- and *β*-tubulin were then determined by Western blotting the samples with anti-phosphotyrosine antibodies. Shown is one of three independent experiments.

onstrated that iodoacetamide treatment produced two distinct protein bands (Figure 5C). Western blot analysis confirmed that the higher and lower bands were α- and *β*-tubulin, respectively (data not shown).

We next repeated the kinase assay shown in Figure 5A by allowing Jak2 to tyrosine phosphorylate tubulin in vitro. Upon completion of the phosphorylation reaction, the samples were alkylated, separated by SDS−PAGE, and Western blotted with anti-phosphotyrosine monoclonal antibodies (Figure 5D). The results indicated that both the α- and the *β*-isoforms of tubulin were tyrosine phosphorylated by Jak2.

Collectively, the data demonstrate that Jak2 tyrosine kinase can readily tyrosine phosphorylate both the α- and the *β*-isoforms of tubulin in vitro.

Jak2 Promotes Tyrosine Phosphorylation of Both Soluble Tubulin and Microtubule Polymers. Previous reports have shown that several different tyrosine kinases can bind to and phosphorylate tubulin on tyrosine residues (*14*−*16*). An important question therefore is what role does Jak2 have tubulin phosphorylation in the context of a complex cellular milieu containing numerous tyrosine kinases? To answer this, we prepared lysates from cells that expressed varying levels of Jak2. *γ*2A cells are human fibrosarcoma-derived cells that completely lack Jak2 expression (*5*). The *γ*2A+Jak2 cells express Jak2 at roughly physiological levels (*6*). Finally, the *γ*2A+vJak2 cells express Jak2 at supraphysiological levels via vaccinia virus-mediated overexpression (*9*). Thus, the only difference between each cell line is the relative amount of expressed Jak2 protein as the *γ*2A background is the same for all three conditions.

Lysates were prepared from each of the three cellular conditions and incubated either in the presence or the absence of ATP. After completion of the kinase reaction, the lysates were immunoprecipitated with anti-α-tubulin monoclonal antibody, separated by SDS−PAGE, and then Western blotted with anti-phosphotyrosine antibodies to measure the relative tyrosine phosphorylation levels of the proteins in the immunoprecipitates (Figure 6A, top panel). Two predominant phosphorylated protein bands of 130 and 55 kDa were observed, and this phosphorylation was ATP-dependent. We found that addition of ATP resulted in increased tubulin tyrosine phosphorylation levels in all three cell lines. These data indicated that Jak2, per se, is not essential for tubulin tyrosine phosphorylation as the *γ*2A cells are Jak2 null. As the relative amount of expressed Jak2 was moderately increased in the *γ*2A+Jak2 cells, however, there was a corresponding increase in tubulin tyrosine phosphorylation levels (lanes 4). Finally, there was a marked increase in the tyrosine phosphorylation levels of tubulin in the *γ*2A+vJak2 cells expressing Jak2 at supraphysiological levels (lanes 6).

The membrane was subsequently stripped and reprobed with either anti-Jak2 (Figure 6A, middle) or anti-α-tubulin (Figure 6A, bottom) antibody to determine the relative levels of these two proteins. Interestingly, while lane 6 showed the greatest levels of tubulin tyrosine phosphorylation, it also showed the lowest levels of total tubulin protein. As such,

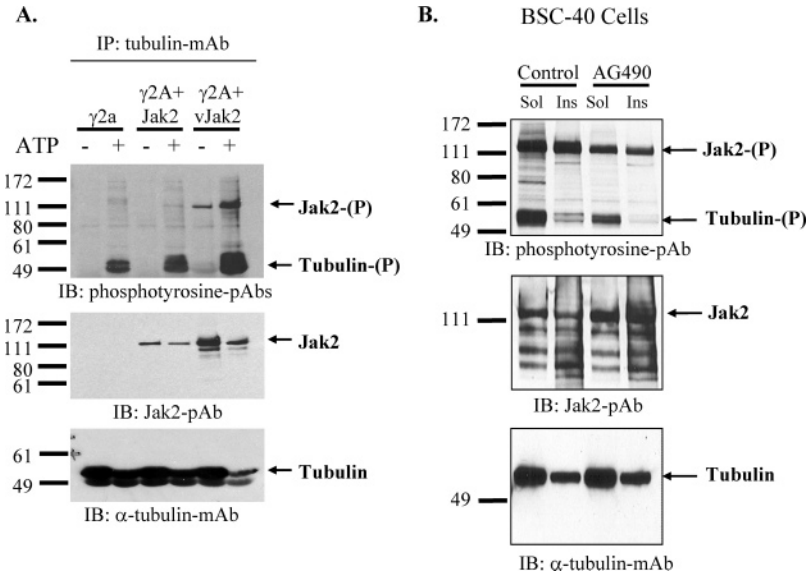

FIGURE 6: Tubulin tyrosine phosphorylation levels increase in a Jak2-dependent manner. (A) Whole cell protein lysates expressing increasing amounts of Jak2 were prepared from the indicated cell lines and ATP was added as indicated. Following termination of the kinase reactions, the samples were immunoprecipitated with anti-α-tubulin antibody. The immunoprecipitates were then separated by SDS−PAGE and sequentially immunoblotted (IB) with anti-phosphotyrosine, anti-Jak2, and anti-α-tubulin antibodies. (B) BSC-40 cells infected with the Jak2-HA expressing vaccinia virus were treated with either DMSO or with 100 μM AG490 for 16 h. The cells were then subjected to selective detergent extraction. Equivalent volumes of soluble (Sol) and insoluble (Ins) protein extracts were resolved by SDS−PAGE and Western blotted as indicated. Shown is one of three independent experiments.

the relative ability of Jak2 to tyrosine phosphorylate tubulin is much greater when the data are expressed as a ratio of tyrosine phosphorylated tubulin to total tubulin.

We next examined whether Jak2 phosphorylates soluble tubulin dimers and/or insoluble microtubule polymers. For this, BSC-40 cells overexpressing HA-tagged Jak2 were pretreated with either DMSO or with the Jak2 pharmacological inhibitor, AG490. Soluble and insoluble cellular fractions were generated similar to that shown in Figure 3. The samples were then separated by SDS−PAGE and Western blotted with anti-phosphotyrosine antibodies. Two predominant protein bands representing phosphorylated Jak2 and phosphorylated tubulin were detected (Figure 6B, top panel). AG490 treatment decreased the tyrosine phosphorylation levels of both Jak2 and tubulin, thereby suggesting that tubulin phosphorylation was dependent on Jak2 kinase activity within these cells. The data also revealed that both soluble tubulin and insoluble microtubule polymers were phosphorylated in these cells, and this too was Jak2-dependent. Finally, we determined the levels of Jak2 and tubulin by Western blotting the same membrane with either anti-Jak2 (Figure 6B, middle panel) or anti-α-tubulin antibodies (Figure 6B, bottom panel).

Taken together, the data in Figure 6 demonstrate that while Jak2 tyrosine kinase is not required for tubulin tyrosine phosphorylation, it is nonetheless sufficient. Furthermore, there is a direct correlation between the catalytic state of Jak2 and the tyrosine phosphorylation levels of tubulin, thereby indicating that tubulin is being phosphorylated by Jak2 in these cells.

Ligand-Dependent Activation of Jak2 Results in Increased Tubulin Tyrosine Phosphorylation and Tubulin/STAT1 Coassociation. To further establish a role for Jak2 in the phosphorylation of tubulin within intact cells under physiological conditions, we tested whether Jak2 could phosphorylate tubulin in response to a known, Jak2-activating, ligand stimulus.

γ2A-GHR-Jak2 cells stably express both the growth hormone receptor (GHR) and Jak2. Previous work has shown that treatment of these cells with growth hormone (GH) results in the rapid and marked activation of Jak2 (*7*). Here, we used these cells to determine if Jak2 could phosphorylate tubulin under conditions in which Jak2 was activated in a ligand-dependent manner. The cells were treated with GH for times ranging from 0 to 40 min. Cellular lysates were immunoprecipitated with an anti-tubulin antibody and immunoblotted with an anti-phosphotyrosine antibody to detect tyrosine phosphorylated tubulin. The results demonstrate that GH induced tubulin tyrosine phosphorylation in a time-dependent manner (Figure 7A, top panel). The tubulin band was just above the IgG heavy chain band. Equal loading of tubulin and its relative position was confirmed by reprobing the membrane with anti-tubulin antibody (Figure 7A, bottom panel). Although GH treatment of these cells is known to largely activate Jak2 tyrosine kinase, it is possible that other kinases might be mediating the GH-dependent tyrosine phosphorylation of tubulin. To clarify the role of Jak2 in this process, the experiment was repeated, but this time we analyzed tubulin phosphorylation from Jak2 immunoprecipitates. After GH treatment, cellular lysates were prepared and then immunoprecipitated with an anti-Jak2 antibody. This would allow for tubulin to coprecipitate with Jak2. The immunoprecipitates were then immunoblotted with anti-phosphotyrosine antibodies to measure both Jak2 and tubulin tyrosine phosphorylation levels (Figure 7B). Two major phosphorylated proteins were observed. After ligand addition, we observed an increase in the tyrosine phosphorylation levels of both Jak2 and tubulin that were present in the Jak2 immunoprecipitates. We subsequently confirmed that the upper and lower bands were Jak2 and tubulin, respectively,

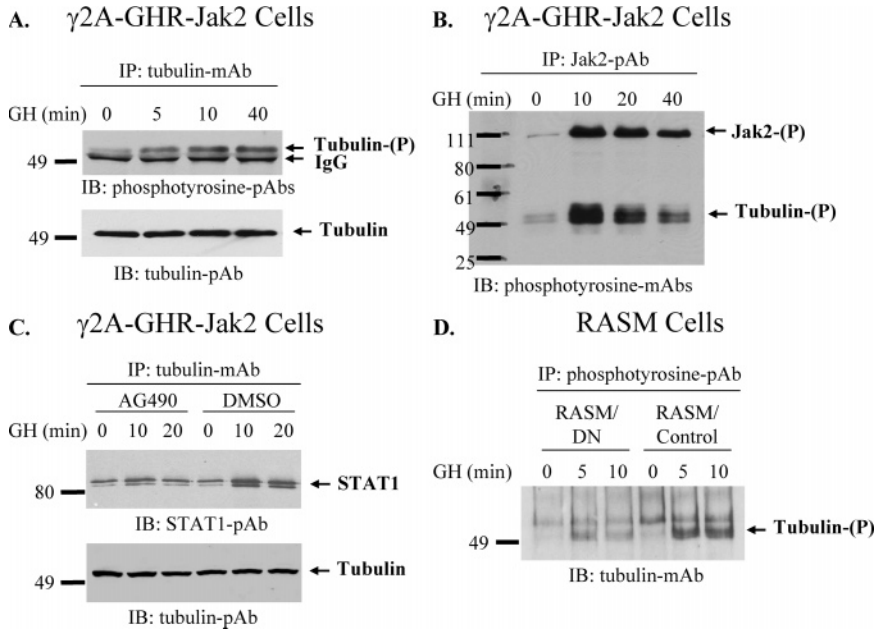

FIGURE 7: Tubulin is phosphorylated in cells in both a ligand- and Jak2-dependent manner. γ2A-GHR-Jak2 cells were stimulated with 250 ng/mL growth hormone for the indicated times. Lysates were prepared and immunoprecipitated with either anti-α-tubulin antibody (A) or anti-Jak2 antibody (B) and Western blotted with the indicated antibodies to measure GH-dependent tubulin phosphorylation. (C) γ2A-GHR-Jak2 cells were treated with either 100 μM AG490 or vehicle control (DMSO) for 16 h and then treated with 250 ng/mL GH for the indicated times. The protein lysates were immunoprecipitated with anti-α-tubulin antibody and Western blotted with anti-STAT1 antibody to measure Jak2-dependent tubulin/STAT1 coassociation. Tubulin protein levels were verified by blotting the same membrane with anti-tubulin antibody. (D) RASM cells expressing a Jak2 dominant negative allele (RASM/DN) or an empty vector control plasmid (RASM/control) were treated with 500 ng/mL GH. Tubulin tyrosine phosphorylation levels were measured by immunoprecipitating the lysates with anti-phosphotyrosine antibody and Western blotting with anti-α-tubulin antibody. Shown is one of three (A, B) or two (C, D) independent experiments.

as they were detected with their respective antibodies (data not shown).

Our data thus far demonstrate that Jak2 and tubulin physically coassociate with one another and Jak2 in turn phosphorylates tubulin. Since Jaks and STATs are known to also bind with one another (*1*), we hypothesized that tubulin and STAT1 similarly coassociate, and this event is Jak2-dependent. To test this, γ2A-GHR-Jak2 cells were pretreated with either 100 μM AG490 or vehicle control (DMSO) for 16 h and then treated with 250 ng/mL GH for the indicated times. Protein lysates were immunoprecipitated with anti-α-tubulin antibody and Western blotted with anti-STAT1 antibody to measure Jak2-dependent tubulin/STAT1 coassociation (Figure 7C, top panel). We found that inhibition of Jak2 via AG490 reduced the levels of GH-mediated tubulin/STAT1 coassociation when compared to DMSO controls, thus suggesting that the Jak2-dependent phosphorylation of tubulin facilitates tubulin/STAT1 protein association. Equal loading of tubulin was confirmed by reprobing the membrane with anti-tubulin antibody (Figure 7C, bottom panel).

While AG490 is a potent inhibitor of Jak2, it is known to inhibit other tyrosine kinases such as c-Src (*17*). This therefore raises the question of whether the inhibitory effect of AG490 is solely due to the absence of Jak2 kinase function. To circumvent this problem, we utilized rat aortic smooth muscle (RASM) cells that stably express either a Neo cassette alone (RASM/control) or the Neo cassette along with a dominant negative form of Jak2 (RASM/DN). The creation and characterization of these cells has been described (*8*). The net affect of the Jak2 dominant negative mutation is that endogenous Jak2-dependent signaling is greatly, yet specifically, reduced. For instance, we have shown that hydrogen peroxide-induced Jak2 activation in the RASM/DN cells is reduced by ~90% when compared to the RASM/control cells, while Jak2-independent signaling between the two cell types is virtually identical (*8, 18*). Here, the two cell types were treated with GH for 0, 5, and 10 min to activate Jak2. Cellular lysates were immunoprecipitated with an anti-phosphotyrosine antibody and immunoblotted with anti-tubulin antibody to detect the levels of tyrosine phosphorylated tubulin protein (Figure 7D). We found that the ability of GH to induce tubulin phosphorylation was greatly reduced in the RASM/DN cells when compared to the RASM/control cells, suggesting that Jak2 phosphorylates tubulin.

Collectively, the data in Figure 7 demonstrate that activation of Jak2 by a ligand results in increased tubulin tyrosine phosphorylation levels and suggest that tubulin is a substrate of Jak2 in cells that express Jak2. Additionally, the data demonstrate that tubulin and STAT1 associate in a Jak2 phosphorylation dependent manner.

STAT1 Colocalizes with Tubulin and Microtubule Depolymerization Impairs GH-Induced STAT1 Nuclear Transport. The phosphorylation of tubulin by Jak2 implies that tubulin may be involved in Jak2-dependent signal transduction. We hypothesized that tubulin participates in Jak/STAT signaling by mediating STAT nuclear transport. To test this hypothesis, we transfected a STAT1-GFP cDNA construct into the γ2A-GHR-Jak2 cells and monitored STAT1 nuclear translocation in response to GH. In the absence of GH, STAT1-GFP (green) colocalized with the cytoplasmic microtubule network (red), as shown by the merged overlay of the two independent images (Figure 8 A, top panels). Not surprisingly, in response

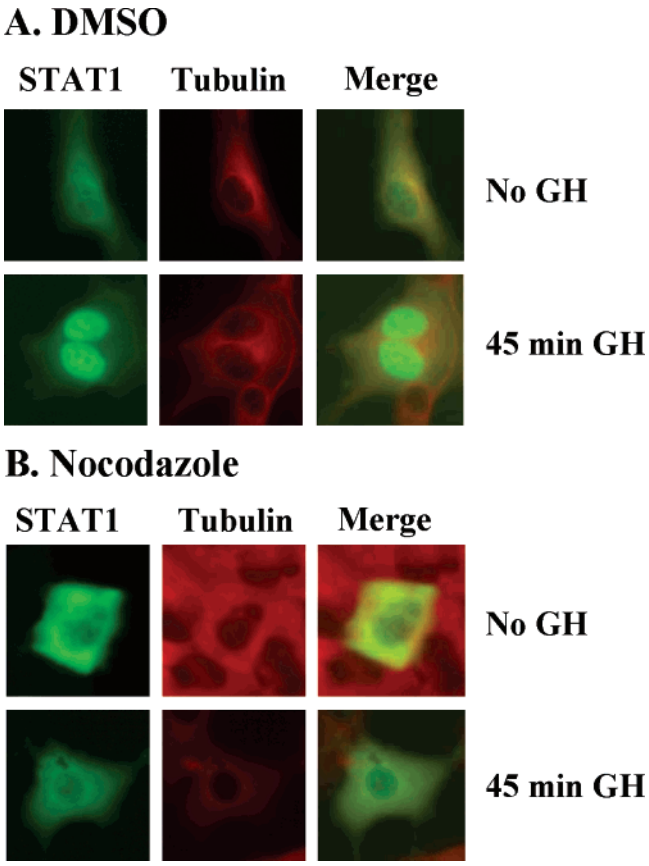

FIGURE 8: STAT1 colocalizes with tubulin and disruption of the microtubule network impairs GH-induced STAT1 nuclear transport. (A) γ2A-GHR-Jak2 cells were transfected with a GFP tagged STAT1 cDNA expression plasmid and then allowed to recover. The cells were subsequently treated with 250 ng/mL growth hormone for the indicated times, fixed, and permeabilized. The cells were immunostained for α-tubulin, and analysis was performed via fluorescent microscopy. The cellular distribution of GFP-STAT1 (green) and α-tubulin (red) were merged digitally. (B) Same as above, but 1.5 h prior to GH treatment, the cells were pretreated with 10 μM nocodazole. Shown is one of three representative results.

to GH, STAT1-GFP had a marked nuclear localization, while tubulin staining was still largely cytosolic (Figure 8A, bottom panels). However, when the microtubule network was disrupted via nocodazole treatment, the ability of STAT1-GFP to translocate into the nucleus in response to GH was dramatically impaired (Figure 8B). These results indicate that the GH-dependent transport of STAT1 from the cytoplasm to the nucleus requires an intact microtubule network.

DISCUSSION

Jak2 has traditionally been viewed as a kinase acting within the classical Jak/STAT signaling paradigm. Here, we demonstrate that Jak2 specifically associates with an important cytoskeletal protein, namely, tubulin. The consequence of this association is that Jak2 phosphorylates tubulin on tyrosine residues. Additionally, we show that tubulin itself is an important component of the Jak/STAT signaling system as disruption of microtubule polymers blocks GH-mediated, STAT1 nuclear translocation. As such, this work suggests that the interdependence between Jak2 and tubulin are important for normal Jak/STAT signal transduction processes to occur.

Our work indicates that the JH2 domain of Jak2 mediates the coassociation between Jak2 and tubulin. This important finding not only specifies the interaction between Jak2 and tubulin but also provides evidence for a better understanding of the function of the JH2 domain. Recent reports have shown that a V617F gain-of-function point mutation in the JH2 domain of Jak2 is the cause of ~90% of all polycythemia vera cases and ~35% of all essential thrombocythemia cases (*19*, *20*). The JH2 domain of Jak2 is the pseudokinase domain. While it shares conserved motifs with other protein kinases, this region is catalytically inactive. Previous reports suggest that the JH2 domain acts as a negative regulator of overall Jak2 kinase function as *Janus* kinases lacking this domain have increased catalytic activity (*21*−*23*). Here, however, our report is the first to indicate that the JH2 domain is capable of mediating inter- as well as intramolecular interactions. Two pieces of evidence support the notion that the association of Jak2 and tubulin is direct. First, the Jak2 immunoaffinity purified eluates contained only two proteins: Jak2 and tubulin. If the association was mediated by a tertiary protein, then three major protein bands should have been detected in Figure 1A. Second, as shown in the GST pull down data (Figure 2B), when pure tubulin is incubated with just the pseudokinase domain of Jak2, they are able to coprecipitate, independent of all other cellular proteins. Collectively, future examinations of JH2 function and malfunction may not only require an analysis of overall kinsae function but also an analysis of changes in the ability by which Jak2 binds other cellular proteins, as Jak2 is known to bind to over 50 different signaling molecules (*24*).

This work demonstrates that Jak2 colocalizes with assembled microtubules within intact cells (Figure 4), and Jak2 is able to phosphorylate tubulin under both ligand-independent (Figure 5) and ligand-dependent signaling systems (Figure 7). Therefore, an important question is, what is the biological relevance of tubulin as a substrate of Jak2? Previous work reported that phosphorylated tyrosine residue(s) on tubulin may provide binding sites for SH2 domain proteins such as c-Src and Fyn and thus mediate the distribution of various signaling proteins within the cell (*15*, *25*−*27*). STAT1 contains an SH2 domain, and our data (Figure 8) shows that STAT1 and tubulin colocalize within the cell. Thus, Jak2 may play a role in the docking of STAT1 protein onto tubulin by providing the phosphotyrosine binding site(s). A recent report demonstrated that STAT5B interacts with microtubules and suggested that microtubules may function as cellular tracks to transport STAT5B into the nucleus (*28*). It was also reported that other proteins, such as p53, associate with cellular microtubules and are transported into the nucleus by dynein (*29*). Our data demonstrate that STAT1 and tubulin colocalize and microtubule destabilizing agents such as nocodazole impair STAT1 nuclear translocation (Figure 8). Taken together, these data suggest that tubulin is an integral part of the Jak/STAT signaling pathway.

In closing, tubulin is involved in many different cellular processes such as cell motility, intracellular transport, chromosome segregation, mitosis, cell shape, and organelle distribution within the cell. It has been hypothesized that these diverse cellular events are mediated by different post-translational modifications of tubulin such as acetylation,

polyglycylation, polyglutamylation, palmitoylation, and/or phosphorylation (*30*). This study suggests that Jak2 is a major tyrosine kinase for tubulin phosphorylation, and in turn, tubulin is an important component of growth hormone-mediated STAT1 nuclear transport.

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SUPPORTING INFORMATION AVAILABLE

Mass spectrometry data indicating that p55 is α- and β-tubulin. This material is available free of charge via the Internet at http://pubs.acs.org.

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