

Cell Death Induced by the Jak2 Inhibitor, G6, Correlates with Cleavage of Vimentin Filaments

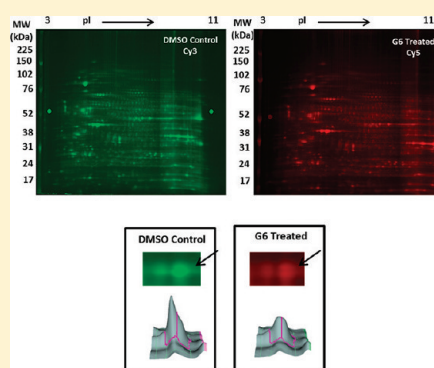
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ABSTRACT: Hyperkinetic Jak2 tyrosine kinase signaling has been implicated in several human diseases including leukemia, lymphoma, myeloma, and the myeloproliferative neoplasms. Using structure-based virtual screening, we previously identified a novel Jak2 inhibitor named G6. We showed that G6 specifically inhibits Jak2 kinase activity and suppresses Jak2-mediated cellular proliferation. To elucidate the molecular and biochemical mechanisms by which G6 inhibits Jak2-mediated cellular proliferation, we treated Jak2-V617F expressing human erythroleukemia (HEL) cells for 12 h with either vehicle control or 25 μ M of the drug and compared protein expression profiles using two-dimensional gel electrophoresis. One differentially expressed protein identified by electrospray mass spectroscopy was the intermediate filament protein, vimentin. It was present in DMSO treated cells but absent in G6 treated cells. HEL cells treated with G6 showed both time- and dose-dependent cleavage of vimentin as well as a marked reorganization of vimentin intermediate filaments within intact cells. In a mouse model of Jak2-V617F mediated human erythroleukemia, G6 also decreased the levels of vimentin protein, *in vivo*. The G6-induced cleavage of vimentin was found to be Jak2-dependent and calpain-mediated. Furthermore, we found that intracellular calcium mobilization is essential and sufficient for the cleavage of vimentin. Finally, we show that the cleavage of vimentin intermediate filaments, *per se*, is sufficient to reduce HEL cell viability. Collectively, these results suggest that G6-induced inhibition of Jak2-mediated pathogenic cell growth is concomitant with the disruption of intracellular vimentin filaments. As such, this work describes a novel pathway for the targeting of Jak2-mediated pathological cell growth.



Jak2 is a member of the *Janus* family of cytoplasmic tyrosine kinases. Other members of this family include Jak1, Jak3, and Tyk2.¹ Jak2 is activated by a variety of cytokines, growth factors, and G protein-coupled receptor (GPCR) ligands, resulting in signaling cascades that regulate cell growth, proliferation, and death.¹ Upon binding of the ligand to its specific receptor, the receptor-associated Jak proteins are activated via a phosphorylation event. An activated Jak can in turn phosphorylate and activate the signal transducers and activators of transcription (STAT) family of transcription factors. Phosphorylated STATs dimerize and translocate to the nucleus where they modulate gene transcription.^{2,3} Thus, the Jak/STAT pathway results in a signal cascade from binding and activation at the plasma membrane to changes in gene transcription in the nucleus.

Hyperkinetic Jak2 promotes cell growth and prevents apoptosis. Hence, constitutively active Jak/STAT signaling pathway has been implicated in a variety of neoplastic disorders. Jak2 can become constitutively active by several different gene alterations including specific chromosomal translocations and point mutations. Jak2 chromosomal translocations such as TEL-Jak2, REL-Jak2, BCR-Jak2, and PCM1-Jak2 lead to the development of a variety of leukemias, lymphomas, and

myelomas.^{4–10} Additionally, an activating Jak2 point mutation (Jak2-V617F) has been linked to the myeloproliferative neoplasms (MPN) such as polycythemia vera, essential thrombocythemia, and primary myelofibrosis.^{11–15} This valine to phenylalanine substitution mutation present in codon 617 of the autoinhibitory pseudokinase domain of Jak2 allows the kinase to evade negative regulation, thereby making it constitutively active. MPN patients bear this mutation in their marrow derived stem cells and are characterized by the overproduction of terminally differentiated blood cells of the myeloid lineage such as red cells or platelets. Current therapies for MPN patients include phlebotomy and hydroxyurea. While these treatments alleviate some disease symptomologies, they are not curative in any way. Therefore, there is an unmet clinical need for these patients.

Using structure-based virtual screening, our group recently identified a novel stilbenoid small molecule inhibitor of Jak2 named G6.¹⁶ We subsequently showed that G6 specifically inhibits Jak2-mediated human pathologic cell growth *in vitro*,

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ex vivo, and *in vivo*.^{17,18} We also demonstrated that G6 inhibits Jak2-mediated cell proliferation via the suppression of key signaling molecules of the Jak/STAT pathway; the consequence of this inhibition is G1/S cell cycle arrest and apoptosis.^{17,18}

Here, we sought to elucidate the molecular and biochemical mechanisms by which G6 inhibits Jak2-mediated cellular proliferation. For this, we compared protein expression profiles between vehicle treated and G6 treated cells using two-dimensional gel electrophoresis. The intermediate filament protein, vimentin, was one protein that was differentially expressed between the two conditions. We therefore hypothesized that the mechanism by which G6 inhibits Jak2-dependent cell proliferation involves modification of this protein. In this study, our data support this hypothesis as we show that G6-induced inhibition of Jak2-mediated pathogenic cell growth correlates with the specific cleavage and cellular reorganization of vimentin.

EXPERIMENTAL PROCEDURES

Drugs. G6, obtained from the National Cancer Institute/Developmental Therapeutics Program (NCI/DTP), was solubilized in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stored at -20°C .

Reagents. AG490, Jak Inhibitor I, PD98059, and PP2 were purchased from Calbiochem. Cycloheximide was purchased from Fisher Scientific. Caspase Inhibitor I (Z-VAD (OMe)-FMK), Calpain Inhibitor V (Mu-Val-HPh-CH₂F, Mu = morpholinoureidyl; HPh = homophenylalanyl), Verapamil, BAPTA-AM, A23187, and 3',3'-iminodipropionitrile (IDPN) were also purchased from Calbiochem.

Cell Culture. Human erythroleukemia (HEL) cells were purchased from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine at 37°C and 5% CO₂.

2-D Differential in Gel Electrophoresis (2-D DIGE). HEL cells were treated with either vehicle control DMSO or 25 μM G6 for 12 h. The cell pellets were resuspended in ice cold buffer containing 0.3% SDS, 20 mM Tris pH 8.0, 100 mM DTT, 10 μL protease inhibitor cocktail III (Calbiochem), 5 mM MgCl₂, and 250 units of benzonase. The cell suspension was homogenized by sonication. Protein was precipitated with 9 volumes of ice cold 10% trichloroacetic acid in acetone overnight at 4°C . Precipitated proteins were then dissolved in solubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.2% SDS, and 20 mM Tris, pH 8.0). After centrifugation at 43 000 rpm for 30 min, solubilized protein in the supernatant was quantified using the EZQ Protein Assay Kit (Invitrogen). Proteins (100 μg per sample) were minimally labeled with CyDye (GE Healthcare) as per the manufacturer's protocol. An internal standard, which is loaded on every gel, was created by mixing equal amounts of protein from all samples. Proteins from the DMSO treated samples were labeled with Cy3 (green), and the G6 treated samples were labeled with Cy5 (red). The internal standard was labeled with Cy2 (blue).

100 μg of Cy2-labeled internal standard, 100 μg of Cy3-labeled sample, and 100 μg of Cy5-labeled sample were mixed with 200 μg of unlabeled internal standard. The mixture was used to rehydrate a 24 cm pH 3 to 11 nl IPG strip (GE Healthcare) overnight in a rehydration buffer (solubilization buffer with 100 nM DTT containing Orange G as tracking dye)

in the dark at room temperature. Three independent replicates of each sample were run on three strips. IEF was carried out in IPGphor3 unit (GE Healthcare) as per manufacturer's recommendation. Temperature was maintained at 19°C throughout focusing.

After completion of IEF, strips were first reduced in 15 mL of 50 mM Tris-HCl pH 6.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 100 mM DTT for 20 min in the dark at room temperature and then alkylated in 15 mL of 50 mM Tris-HCl pH 6.8, 6 M urea, 30% (v/v) glycerol, 2% SDS, and 2.5% iodoacetamide for 20 min. After equilibration, strips were transferred and mounted on an 8%–16% precast Tris-Glycine polyacrylamide gel (Jule). Electrophoresis was carried out initially at 12°C at 10 mA/gel for 1 h and then at constant current overnight at 12 mA/gel and a limit of 150 V until dye front reached the bottom of the plate.

Gels were then scanned with Typhoon 9400 variable mode imager (GE Healthcare). The excitation/emission wavelengths for Cy2, Cy3, and Cy5 were 488/520, 532/580, and 633/670 nm, respectively. For each gel, images for the internal standard as well as the control and experimental conditions were acquired. The digital images were then analyzed with DeCyder 2D version 7.0 (GE Healthcare). Information from replicate gels was analyzed with BVA Module (Biological Variation Analysis). Spots were selected by setting the fold difference threshold to 1.6-fold. Statistical significance was estimated using Student's *t*-test. Protein identification using electrospray mass spectroscopy was done at the Scripps Research Institute.

Cell Lysis. Cells ($\sim 10^7$) were washed with two volumes of ice-cold PBS and then lysed in 0.8 mL of ice-cold RIPA buffer (20 mM Tris pH 7.5, 10% glycerol, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 2.5 mM EDTA, 50 mM NaF, 10 mM Na₄P₂O₇, 4 mM benzamide, and 10 $\mu\text{g}/\text{mL}$ aprotinin). Protein concentrations in the whole cell lysates were determined using a Bradford assay (Bio-Rad). Cell lysates were then resuspended in SDS sample buffer. Whole cell lysates (~ 30 μg) were separated by SDS-PAGE and then transferred onto nitrocellulose membranes for analysis by Western blotting.

Western Blotting. Nitrocellulose membranes were first blocked with 5% milk/TBST solution (20 mM Tris pH 7.2, 150 mM NaCl, 0.05% Tween 20) at room temperature and then probed first with the different indicated primary antibodies overnight at 4°C followed by the respective secondary antibodies (1:4000, GE Healthcare). The immuno-reactive bands were then visualized using the enhanced chemiluminescence system (Western Lightning Ultra, Perkin-Elmer). The following antibodies were used at the indicated dilutions: vimentin (Abcam and BD Biosciences, each at 1:500), STAT1 (Santa Cruz Biotechnology, 1:1000), and β -actin (Cell Signaling, 1:500).

Immunofluorescence. HEL cells were cultured in RPMI in 100 mm dishes and treated with 25 μM G6 for 24 h. Following treatment, the cells were centrifuged, washed, and resuspended in 1X PBS. Cells were then plated onto poly-L-lysine coated 8-chamber slides (Santa Cruz Biotechnology) and fixed at -20°C in a mixture of 50% methanol and 50% acetone for 10 min. The fixed cells were then permeabilized with 0.2% Triton X-100 and blocked with 5% goat serum for 30 min at room temperature. The samples were incubated overnight at 4°C with a primary antibody of mouse anti-vimentin (BD Biosciences, 1:100) or rabbit anti- β -actin (Cell Signaling, 1:100) and washed 4 times with PBS the following morning. The samples were then incubated with a FITC-conjugated

anti-mouse secondary antibody or a FITC-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The cells were again washed with PBS, mounted with UltraCruz DAPI containing mounting media (Santa Cruz Biotechnology), and sealed with a coverslip. These cells were imaged using a 100× objective on an inverted fluorescence microscope (Olympus).

Cell Proliferation Assay. HEL cells were plated in 96-well plates and treated with either 0.25% DMSO, 30 μ M G6, or 2% IDPN for the indicated periods of time. Cell viability was then assessed for each sample by trypan blue exclusion staining and a hemocytometer.

In Vivo Animal Model. The xenograft model of Jak2-V617F expressing HEL cells in NOD/SCID mice has been described previously.¹⁸ Briefly, 3 months old NOD/SCID mice were randomized into five groups ($n = 6$ mice per group). One group consisted of naive animals that did not receive any treatment. All other groups received a single tail vein injection of 2×10^6 Jak2-V617F-positive HEL cells. Three weeks after HEL cell injection, the mice developed symptoms of a fully penetrant bone marrow malignancy. The mice then began receiving intraperitoneal injections of either vehicle control (DMSO) or G6 at doses of 0.1, 1, and 10 mg/kg/day for the next 21 days. At the end of the 3 week treatment period, all groups of mice were euthanized and bone marrow tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin.

Bone Marrow Immunohistochemistry. Paraffin embedded bone marrow sections from each treatment group were analyzed by anti-vimentin immunohistochemistry. Antigen retrieval was carried out first by microwaving at 95 °C for 20 min in 1 mM EDTA–NaOH solution, pH 8.0. The section were then cooled, blocked with Protein Block (DAKO), and incubated with anti-vimentin antibody (Abcam, 1:100) for 2 h at room temperature. Antigen–antibody complexes were detected using biotinylated secondary antibodies and streptavidin-peroxidase substrate (DAKO). Stained sections were then analyzed via a standard light microscope (Nikon) at 40× and 100× magnifications.

Statistical Analysis. For statistical evaluation of time-dependent response of HEL cell viability to G6 and IDPN, a two-way analysis of variance was used. For analysis of differential expression of proteins in 2-D DIGE and G6-induced degradation of vimentin using densitometry, a Student's *t*-test was employed. Data were assumed to be statistically significant when $p < 0.05$.

RESULTS

G6 Treatment Induces Time- and Dose-Dependent Degradation of Vimentin. The human erythroleukemia (HEL 92.1.7) cell line is homozygous for the Jak2-V617F mutation.^{19,20} The presence of this mutation induces constitutively active Jak/STAT signaling and promotes a G1/S phase transition, thereby driving increased cellular proliferation.²¹ We previously demonstrated that the Jak2 inhibitor, G6, inhibits Jak2-V617F-mediated HEL cell proliferation and induces apoptosis.^{16,17} However, the specific mechanisms by which G6 does this are not known.

To gain some insight into the mechanism by which G6 reduces cell viability, HEL cells were treated for 12 h with either vehicle control (0.25% DMSO) or 25 μ M G6. The protein expression profiles of these two treatment conditions

were compared using two-dimensional gel-electrophoresis (Figure 1A–D). The two-dimensional gel images were then scanned, and the staining intensities of the various protein spots were compared between the two treatment conditions. One spot in particular, identified from the scanning results, was highly expressed in the DMSO treated cells, but significantly reduced in the G6 treated cells (Figure 1E; circled and marked by arrows). That spot was excised and identified using electrospray mass spectrometry as vimentin. In a separate two-dimensional gel electrophoresis study, we compared the protein expression profiles between HEL cells that had been treated with either DMSO or 25 μ M G6 for 24 h. Even at this longer time point, the spot representing vimentin was still significantly reduced in the G6 treated cells when compared to the DMSO treated cells (data not shown).

To confirm that vimentin protein levels were decreasing with G6 treatment, protein samples from both conditions were subjected to Western blot analysis with an anti-vimentin antibody. Consistent with the mass spectroscopy data, treatment of HEL cells with G6 resulted in the disappearance of full-length vimentin (Figure 1F). Of note, we also observed the appearance of low molecular weight fragments of vimentin in the G6 treated cells (Figure 1F).

To determine whether this effect was time- and dose-dependent, HEL cells were treated either with 25 μ M of G6 for increasing lengths of time or with varying doses of G6 for 24 h. Whole cell lysates were then separated on SDS-PAGE and examined by immunoblotting with an anti-vimentin antibody. Figure 2A is a representative blot showing that full-length vimentin was cleaved into low molecular weight fragments with G6 treatment as a function of time. The same samples were then reprobbed with an anti- β -actin antibody to confirm equal protein loading and also to demonstrate the specificity of G6 for vimentin over other cytoskeletal proteins such as β -actin. Quantification of all blots using densitometry confirmed the total loss of full-length vimentin protein in response to G6 treatment over time (Figure 2B). Similarly, Figure 2C is a representative blot showing a dose-dependent cleavage of full-length vimentin in response to G6 and Figure 2D is a quantification of all dose-dependent blots.

Collectively, the data in Figures 1 and 2 demonstrate the ability of G6 to induce specific cleavage of the intermediate filament protein vimentin. Furthermore, this effect is both time- and dose-dependent.

G6 Treatment Induces Marked Reorganization of Vimentin Intermediate Filaments within Cells. We next wanted to study the effect of G6 treatment on structure and cellular distribution of intracellular vimentin filaments. For this, HEL cells were treated with 25 μ M G6 for 0 and 24 h, and then vimentin expression was analyzed via indirect immunofluorescence. For the 0 h time point, we found that vimentin was largely distributed over the cytoplasm (Figure 3A, C, E). However, after 24 h of G6 treatment, vimentin had an irregular staining pattern in the perinuclear region of the cell (Figure 3B, D, F). As a control, similarly treated HEL cells were examined for changes in β -actin expression. We found that β -actin was uniformly distributed across the cytoplasm of the cell at the 0 h time point, and this pattern did not change with G6 treatment (Figure 3G–L).

As such, the data in Figure 3 indicate that G6 treatment specifically induces cellular redistribution of vimentin intermediate

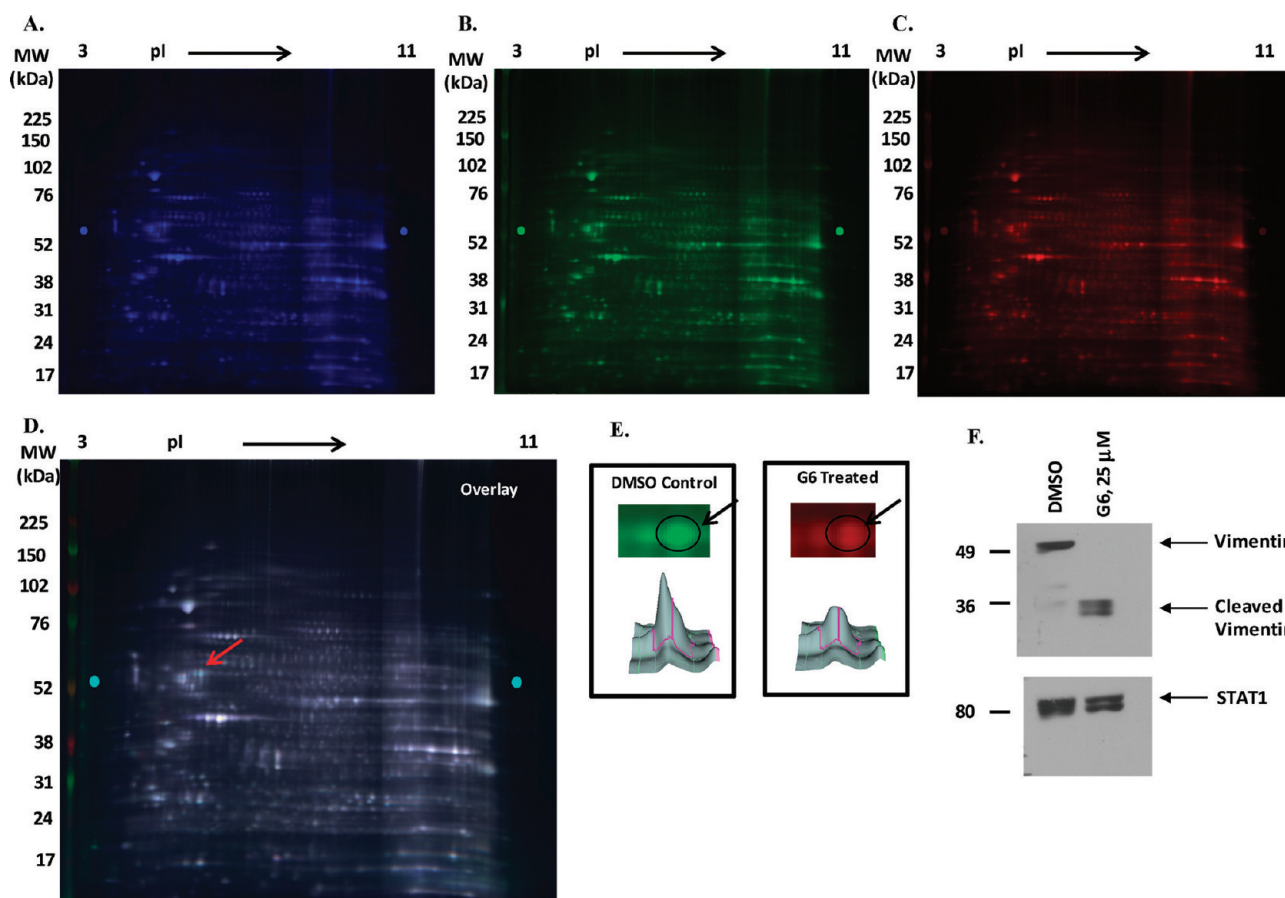


Figure 1. Identification of vimentin as a differentially expressed protein between vehicle treated and G6 treated HEL cells. HEL cells were treated with either 0.25% DMSO or with 25 μ M of G6 for 12 h. Proteins from the internal standard (A), DMSO treated (B), and G6 treated (C) were labeled with Cy2 (blue), Cy3 (green), and Cy5 (red), respectively. (D) An overlay of the three colored images is shown. One protein spot, indicated by the arrow, was differentially expressed between the vehicle treated and G6 treated samples. (E) Analysis of the images obtained from the 2-D DIGE using DeCyder 2D predicted this differentially expressed protein to be significantly downregulated in the G6 treated samples ($p = 0.01$). The indicated protein was excised and identified using electrospray mass spectrometry as vimentin. (F) HEL cells were treated either with DMSO or 25 μ M of G6 for 24 h and analyzed by Western blotting using an anti-vimentin antibody. The same samples were also blotted with an anti-STAT1 antibody to confirm equal loading across all lanes. Shown is one of three representative images.

filaments within HEL cells while having no effect on the cellular distribution of β -actin microfilaments.

G6-Induced Cleavage of Vimentin Is Jak2-Mediated.

Having already demonstrated the ability of G6 to induce specific cleavage of vimentin (Figure 2), we next wanted to determine if this G6-induced cleavage was Jak2-dependent. For this, HEL cells were treated for 24 h with increasing concentrations of three different Jak2 inhibitors; G6, AG490, and Jak Inhibitor I. As a control, HEL cells were also treated with non-Jak2 inhibitors, namely, the MAPK inhibitor, PD98059, and Src family kinase inhibitor, PP2. Whole cell lysates were separated by SDS-PAGE and immunoblotted with an anti-vimentin antibody. We observed that the Jak2-specific inhibitors induced cleavage of vimentin dose dependently (Figure 4A), whereas the non-Jak2 inhibitors had no effect on full-length vimentin (Figure 4B). The loading of total protein was determined by immunoblotting the same cellular lysates with an anti-STAT1 antibody (Figure 4A,B). Treatment of HEL cells with higher concentrations of the different Jak2 inhibitors (such as 100 μ M G6, 100 μ M AG490, or 30 μ M Jak Inhibitor I) potentially induces apoptosis in these cells, thereby leading to the degradation of all cellular proteins, including vimentin and STAT1. Hence, there is a general reduction in the

total protein that is extracted from these cells, which explains the lower level of expression or total absence of proteins observed in the samples that had been treated with high concentrations of the different Jak2 inhibitors.

Thus, from Figure 4, we conclude that G6-induced vimentin degradation is Jak2-mediated.

G6-Induced Cleavage of Vimentin Is Independent of *de novo* Protein Synthesis and Caspase Activity, but Calpain-Dependent.

Given that G6 induces specific cleavage of vimentin (Figure 2), we next wanted to determine whether this G6-induced vimentin cleavage is dependent on *de novo* protein synthesis. To assess this, HEL cells were first pretreated for 4 h with increasing doses of cycloheximide (CHX), an inhibitor of protein biosynthesis, and then treated with increasing concentrations of G6 for 24 h. Cycloheximide inhibits protein synthesis by interfering with the translation elongation process of protein biosynthesis.²² Western blot analysis of the cell lysates from the different treatment groups showed that exposure to increasing doses of G6 induced a dose dependent cleavage of vimentin in HEL cells which was not blocked by pretreatment with cycloheximide (Figure 5A), indicating that this G6-induced cleavage process does not require *de novo* protein synthesis.

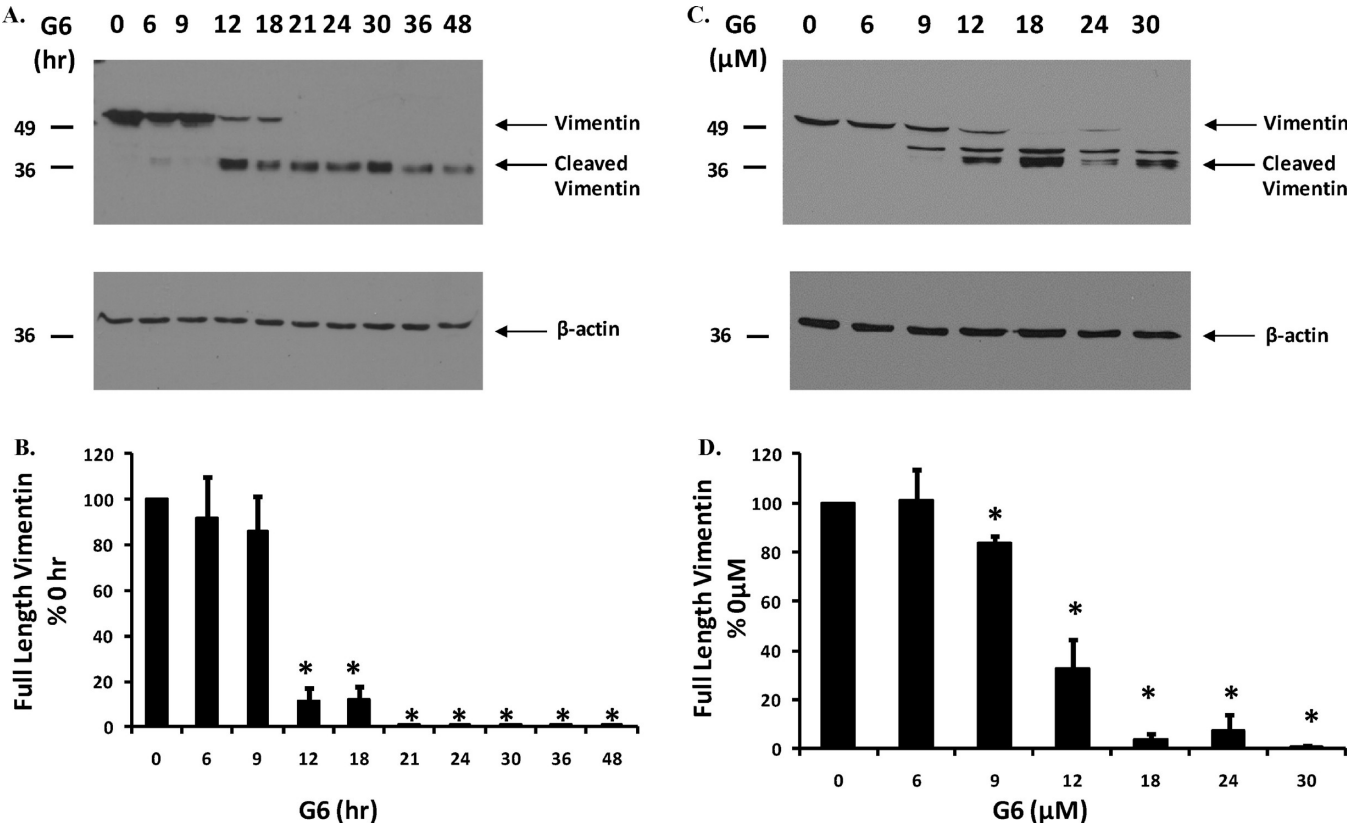


Figure 2. G6 treatment induces time- and dose-dependent degradation of vimentin. HEL cells were treated either with 25 μ M of G6 for varying lengths of time (A) or with increasing doses of G6 for 24 h (C). Cell lysates were then separated on SDS-PAGE and immunoblotted with an anti-vimentin antibody. The same samples were then reprobed with an anti- β -actin antibody to confirm equal protein loading and also to demonstrate the specificity of G6 for vimentin over other cytoskeletal proteins such as β -actin. Shown is one of three independent results for each. Expression of full-length vimentin was quantified using densitometry and plotted as a function of either time (B) or dose (D) of G6 treatment. Data shown are the mean \pm SE from three independent experiments. * p < 0.05 with respect to 0 h (B) or 0 μ M (D).

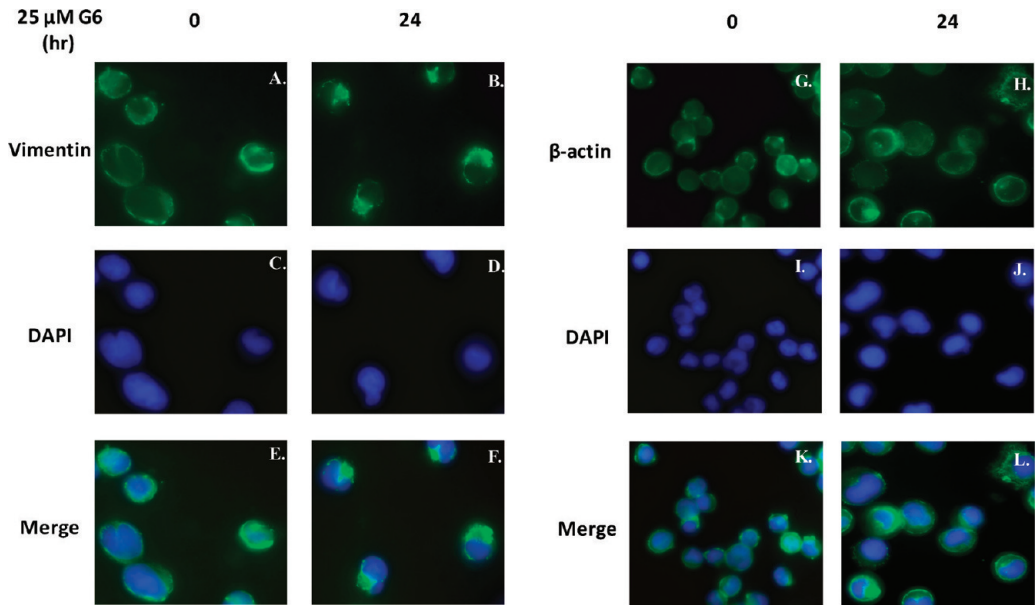


Figure 3. G6 treatment induces marked reorganization of vimentin intermediate filaments within cells. HEL cells, treated with 25 μ M of G6 for 0 or 24 h, were analyzed via indirect immunofluorescence for changes in the cellular distribution of vimentin and β -actin in response to drug treatment. Vimentin (A and B) and β -actin (G and H) were indirectly labeled with a FITC-conjugated secondary antibody. The nuclei were counter stained with DAPI (C, D and I, J). The images were then merged (E, F and K, L). Shown is one of two representative results.

Vimentin is cleaved in response to G6 treatment into low molecular weight fragments of vimentin (Figure 2), suggesting

that this process is mediated by a protease/preteolytic enzyme. Caspases are a class of intracellular cysteine proteases

with roles in cytokine maturation, inflammation, and apoptosis.²³ We previously showed that G6 induces caspase

3/7 activation in a time-dependent manner in HEL cells.¹⁸ It has also been reported that vimentin is a caspase substrate and can be cleaved by some caspases *in vitro*.²⁴ Therefore, we wanted to determine if G6-induced vimentin cleavage is caspase-mediated. For this, we first pretreated HEL cells with the pan-caspase inhibitor, Caspase Inhibitor I (zVAD-fmk), for 4 h before treating them with 30 and 60 μ M G6 for 24 h. The effect of caspase inhibition on G6-dependent vimentin cleavage was then studied by Western blotting the cell lysates with an anti-vimentin antibody. We found that inhibition of caspases by zVAD-fmk was unable to prevent G6-induced cleavage of vimentin (Figure 5B) but was able to significantly reduce the G6-induced cleavage of PARP (Figure 5B), a substrate known to be cleaved by caspases,²⁵ thereby indicating that G6-induced cleavage of vimentin is caspase-independent.

Calpain, a calcium-dependent neutral cysteine protease,²⁶ is yet another protease that is known to cleave vimentin.^{27,28} Hence, to examine whether G6-induced vimentin cleavage is calpain-mediated, we pretreated HEL cells with a calpain inhibitor, Calpain Inhibitor V (Mu-Val-HPh-CH2F), for 4 h before exposing them to increasing doses of G6 for 24 h. Immunoblotting analysis of the HEL cell lysates showed that calpain inhibition prevented G6-induced cleavage of vimentin in a dose-dependent manner (Figure 5C), demonstrating that the protease involved in the cleavage of vimentin in response to G6 treatment is calpain.

Overall, the data in Figure 5 indicate that the G6-induced cleavage of intermediate filament protein vimentin is independent of *de novo* protein synthesis and caspase activity but dependent on calpain protease activity.

Mobilization of Calcium Is Essential and Sufficient for the Cleavage of the Intermediate Filament Protein Vimentin.

Given that calpain is a calcium-dependent cysteine

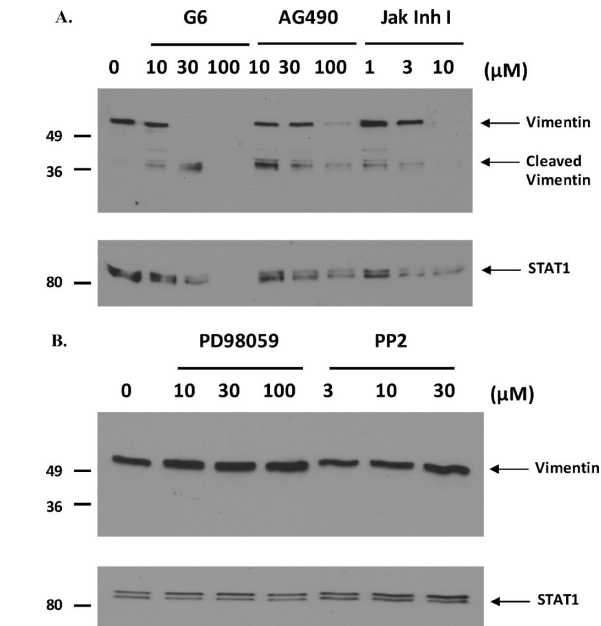


Figure 4. G6-induced cleavage of vimentin is Jak2-mediated. HEL cells were treated for 24 h with increasing concentrations of Jak2 specific inhibitors (G6, AG490, and Jak Inhibitor I) (A) or non-Jak2 inhibitors (MAPK inhibitor, PD98059 and c-Src inhibitor, PP2) (B). Whole cell lysates were separated by SDS-PAGE and immunoblotted with an anti-vimentin antibody. Loading of total protein across all lanes was determined using an anti-STAT1 antibody. Shown is one of three representative blots.

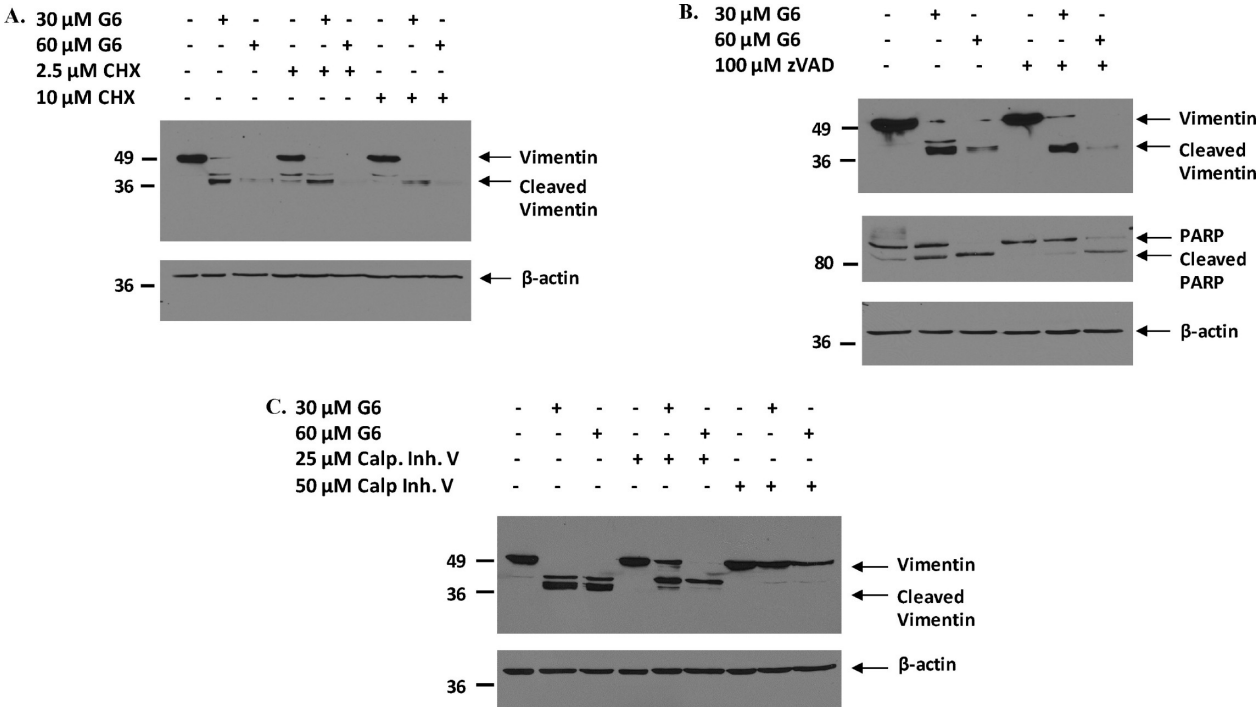


Figure 5. G6-induced cleavage of vimentin is independent of *de novo* protein synthesis and caspase activity, but calpain-dependent. HEL cells were pretreated for 4 h with either cycloheximide (CHX) (A), caspase inhibitor I (zVAD) (B), or calpain inhibitor V (C) and then treated with increasing doses of G6 for 24 h. Whole cell lysates from the different treatment groups were then analyzed by Western blotting with an anti-vimentin antibody. The same lysates were also probed with an anti- β -actin antibody to confirm equal protein loading across all lanes. Shown is one of three representative results for each.

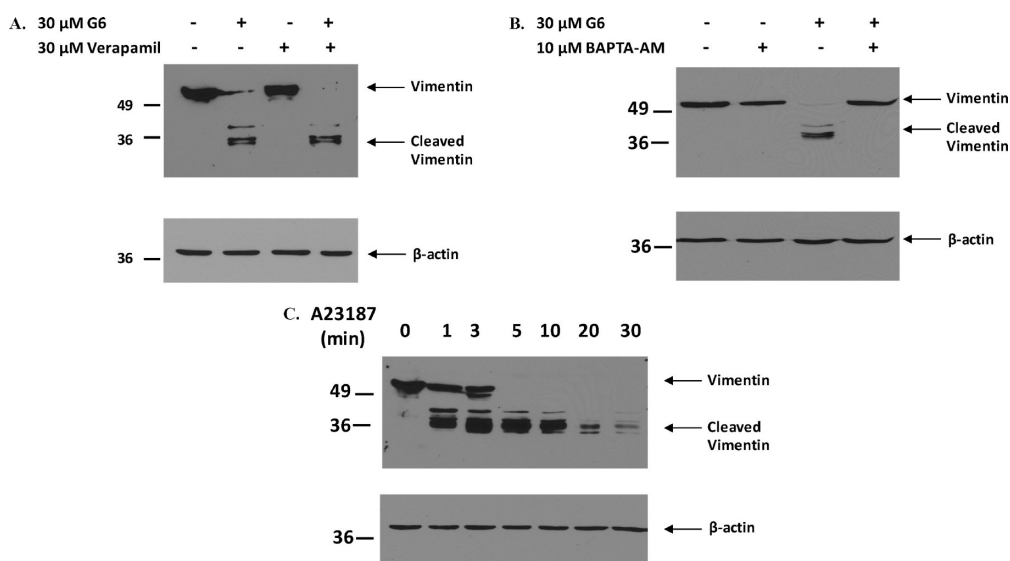


Figure 6. Mobilization of calcium is essential and sufficient for the cleavage of vimentin. HEL cells were first pretreated with either 30 μ M verapamil for 4 h (A) or 10 μ M BAPTA-AM for 2 h (B) and then treated with 30 μ M G6 for 24 h. Post-treatment, the cells were lysed, and proteins were separated by gel electrophoresis and then immunoblotted with an anti-vimentin antibody (upper panel) or an anti- β -actin antibody (lower panel) to confirm equal protein across all lanes. (C) HEL cells were treated with 10 μ M A23187 for the indicated periods of time. Cellular lysates were then probed with an anti-vimentin antibody (top panel). An anti- β -actin antibody was used as a loading control (bottom panel). Shown is a representative blot from three independent experiments for each.

protease, we next investigated the role of calcium in the G6-induced vimentin cleavage process. Specifically, we first examined the effect of inhibiting the flux of extracellular calcium into cells by pretreating the cells with verapamil. Verapamil blocks Ca^{2+} channels, principally the L-type channel, thereby interfering with the extracellular influx of calcium ions. HEL cells were pretreated with 30 μ M verapamil for 4 h before exposure to 30 μ M G6 for 24 h. Cell lysates were then immunoblotted with an anti-vimentin antibody. We found that inhibition of extracellular calcium ion influx into the cell via blockage of L-type calcium channels did not have any effect on G6-induced cleavage of vimentin (Figure 6A). Therefore, we next studied the effect of chelating intracellular calcium on G6-mediated vimentin cleavage. For this, we pretreated HEL cells with 10 μ M BAPTA-AM for 2 h before treatment with 30 μ M G6 for 24 h. BAPTA-AM is membrane-permeable ester form of the calcium chelator BAPTA. Once inside the cell, it is hydrolyzed by cytosolic esterases into its active form and can chelate intracellular calcium. Results from the Western blot analysis showed that chelation of intracellular calcium protected vimentin from G6-induced cleavage (Figure 6B), indicating that intracellular calcium has a critical role to play in mediating the G6-induced cleavage of vimentin.

In the next experiment, we examined the effect of the calcium ionophore, A23187, on vimentin protein levels within HEL cells. A23187 is a mobile ion-carrier that forms stable complexes with divalent cations, such as calcium, and can hence be used for increasing intracellular levels of calcium ions. Accordingly, HEL cells were treated with 10 μ M A23187 for increasing periods of time and the cellular lysates were then Western blotted using an anti-vimentin antibody. We found that increasing intracellular calcium levels via exposure to an ionophore is sufficient to induce cleavage of vimentin in HEL cells (Figure 6C), further confirming the essential role that calcium ions play in the vimentin cleavage process.

Collectively, data in Figure 6 demonstrate that mobilization of intracellular calcium ions is both essential and

sufficient for the cleavage of the intermediate filament protein, vimentin.

Cleavage of Vimentin Is Sufficient To Reduce HEL Cell Viability. To determine how critical vimentin is to the viability of cells, we studied the effect of vimentin cleavage on the survival of HEL cells. The drug 3,3'-iminodipropionitrile (IDPN) selectively disrupts vimentin intermediate filaments.²⁹ Therefore, we treated HEL cells with either vehicle control DMSO, 30 μ M G6, or 2% IDPN for 0, 6, 12, 24, or 48 h. At each time point, the number of viable cells in each condition was determined, and cell lysates from those same conditions were immunoblotted with an anti-vimentin antibody in order to correlate decreased cell numbers with increased vimentin cleavage. We found that treatment with both G6 and IDPN time dependently decreased viable cell numbers (Figure 7A), and this decrease in cell viability correlated with a corresponding time-dependent cleavage of full-length vimentin in the G6 and IDPN treated cells (Figure 7B).

Overall, the data in Figure 7 demonstrate that the cleavage of vimentin intermediate filaments is sufficient to reduce the viability of Jak2-V617F expressing HEL cells.

G6 Treatment Decreases the Levels of Vimentin Protein, *in vivo*. Our data thus far indicate that treatment of HEL cells with G6 results in the degradation and subsequent loss of vimentin protein, *in vitro*. To determine if this is conserved *in vivo*, HEL cells were injected into the tail vein of NOD/SCID mice and allowed to engraft into the bone marrow over the ensuing 21 days, at which time the mice began receiving daily intraperitoneal injections of either vehicle control (DMSO) or G6 at doses of 0.1, 1, and 10 mg/kg/day for the next 21 days. At the end of the 3 week treatment period, all groups of mice were euthanized and bone marrow was analyzed for vimentin protein levels via anti-vimentin immunohistochemistry. Representative stained sections from each treatment group are shown at 40 \times (Figure 8A) and 100 \times (Figure 8B) magnification. We found that when a negative

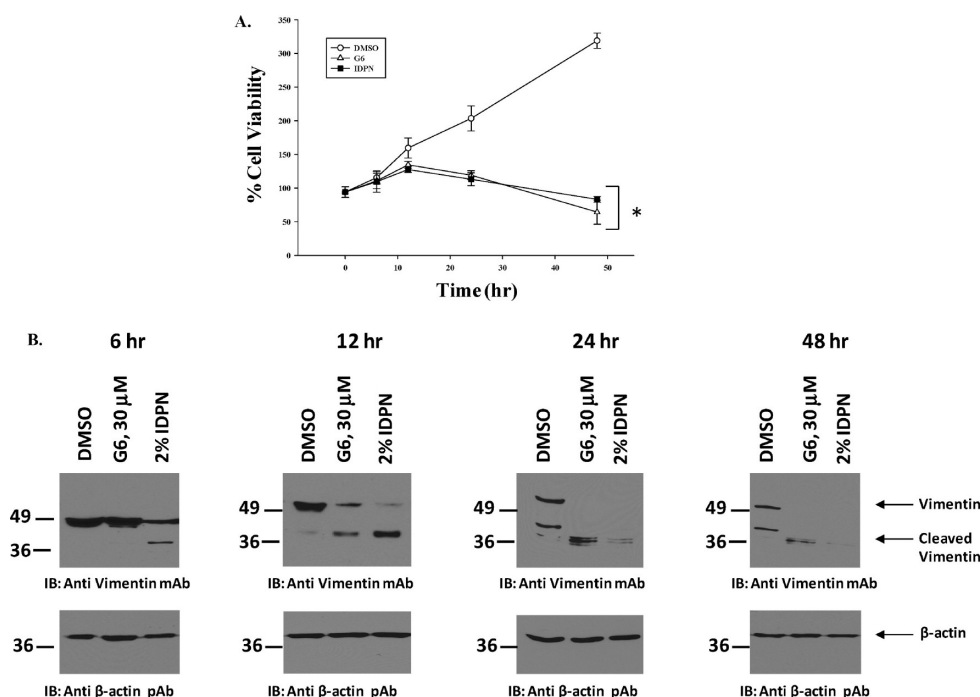


Figure 7. Cleavage of vimentin is sufficient to reduce HEL cell viability. HEL cells were exposed to either vehicle control (DMSO), 30 μ M G6, or 2% IDPN for 0, 6, 12, 24, or 48 h. (A) The numbers of viable cells at each time point were determined and plotted as a function of treatment condition. (B) Cell lysates from each treatment group were collected simultaneously and analyzed by immunoblotting with either an anti-vimentin antibody or an anti- β -actin antibody. Shown is one of three representative results. * $p < 0.05$ with respect to DMSO.

control IgG antibody was used in place of the anti-vimentin primary antibody in the immuno-histochemical procedure, only the hematoxylin counter stain was observed. Probing the naive bone marrow with the anti-vimentin antibody revealed strong staining in erythroid cells, but not myeloid cells. HEL cell injection followed by DMSO treatment resulted in a dramatic increase in the expression of vimentin protein when compared to naive animals. Treatment with 0.1 mg/kg/day of G6 did not produce any observable change in the expression level of vimentin when compared to DMSO treated mice. However, treatment with 1 and 10 mg/kg/day of G6 clearly reduced the levels of vimentin protein to those seen in the completely naive animals.

Hence, the data in Figure 8 indicate G6 treatment reduces HEL cell-induced vimentin expression in a dose-dependent manner, *in vivo*.

DISCUSSION

Our group recently identified a novel stilbenoid Jak2 small molecule inhibitor named G6.¹⁶ We subsequently showed that it specifically inhibits Jak2-mediated human pathologic cell growth *in vitro*, *ex vivo*, and *in vivo*.^{17,18} We have also demonstrated that G6 inhibits Jak2-mediated cell proliferation via the suppression of key signaling molecules of the Jak/STAT pathway and with the induction of G1/S cell cycle arrest and apoptosis.^{17,18} In this report, our objective was to determine the mechanisms by which G6 exerts its inhibitory actions. To this end, we found that G6 treatment induced a time- and dose-dependent cleavage of the intermediate filament protein, vimentin (Figure 2). G6 treatment of HEL cells resulted in the movement of vimentin from a mostly cytoplasmic to a predominantly perinuclear distribution within the cell (Figure 3). The G6-mediated cleavage of vimentin is Jak2-dependent

(Figure 4) and calpain-mediated (Figure 5). The mobilization of intracellular calcium is critical for G6-mediated vimentin cleavage (Figure 6), and the cleavage of vimentin, *per se*, is sufficient to reduce HEL cell viability (Figure 7). Lastly, the ability of G6 to reduce vimentin levels is conserved *in vivo* (Figure 8). Taken together, these results describe a novel mechanism by which G6 exerts its inhibitory actions.

Vimentin, a member of the type III intermediate filament protein family, is a key component of the cytoskeleton of the cell. It plays an important role in maintaining cell shape and integrity as well as stabilizing cytoskeletal interactions such as adhesion, migration, and signaling. Phosphorylation is a key regulator of the dynamics of vimentin assembly/disassembly and modulates the organization, subcellular distribution, and function of these intermediate filaments. Vimentin can be phosphorylated on distinct serine/threonine residues by various protein kinases, including protein kinase A, protein kinase C, cyclin-dependent kinase 1, Rho kinase, p21-activated kinase 1, and Aurora-B kinase. Site-specific phosphorylation is usually associated with disassembly of vimentin intermediate filaments. For example, phosphorylation of vimentin by protein kinase A or protein kinase C on specific serine residues results in disassembly of the vimentin filaments.^{30,31} Site-specific phosphorylation of vimentin by other kinases during the various stages of mitosis plays a critical role in the regulation/progression of mitotic events. Specifically, it has been reported that cyclin-dependent kinase 2 phosphorylates vimentin from prometaphase to metaphase,³² while Aurora-B kinase³³ and Rho-kinase³⁴ phosphorylate vimentin specifically at the cleavage furrow from anaphase to the end of mitosis. Disrupting the interaction between these protein kinases and vimentin filaments prevents effective separation of filaments during cytokinesis, thereby resulting in abnormal cell division.^{35,36} Another study³⁷ demonstrated that stimulation of cultured smooth muscle cells

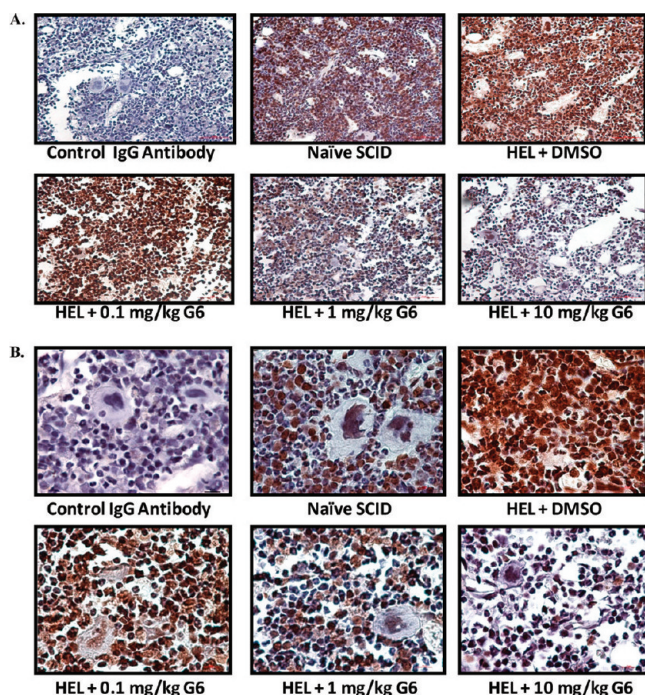


Figure 8. G6 treatment decreases the levels of vimentin protein, *in vivo*. NOD/SCID mice were randomized into 5 groups ($n = 6$ per group). One group consisted of naive animals that did not receive any treatment whatsoever. All other mice received 2×10^6 HEL cells via a single tail vein injection. Three weeks after injection, the mice began receiving vehicle control solution (DMSO) or G6 at doses of 0.1, 1, and 10 mg/kg/day. After 3 weeks of treatment, the mice were euthanized. Anti-vimentin immuno-histochemistry was then carried out on bone marrow sections from the indicated groups of animals. Shown are representative stained bone marrow sections from each treatment group at 40 \times (A) and 100 \times (B) magnifications. Also shown is a negative control in which an IgG antibody was used in place of the anti-vimentin primary antibody during the immune-histochemical procedure.

with serotonin resulted in p21-activation kinase 1-mediated phosphorylation of vimentin at serine-56. This phosphorylation event leads to the disassembly of vimentin intermediate filaments and alters the migratory and contractile properties of smooth muscle cells.³⁸ Vimentin was recently identified as a protein which, in response to catecholamine stimulation, can directly interact with β -adrenergic receptor and activate extracellular signal-regulated kinases (Erk)-1 and -2 via direct recruitment and activation of Src kinases.²⁹ It has also been suggested that the vimentin intermediate filament network can serve as platforms/scaffolds for signaling molecules.³⁹ A group from France⁴⁰ previously reported that the Src family kinase, Yes, associates and localizes with vimentin filaments in amoeboid microglia. This suggests that vimentin might serve as a molecular support for Yes kinase and regulate its phosphorylation and subsequent signal transduction. Vimentin has also been shown to stabilize the phosphorylated form of Erk by protecting it from phosphatases via steric hindrance.⁴¹ Our lab has previously reported that Jak2 can associate with and directly phosphorylate the cytoskeleton protein tubulin.⁴² These studies suggest that it is possible that vimentin might have yet to be determined regulatory/stabilization associations with several other membrane associated protein complexes, including Jak2.

Epithelial–mesenchymal transition (EMT), a reprogramming process by which cells undergo a morphological switch

from the epithelial phenotype to the mesenchymal fibroblast-like phenotype, is associated with normal embryonic development and is also activated during cancer invasion and metastasis.⁴³ At a molecular level, during EMT, cells lose epithelial markers, such as E-cadherin, and start expressing mesenchymal markers, such as N-cadherin and vimentin.⁴⁴ As a result, epithelial cells lose their well-defined cell–cell/cell–substratum contacts/adhesion as well as their structural/functional polarity and gradually assume a spindle-shape morphology.⁴⁵ Acquisition of mesenchymal characteristics allows the epithelial cells within a tumor to metastasize by migrating into surrounding tissues.⁴⁵ EMT is also marked by an increase in nuclear localization of β -catenin,⁴⁶ which in turn transcriptionally activates the expression of EMT-inducing genes, such as matrix metalloproteinase 7,⁴⁷ fibronectin,⁴⁸ and vimentin.⁴⁹ Vimentin overexpression is known to promote migration in epithelial cells that are involved in normal physiological processes, such as organogenesis, placentation, and wound healing.⁵⁰ Increased *de novo* expression of this protein is also associated with invasive cancer cells that have higher chances of metastasizing and poor prognosis.^{51–54} Knockdown of vimentin expression in vimentin-expressing breast cell lines using antisense resulted in a corresponding decrease in the *in vitro* invasiveness/migration of these cells.⁵³ It has also been reported that vimentin knockout mice show impaired wound healing ability.⁵⁵ These studies strongly emphasize that vimentin has a functional role in inducing epithelial cell migration/invasion and EMT. However, the exact mechanism by which vimentin induces EMT is still elusive.

It is hypothesized that vimentin may destabilize E-cadherin mediated cell adhesion complexes, thereby leading to an increased migratory ability of cells overexpressing vimentin.⁴⁹ There is evidence that phosphorylation of β -catenin on critical tyrosine residues can disrupt the interaction of the protein with E-cadherin, thereby rendering it free to translocate to the nucleus and regulate transcription of downstream target genes.⁵⁶ For example, phosphorylation of β -catenin by Src kinase on tyrosine 654 causes a decrease in the binding affinity of the protein for E-cadherin.⁵⁷ Similarly, studies have shown that blockade of Jak2 tyrosine kinase activity can suppress the accumulation of β -catenin in leukemic cells.⁵⁸ β -Catenin can transactivate vimentin by directly interacting with the vimentin promoter,⁴⁹ thereby suggesting that decreased accumulation of this protein can cause a corresponding decrease in the expression levels of vimentin protein. Another recent study has reported that blockade of Jak2 by AG490 inhibits migration and proliferation of human colon cancer cells.⁵⁹ Specifically, AG490 decreased STAT3 phosphorylation and vimentin expression, thereby suggesting that Jak2/STAT3/vimentin signaling participates in regulating the proliferation and migration of colon cancer cells.⁵⁹ The group led by Ahmed^{60,61} demonstrated that, in response to the stimulation of epidermal growth factor receptor, there is an activation of Jak2/STAT3 signaling pathway in high grade ovarian carcinomas. A corresponding transition to a migratory phenotype, marked by increased expression of mesenchyme-associated N-cadherin, vimentin, and nuclear translocation of β -catenin, was also observed in these cells.^{60,61} A separate study reported the role of STAT5a in RhoA-induced epithelial-to-mesenchymal transition. RhoA induces Jak2-dependent tyrosine phosphorylation of STAT5a with a concomitant increase in vimentin expression and cell motility.⁶² It is also known that STAT3 can directly regulate the expression of vimentin protein by interacting with its promoter region.⁶³ When taken together, it is possible that the inhibition

of Jak2 via G6 and the subsequent loss of vimentin expression that we report in this paper can be due to either a Jak2-mediated modulation of β -catenin activity/stability or a Jak2-mediated alteration in STAT3 transcriptional activity.

Apoptosis is associated with disruption of the cytoskeletal network and caspase and/or calpain-induced cleavage of cytoskeletal proteins, such as vimentin, is known to occur in response to various inducers of apoptosis.^{64–68} However, studies have reported that knockdown of vimentin does not induce significant apoptosis *per se*, but cleavage or degradation of vimentin potentiates the therapeutic effects of a drug.^{69,70} These studies also report that higher levels of vimentin expression render cells more susceptible to drug-induced apoptosis. In agreement with these previous reports, we found that Jak2-V617F expressing HEL cells have readily detectable levels of vimentin protein and are very susceptible to drug inhibition and subsequent loss of cell viability. However, in contrast to the earlier reports, we found that the loss of vimentin, either by G6 or by IDPN treatment, was sufficient to significantly decrease cell viability (Figure 7). Possible explanations for these observed differences include the fact that the earlier reports used siRNA to knockdown vimentin mRNA levels, whereas we used pharmacological inhibitors that resulted in decreased vimentin protein levels. In addition, G6 also reduces Jak2 kinase activity^{17,18} which presumably vimentin siRNA treatment does not. These differences notwithstanding, our work here is significant in that we demonstrate that G6 treatment results in vimentin cleavage and the subsequent loss of cell viability.

With respect to the signaling pathway that facilitates G6-mediated vimentin cleavage, our data indicate that calcium plays a critical role in this process. For example, the G6-induced cleavage of vimentin is mediated by the calcium-dependent protease, calpain (Figure 5C). Furthermore, we show that mobilization of intracellular calcium is both essential and sufficient for cleavage of vimentin. Our data therefore suggest that there is a close correlation between Jak2 kinase activity and the levels of intracellular calcium ions. This is supported by previous work which indicates that erythropoietin (Epo)-induced activation of its cognate receptor, EpoR, inhibits calcium-induced neurotransmitter release via the activation of Jak2.⁷¹ This report also shows that this Epo-induced inhibition of calcium activity can be blocked by treatment with a tyrosine kinase inhibitor, such as genistein, further confirming the importance of Jak2 in mediating calcium-induced responses. Overall, these studies demonstrate that Jak2 can modulate intracellular calcium levels. However, the exact protein target(s) that Jak2 may phosphorylate in the calcium signaling pathway are not known.

We have previously reported that G6 treatment potently induces apoptosis in HEL cells via the modulation of various Bcl-2 family proteins, such as Bcl-xL, Bim, and Bid.^{17,18} Previous studies have reported that elevation of intracellular calcium levels can trigger apoptosis via the destabilization of the mitochondrial membrane and subsequent activation of calcium-dependent calpain proteases.^{72–74} Activation of calpains can further lead to the cleavage and activation of apoptotic regulators of the Bcl2 family, such as Bid.⁷⁵ Therefore, these studies suggest that calcium and calcium-dependent proteases may have an important role to play in the G6-mediated cell death/apoptosis process.

The Jak2-V617F mutation is found in a large percentage of MPN patients.^{11–15} A previous study, which compared the

mRNA expression profiles between healthy individuals and MPN patients, reported vimentin to be one gene that was differentially expressed in these two groups.⁷⁶ Specifically, MPN patients were found to have elevated levels of vimentin mRNA when compared to nondiseased individuals. This increase in vimentin gene expression correlated positively with the presence of the Jak2-V617F mutation. In other words, significant overexpression of vimentin was only observed in patients that were homozygous for the Jak2-V617F mutation. Thus, our data here, which describes vimentin as one protein that is downregulated in response to Jak2-V617F inhibition by G6, is noteworthy. Furthermore, our data which show that the cleavage of vimentin is Jak2-dependent (Figure 4) imply that pharmacological inhibition of Jak2 is sufficient to induce cleavage of vimentin and subsequent loss of cell viability (Figure 7). When taken together with the MPN microarray data,⁷⁶ our work here suggests that there may be a link between hyper activation of Jak2 kinase and overexpression of vimentin. Furthermore, vimentin expression might be a potential biomarker for the progression of Jak2-V617F-mediated pathogenesis and for disease regression via Jak2 inhibitory therapy.

In summary, our data show that G6-induced inhibition of Jak2-mediated pathogenic cell growth correlates with decreased expression of vimentin *in vitro* and *in vivo*. As such, this work describes a novel mechanistic pathway for the targeting of Jak2-mediated pathological cell growth.

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ABBREVIATIONS

Jak2, Janus kinase 2; GPCR, G protein-coupled receptor; STAT, signal transducers and activators of transcription; BCR, B-cell receptor; MPN, myeloproliferative neoplasms; G1/S phase, Gap1/synthesis phase; HEL, human erythroleukemia; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; FITC, fluorescein isothiocyanate; DAPI, 4',6-diamidino-2-phenylindole; IDPN, β,β' -iminodipropionitrile; EMT, epithelial–mesenchymal transition.

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