Multiple Cysteine Residues Are Implicated in Janus Kinase 2-Mediated Catalysis†

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ABSTRACT: The redox regulation of Janus kinase 2 (JAK2) is poorly understood, and there are contradictory reports as to whether the enzyme's activity is inhibited or stimulated by oxidizing conditions in the cell. Here we demonstrate that multiple cysteine residues within the JAK2 catalytic domain may be crucial for enzymatic activity. The enzyme is catalytically inactive when oxidized; activity can be restored via reduction to the thiol state. A series of recombinant variants of JAK2 were overproduced using the baculoviral expression vector system. A truncated variant of JAK2, GST/(NΔ661)rJAK2, provided evidence that the amino-terminal autoinhibitory domain was not essential for direct redox regulation and that only nine cysteine residues were potentially involved. The effect of individually and combinatorially altering these nine cysteines was examined via cysteine-to-serine mutagenesis. This identified four cysteine residues in the catalytic domain (Cys866, Cys917, Cys1094, and Cys1105) that cooperatively maintain JAK2's catalytic competency. Our data are consistent with a direct mechanism for redox regulation of JAK2 via oxidation and reduction of critical cysteine residues.

Molecular redox (reduction/oxidation) reactions are important mechanisms for the modulation of intracellular signal transduction pathways. Oxidants such as hydrogen peroxide and nitric oxide are important second messengers in the immune system (*1*, *2*). Severe oxidative stress, however, has been associated with T cell hyporesponsiveness and immunosuppression in various pathological conditions (*3*−*5*), reflecting the complex outcomes of a deceptively simple chemical phenomenon.

The Janus protein-tyrosine kinases (JAKs)[1] are crucial signal transducers in numerous autocrine, paracrine, and endocrine pathways (*6*, *7*). However, the redox regulation of Janus protein-tyrosine kinases is poorly understood. The primary point of confusion stems from seemingly contradictory evidence as to whether JAK's activity is inhibited or stimulated by oxidizing environments. One body of evidence suggests that JAKs are activated via reduction and inactivated via oxidation. For example, pretreatment of JAK2 and JAK3 with low molecular weight oxidants (nitric oxide or *o*-iodosobenzoate) inhibited the in vitro radiolabeling autokinase activities of these enzymes, and this inhibition was reversible by retreatment with the low molecular weight reductant dithiothreitol (DTT). In that study (*8*), preexposure of intact cells to nitric oxide prior to cytokine stimulation resulted in the loss of cytokine-induced JAK activation, which correlated with the loss of cytokine-induced proliferation. Other investigators observed that the production of physiologically relevant levels of nitric oxide by macrophages or by myeloid suppressor cells inhibited the activities of JAKs in neighboring T cells (*9*, *10*). The inhibition of JAK activity in an oxidizing environment would provide a simple explanation for the reported immunosuppressive effects of high levels of reactive oxygen species and of nitric oxide (*11*−*15*), since impaired JAK activity should impair the efficiency of cytokine-initiated signal transduction. Conversely, the reductive enhancement of JAKs would partially explain the requirement for intracellular thiol reducing agents, such as thioredoxin, in promoting T cell and NK cell proliferation (*16*−*18*), since highly efficient cytokine-initiated signal transduction would be expected under conditions of full JAK activity. This response to redox regulation would also be consistent with a variety of other observations, ranging from the blockade of JAK/STAT signal transduction in oxidatively stressed neuronal cells to the unexpected observation that thioredoxin was essential for interferon-γ-mediated growth arrest in HeLa cells (*19*−*21*).

However, the model of oxidative inhibition and reductive enhancement of JAK activity appears to be at odds with another substantial body of data suggesting that mediators of oxidative stress, most notably hydrogen peroxide, stimulate JAK activity or activate the JAK/STAT pathway (*22*−*27*). It is conceivable that the apparent JAK stimulation is indirect. The oxidative inhibition of protein tyrosine phosphatases (*28*, *29*) can dramatically increase the phosphotyrosine content of JAK1, JAK2, and TYK2 (*30*, *31*). Alternatively, the indirect activation of JAKs by hydrogen peroxide might involve the intervention of a transphosphorylating protein tyrosine kinase such as FYN (*27*).

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[1] Abbreviations: JAK, Janus kinase; *o*-IBZ, *o*-iodosobenzoate; DTT, dithiothreitol; STAT, signal transducers and activators of transcription; GST, glutathione S-transferase; Sf21, *Spodoptera frugiperda* cell line 21; rJAK2, rat JAK2; MOI, multiplicity of infection; Trx1, thioredoxin.

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Whether the mechanism is direct or indirect, a clear mechanistic characterization of the redox regulation of JAKs is sorely needed. Because cysteine residues are chemically versatile and known to serve as reversible covalent regulatory switches (*32*, *33*), we examined whether specific cysteine residues within JAK2 are critical to the enzyme's activity. If so, these critical cysteines might provide a redox sensor through which enzymatic activity could be directly modulated.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Baculoviruses. The recombinant baculoviruses producing the hyperactive (NΔ661)-rJAK2 enzyme (*34*), the GST/rJAK2 chimeric enzyme (*35*), the hyperactive GST/(NΔ661)rJAK2 enzyme, and the inactive GST/(NΔ661)rJAK2(K882E) and GST/rJAK2(K882E) enzymes (*36*) have been previously described. Each of the nine cysteine residues in the GST/(NΔ661)rJAK2 protein was individually converted to a serine residue via site-directed mutagenesis of the pAcGHLT-A:(NΔ661)rJAK2 transfer vector using the QuikChange site-directed mutagenesis kit (Stratagene). Following DNA sequence analysis to verify the mutations, these transfer vectors were used to generate recombinant baculoviruses as before (*36*). The primer sets used for mutagenesis were 5′-CCCTTATTCATGGGAATGTGAGTGCCAAAAATATCCTGC-3′ and 5′-GCAGGATATTTTTGGCACTCACATTCCCATGAATAAGGG3′ for the GST/(NΔ661)rJAK2(C675S) mutant, 5′-CCATGGGTACCACCTGAGAGCATTGAAAACCCTAAAAATC-3′ and 5′-GATTTTTAGGGTTTTCAATGCTCTCAGGTGGTACCCATGG-3′ for the GST/(NΔ661)-rJAK2(C723S) mutant, 5′-CTCTGTGGGAGATCAGCAGTGGAGGAGAC-3′ and 5′-GTCTCCTCCACTGCTGATCTCCCACAGAG-3′ for the GST/(NΔ661)rJAK2(C748S) mutant, 5′-GGCAAACCTTATAAATACTAGCATGGATTATGAGCCAGACTTCAGGC-3′ and 5′-GCCTGAAGTCTGGCTCATAATCCATGCTAGTATTTATAAGGTTTGCC-3′ for the GST/(NΔ661)rJAK2(C787S) mutant, 5′-GGAGTGTGGAGATGAGCCGCTATGACCCG-3′ and 5′-CGGGTCATAGCGGCTCATCTCCACACTCC-3′ for the GST/(NΔ661)-rJAK2(C866S) mutant, 5′-GAAGTACAAGGGAGTGAGCTACAGTGCTGGTCG-3′ and 5′-CGACCAGCACTGTAGCTCACTCCCTTGTACTTC-3′ for the GST/(NΔ661)-rJAK2(C917S) mutant, 5′-CAGTATACATCCCAGATAAGCAAGGGCATGGAGTATC-3′ and 5′-GATACTCCATGCCCTTGCTTATCTGGGATGTATACTG-3′ for the GST/(NΔ661)rJAK2(C961S) mutant, 5′-CTGCCGAGACCAGAAGGGAGCCCAGACGAGATTTATG-3′ and 5′-CATAAATCTCGTCTGGGCTCCCTTCTGGTCTCGGCAG-3′ for the GST/(NΔ661)rJAK2(C1094S) mutant, and 5′-GTGATCATGACAGAAAGCTGGAACAACAATGTCAACCAACGTCCC-3′ and 5′-GGGACGTTGGTTGACATTGTTGTTCCAGCTTTCTGTCATGATCAC-3′ for the GST/(NΔ661)rJAK2(C1105S) mutant.

Cumulative mutations were introduced into a baculoviral transfer vector until all targeted cysteine codons were converted to serine codons. Following sequence verification, recombinant baculoviruses were generated, isolated, and amplified, and the infectious titers were ascertained essentially as previously described (*34*). Because the full nomenclature for the cumulative Cys-to-Ser mutants is cumbersome, an informal nomenclature will be used in this paper, as is defined in Table 1.

Table 1: GST/(NΔ661)rJAK2(Cys-to-Ser) Mutants Used in This Study

Nomenclature	Point Mutations	Schematic
GST/(N661)rJAK2	none	
GST/(N661)rJAK2(K882E)	K882E	
GST/(N661)rJAK2(C675S)	C675S	
GST/(N661)rJAK2(C723S)	C723S	
GST/(N 661)rJAK2(C748S)	C748S	
GST/(N661)rJAK2(C787S)	C787S	
GST/(N661)rJAK2(C866S)	C866S	
GST/(N661)rJAK2(C917S)	C917S	
GST/(N661)rJAK2(C961S)	C961S	
GST/(N661)rJAK2(C1094S)	C1094S	
GST/(N661)rJAK2(C1105S)	C1105S	
GST/(N661)rJAK2(4C:4S)	C866S, C917S, C1094S, C1105S	
GST/(N661)rJAK2(5C:5S)	C675S, C723S, C748S, C787S, C961S	
GST/(N661)rJAK2(9C:9S)	C675S, C723S, C748S, C787S, C866S, C917S, C961S, C1094S, C1105S	

Production of Recombinant JAK2 Proteins. The above recombinant baculoviruses were used singly or in combination to infect Sf21 insect cells at a multiplicity of infection (MOI) of 10; infected Sf21 cells were harvested via centrifugation 72 h postinfection and stored at −80 °C until lysis. Clarified lysate was prepared from 1×10^7 infected Sf21 cells resuspended with 1 mL of insect cell lysis buffer containing 1% Triton X-100, 130 mM NaCl, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium phosphate, 10 mM Tris−Cl, pH 8.0, and protease inhibitors (16 μg/mL benzamidine hydrochloride, 10 μg/mL phenanthroline, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, and 1 mM PMSF) (*35*). rJAK2 variants were recovered from the clarified cell lysates as described below.

Immunoprecipitation and Redox Treatments. Cell lysates were centrifuged at 10000*g* for 15 min; the insoluble fraction was removed, and anti-JAK2 antiserum (Upstate Biotechnology item no. 06-255) was added to the supernatant. After 2 h, protein A Sepharose CL-4B (GE Healthcare) was added and the resulting solution mixed for an additional 2 h. The immobilized JAK2/antibody/protein A Sepharose complexes were pelleted by centrifugation (3000*g* for 5 min at 4 °C) and washed three times with insect cell lysis buffer. In some experiments, the immobilized JAK2/antibody complexes were incubated with 10 mM DTT or 2.5 mM *o*-iodosobenzoate (*o*-IBZ) for 1 h at 4 °C; the samples were then centrifuged and the supernatant discarded. In the redox reversibility experiments, half of the subdivided DTT-

pretreated samples were washed in insect cell lysis buffer and retreated with 2.5 mM *o*-IBZ for 1 h at 4 °C. Likewise, half of the subdivided *o*-IBZ-pretreated samples were washed and retreated with 10 mM DTT for 1 h at 4 °C. In most experiments, the samples were then split into two equal aliquots; one aliquot was assayed by Western blot, and the other aliquot was subjected to the in vitro radiolabeling autokinase assay as described below.

Western Blotting. Sample aliquots were boiled with 30 μL of SDS−PAGE sample buffer for 3 min, resolved by 7.5% SDS−PAGE (1×10^6 cells/lane), and transferred to Immobilon-P transfer membranes (Millipore). Membranes were probed with anti-JAK2 (Upstate Biotechnology item no. 06-255), washed three times, probed with peroxidase-conjugated secondary antibodies (KPL item no. 074-1516), and washed three times again, and the signal was visualized using an enhanced chemiluminescence (ECL) system (GE Healthcare). The relative intensity of the anti-JAK2 signal was estimated from digital scans of the film using ImageQuant 5.2 software (Molecular Dynamics/GE Healthcare). In some experiments the membrane was then "stripped" via 30 min of incubation in 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris−HCl (pH 6.7) at 50 °C. The membrane was then reprobed with anti-phosphotyrosine antibody (4G10, Upstate Biotechnology item no. 05-321), washed three times, probed with peroxidase-conjugated secondary antibodies (KPL item no. 074-1806), and washed three times again and the signal visualized using ECL. In some experiments the membranes were stripped a second time and reprobed with anti-phosphoJAK2 antibody (Upstate Biotechnology item no. 07-606) to demonstrate the extent of phosphorylation of the activation loop tyrosines Tyr1007/1008.

In Vitro Radiolabeling Autokinase Assays. Sample aliquots were centrifuged, the supernatants were discarded, and the pellets were resuspended with 100 μL of radioactive kinase assay cocktail. The cocktail contained 250 μCi/mL [γ-^{32}P]-ATP, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 50 mM NaCl, 100 μM Na_2VO_3, and 10 mM HEPES (pH 7.6). The samples were incubated for 20 min at room temperature with mixing. The reactions were stopped by centrifuging briefly, discarding the kinase cocktail, and washing the pellets three times with insect cell lysis buffer. The pellets were boiled with 30 μL of SDS−PAGE sample buffer for 3 min and resolved by 7.5% SDS−PAGE (1×10^6 cells/lane). The gel was transblotted onto a PVDF membrane, and the relative incorporation of radioactivity was quantified via PhosphoImager analysis. For most experiments, the intensity of each of the autokinase signals was calculated as a percentage of the GST/(NΔ661)rJAK2 autokinase signal and then normalized for the anti-JAK2 signal intensity relative to that of GST/(NΔ661)rJAK2.

Production of Recombinant Thioredoxin Variants. Recombinant redox-active thioredoxin (Trx1) and redox-inactive mutant thioredoxin (C32S/C35S; mTrx1) were produced and purified essentially as described by Oblong and coworkers (*37*). The cDNA source for human thioredoxin was kindly provided by Dr. Garth Powis. The minor modifications involved the substitution of a QuikChange site-directed mutagenesis kit (Stratagene), *Escherichia coli* BL21-Gold (DE3) cells (Stratagene), a DEAE−Sepharose fast flow ion exchange column (GE Healthcare), and a Hi Prep 16/60 Sephacryl S-100 HR gel filtration column (GE Healthcare)

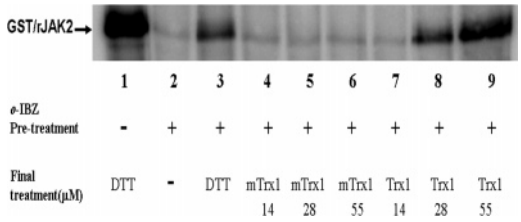

FIGURE 1: Purified recombinant wild-type Trx1, but not redox-inactive mTrx1, can restore in vitro radiolabeling autokinase activity to oxidatively inactivated GST/rJAK2. Sf21 cells were infected with recombinant baculovirus producing GST/rJAK2. Samples in lanes 2−9 were pretreated for 1 h with 2.5 mM *o*-IBZ before retreatment with the indicated reagents and then assayed for autokinase activity. Key: lane 1, 10 mM DTT only (no retreatment); lane 2, *o*-IBZ pretreatment only (no retreatment); lane 3, 10 mM DTT; lane 4, 14 μM mTrx1; lane 5, 28 μM mTrx1; lane 6, 55 μM mTrx1; lane 7, 14 μM Trx1; lane 8, 28 μM Trx1; lane 9, 55 μM Trx1. The experiment represented here was performed three times.

for the comparable materials described by Oblong and coworkers (*37*) and the use of 200 μg/mL rifampicin to inhibit bacterial RNA polymerase following the isopropyl 1-thio-β-D-galactopyranoside induction step. Purified recombinant Trx1 and mTrx1 proteins were analyzed via Western immunoblots using anti-human thioredoxin antibody (Serotec item no. MCA1538). A turbidimetric insulin disulfide reduction assay (*38*) was performed to confirm that Trx1 was enzymatically active while mTrx1 was not. All spectrophotometric measurements were carried out at room temperature using a Cary 100 Bio spectrophotometer (Varian). Because the purified Trx1 variants were stored in the presence of 1 mM DTT, immediately prior to use, DTT was removed with G-25 spin columns and the proteins were eluted in nitrogen-sparged 0.1 M phosphate buffer (pH 7.0).

RESULTS

In an earlier report, we showed that the autokinase activity of JAK2 was reversibly modulated by DTT, a low molecular weight redox reagent, which reduces disulfides to thiols (*8*). However, no further molecular details were proposed. No evidence was provided as to whether physiologically relevant thiol reducing proteins, such as thioredoxin (Trx1), could modulate the redox state of JAK2. Thioredoxin is the central mediator of a thiol oxidoreductase system crucial for the control of intracellular redox status. This 12 kDa protein has two active site cysteines, Cys32 and Cys35, which are crucial for its ability to reduce disulfide bonds in various target proteins. Redox-inactive thioredoxin can be engineered by converting these cysteines to serines via site-directed mutagenesis, and both proteins can be overproduced in *E. coli* and purified to homogeneity (*33*).

In this study, overproduced GST/rJAK2, a glutathione S-transferase-tagged, full-length form of rat JAK2, was recovered from baculovirus-infected Sf21 cell lysates. Prior to conduction of a radiolabeling autokinase assay, one sample was reduced with 10 mM DTT (Figure 1, lane 1). The remaining samples were oxidized by pretreatment with *o*-IBZ, which oxidizes neighboring thiol pairs to disulfides (Figure 1, lanes 2−9). The oxidized enzyme was then retreated with 13.7, 27.5, or 55 μM redox-inactive Trx1 (Figure 1, lanes 4, 5, and 6, respectively), which did not perceptibly increase the autokinase activity of GST/rJAK2. In contrast, when the oxidized GST/rJAK2 was retreated with

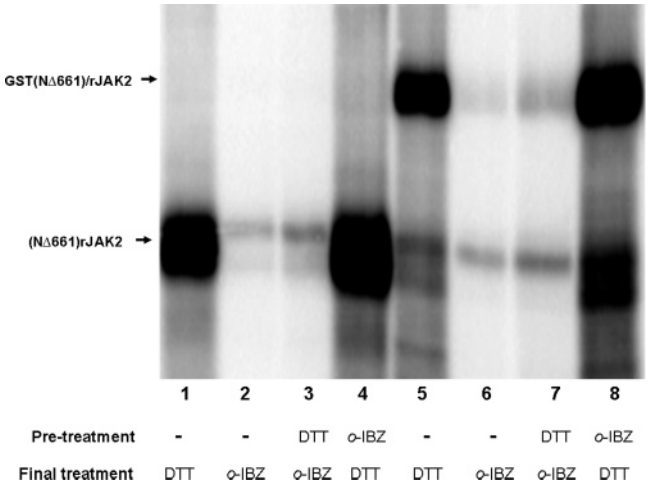

FIGURE 2: *o*-IBZ inhibits the autokinase activity of (NΔ661)rJAK2 and GST(NΔ661)/rJAK2 in a DTT-reversible fashion. Sf21 cells were infected with recombinant baculovirus producing either (NΔ661)rJAK2 (lanes 1−4, lower arrow) or GST(NΔ661)/rJAK2 (lanes 5−8, upper arrow). Following JAK2 immunoprecipitation, half of the enzymes were set aside for control anti-JAK2 Western blots and the other half were processed for in vitro radiolabeling autokinase assay. The aliquots for autokinase assay were divided into two equal aliquots. One aliquot was pretreated with 10 mM DTT (lanes 1, 3, 5, and 7); half of this was retreated with 2.5 mM *o*-IBZ (lanes 3 and 7). The second aliquot was pretreated with 2.5 mM *o*-IBZ (lanes 2, 4, 6, and 8), and half of this was retreated with 10 mM DTT (lanes 4 and 8). Following a brief wash to remove redox reagents, the samples were assayed for radiolabeling in vitro autokinase activity. This experiment was performed three times.

13.7, 27.5, or 55 μM functional Trx1 (Figure 1, lanes 7, 8, and 9, respectively), its autokinase activity was dramatically enhanced. Interestingly, low concentrations (27.5 and 55 μM) of Trx1 were at least as effective at restoring autokinase activity as higher (10 mM) concentrations of DTT (compare Figure 1, lane 3 versus lanes 8 and 9). It is therefore plausible that the regulation of JAK2 may be physiologically controlled by the thioredoxin system.

To elucidate the molecular details of redox regulation of JAK2 catalysis, we first considered the covalent stabilization of the enzyme in an autoinhibited state. Several lines of evidence show that an extracatalytic region in JAK2 behaves as an autoinhibitory domain. For example, deletion or mutation of the amino-terminal domain converts the enzyme into a hyperactive form (*34*−*36*, *39*), which suggests that such forms lack a functional autoinhibitory domain. Therefore, if the oxidative inhibition of JAKs occurred through the covalent stabilization of the autoinhibitory domain which would "lock" the enzyme in an inactive state, then the hyperactive forms of the enzyme, (NΔ661)rJAK2 (*34*) and GST/(NΔ661)rJAK2 (*36*), should be refractive to oxidative inhibition by thiol-selective reagents.

To examine this possibility, (NΔ661)rJAK2 and GST/(NΔ661)rJAK2 enzymes were recovered from baculovirus-infected Sf21 cell lysates. The immunoprecipitated enzymes were pretreated with either 2.5 mM *o*-IBZ or 10 mM DTT. Additionally, *o*-IBZ-pretreated enzymes were retreated with DTT, and DTT-pretreated enzymes were retreated with *o*-IBZ. As shown in Figure 2, pretreatment or retreatment with *o*-IBZ essentially abolished the autokinase activity in both enzyme forms (lanes 2, 3, 6, and 7), while pretreatment or retreatment with DTT greatly enhanced the autokinase activity of both enzyme forms (lanes 1, 4, 5, and 8). These experiments demonstrate that the mechanism of redox regulation is independent of the autoinhibitory domain. These experiments also show that the presence of the GST domain does not interfere with the biochemically reversible redox regulation of JAK2 autokinase activity. (NΔ661)rJAK2 comigrates with the rabbit IgG heavy chain under SDS−PAGE conditions, and the amount of JAK2-immunoreactive protein is therefore obscured in subsequent Western immunoblot analyses (*34*). The use of the chimeric GST/(NΔ661)-rJAK2 form provides a technical advantage in that it can be clearly resolved from the antibodies employed as immunoprecipitants.

One can propose that the fully active forms of JAK2 contain critical cysteine residues which must be present as thiols to maintain JAK2's active state. Upon oxidation of these residues to the disulfide state, JAK2 is no longer catalytically active, although activity can be restored via reduction of the disulfide bond. To propose a more detailed regulatory mechanism, it is necessary to identify which cysteine residues are critical for redox regulation. Of the 1132 amino acids in the rat JAK2 protein, 27 are cysteines. These 27 cysteine residues can theoretically form up to 351 unique disulfide bond combinations. The observation that the (NΔ661)rJAK2 and GST/(NΔ661)rJAK2 isoforms are reversibly regulated by thiol redox reagents fortunately restricts the critical cysteine residues to only nine candidates. The theoretical number of unique disulfide combinations therefore drops to 36. However, it is still not clear whether only one unique dithiol/disulfide interconversion accounts for redox regulation, whether multiple unique dithiol/disulfide interconversions are simultaneously involved, or whether redox regulation can be conferred by degenerate redox conformations. It is also possible that the critical cysteines may be converted to sulfenic acids upon oxidation. To clarify this confusion, a series of single-point mutations were created so that each of the cysteine residues in the GST/(NΔ661)-rJAK2 protein would be conservatively changed to a serine residue. Serine residues should retain the approximate size, geometry, and polarity of the cysteine residues but would be incapable of forming disulfide bonds.

Nine recombinant baculoviruses were created to express individual cysteine-to-serine mutant variants of GST/(NΔ661)-rJAK2. The recombinant proteins were recovered from infected cell lysates by immunoprecipitation with polyclonal antibodies which recognized JAK2, then pretreated with DTT, and divided into two equal aliquots. One aliquot was assayed for in vitro autokinase activity, and the other aliquot was assayed for relative JAK2 abundance via Western immunoblot. As shown in Figure 3, five of the cysteine-to-serine mutants, GST/(NΔ661)rJAK2(C675S), GST/(NΔ661)-rJAK2(C723S), GST/(NΔ661)rJAK2(C748S), GST/(NΔ661)-rJAK2(C787S), and GST/(NΔ661)rJAK2(C961S), exhibited no significant impairment of autokinase activity as compared to the nonmutated GST/(NΔ661)rJAK2 control. In contrast, four of the mutants, GST/(NΔ661)rJAK2(C866S), GST/(NΔ661)rJAK2(C917S), GST/(NΔ661)rJAK2(C1094S), and GST/(NΔ661)rJAK2(C1105S), consistently exhibited significantly lower autokinase activity than GST/(NΔ661)-rJAK2. Of the four mutations, conversion of Cys866 to serine resulted in the lowest level of autokinase activity. The GST/(NΔ661)rJAK2(C866S) variant had 14.7% (±7.5% standard error (SE), $n = 10$) of GST/(NΔ661)rJAK2's autokinase

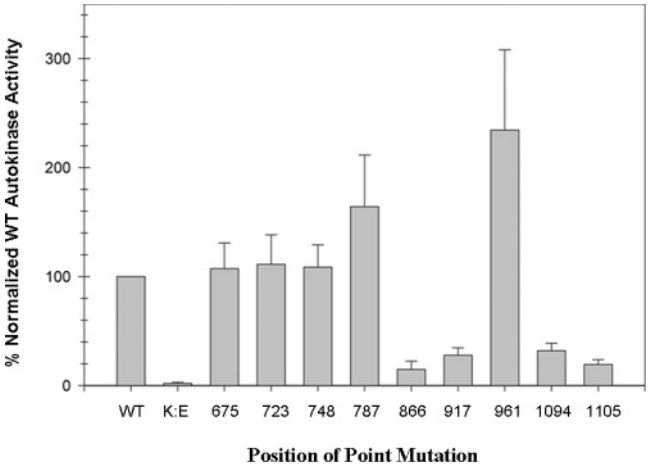

FIGURE 3: In vitro autokinase activity of nine cysteine-to-serine mutants expressed as a normalized percentage of GST/(NΔ661)-rJAK2 activity. A total of 2×10^6 Sf21 cells were infected separately with baculoviruses expressing recombinant GST/(NΔ661)-rJAK2 enzyme variants. Each JAK2 immunoprecipitated sample was divided into two aliquots. One aliquot was analyzed via anti-JAK2 immunoblot. The other aliquot was pretreated with 10 mM DTT and then assayed for radiolabeling autokinase activity. The intensity of each of the autokinase signals was calculated as a percentage of the GST/(NΔ661)rJAK2 autokinase signal and then normalized for the anti-JAK2 signal intensity relative to that of GST/(NΔ661)rJAK2. GST/(NΔ661)rJAK2 is indicated as "WT" on this graph. The bars represent the mean value from at least four measurements ± the standard error of the mean. The *n* values for WT, K:E, 675, 723, 748, 787, 866, 917, 961, 1094, and 1105 are 12, 10, 10, 4, 10, 9, 10, 9, 4, 8, and 7, respectively.

activity. The activities of GST/(NΔ661)rJAK2(C917S), GST/(NΔ661)rJAK2(C1094S), and GST/(NΔ661)rJAK2(C1105S) were 27.8% (±6.7% SE, $n = 9$), 31.9% (±6.9% SE, $n = 8$), and 19.3% (±4.4% SE, $n = 7$) relative to that of GST/(NΔ661)rJAK2, respectively. While the autokinase activities of these four enzyme forms are significantly lower than that of GST/(NΔ661)rJAK2, they are clearly not inactive as indicated by the significant increase in activity they have over the kinase-inactive variant GST/(NΔ661)rJAK2(K882E) (2.0% ± 1.0% SE, $n = 10$).

The observation that no single cysteine-to-serine mutation caused a complete loss of activity suggested that multiple cysteine residues may be involved in a complex redox regulatory mechanism. If so, then a combination of cysteine-to-serine mutations might completely obliterate enzyme activity. When all nine cysteines were converted to serines (designated GST/(NΔ661)rJAK2(9C:9S)), the autokinase activity of the recombinant enzyme was nearly undetectable under in vitro radiolabeling autokinase assay conditions (data not shown). Two additional baculoviruses were engineered to investigate whether specific cysteine residues might be involved in redox-reversible catalysis, whereas others might be uninvolved. GST/(NΔ661)rJAK2(4C:4S) contained serine substitutions for the four cysteine residues (Cys866, Cys917, Cys1094, and Cys1105) which individually impaired the in vitro radiolabeling autokinase activity of the enzyme. GST/(NΔ661)rJAK2(5C:5S) contained serine substitutions for the five cysteine residues (Cys675, Cys723, Cys748, Cys787, and Cys961) which caused no significant impairment of the enzyme's in vitro radiolabeling autokinase activity when introduced individually. The in situ autophosphorylation and redox-reversible in vitro radiolabeling autokinase activities of GST/(NΔ661)rJAK2(4C:4S) and GST/(NΔ661)rJAK2-(5C:5S) were compared to those of the active and inactive enzyme forms (Figure 4). GST/(NΔ661)rJAK2, GST/(NΔ661)rJAK2(K882E), GST/(NΔ661)rJAK2(4C:4S), and GST/(NΔ661)rJAK2(5C:5S) were immunoprecipitated and divided into equal aliquots. Half of the immobilized enzymes were pretreated with 2.5 mM *o*-IBZ and the other half with 10 mM DTT, and then the aliquots were again subdivided. Half of the DTT-pretreated aliquots were retreated with *o*-IBZ, and conversely, half of the *o*-IBZ-pretreated aliquots were retreated with DTT. All aliquots were then divided in half a final time before the assays were conducted, the results of which are shown in Figure 4.

The anti-JAK2 Western immunoblot assay verified that all proteins were present in comparable amounts and that no gross inequities were introduced by the repetitive subdivision process (Figure 4B). The anti-phosphotyrosine Western immunoblot was used to gauge the extent of in situ autophosphorylation (Figure 4C). The anti-phosphoJAK2 Western immunoblot was used to demonstrate the level of phosphorylation of the activation loop tyrosines Tyr1007/1008 (Figure 4D). GST/(NΔ661)rJAK2(K882E) contained no detectable phosphotyrosine and appears to be completely

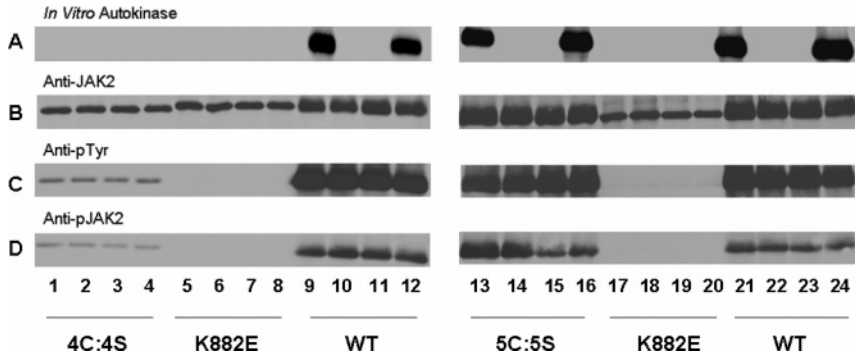

FIGURE 4: Redox-reversible in vitro radiolabeling autokinase activity of the GST/(NΔ661)rJAK2 mutants containing multiple cysteine-to-serine substitutions. Sf21 cells were infected with recombinant baculovirus producing GST/(NΔ661)rJAK2(4C:4S) (lanes 1−4), GST/(NΔ661)rJAK2(5C:5S) (lanes 13−16), GST/(NΔ661)rJAK2(K882E) (lanes 5−8 and 17−20), and GST/(NΔ661)rJAK2 (lanes 9−12 and 21−24). One aliquot of immunoprecipitated enzyme was used for in vitro autokinase analysis (panel A), and the other was analyzed for JAK2, phosphotyrosine, and phospho-JAK2 content via Western immunoblots (panels B, C, and D, respectively). Immunocomplexes were treated with 10 mM DTT (lanes 1, 5, 9, 13, 17, and 21), pretreated with 10 mM DTT and retreated with 2.5 mM *o*-IBZ (lanes 2, 6, 10, 14, 18, and 22), treated with 2.5 mM *o*-IBZ (lanes 3, 7, 11, 15, 19, and 23), or pretreated with 2.5 mM *o*-IBZ and retreated with 10 mM DTT (lanes 4, 8, 12, 16, 20, and 24). This experiment was performed twice.

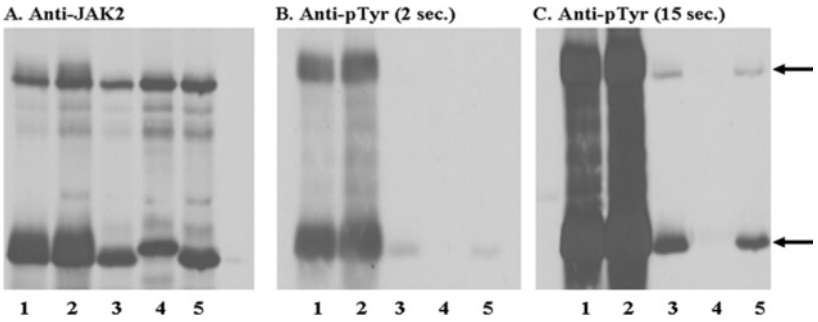

FIGURE 5: In situ exokinase activities of GST/(NΔ661)rJAK2 mutants containing multiple cysteine-to-serine substitutions. Sf21 cells were coinfected with GST/rJAK2(K882E) and GST/(NΔ661)rJAK2 (lane 1), GST/(NΔ661)rJAK2(5C:5S) (lane 2), GST/(NΔ661)rJAK2(4C:4S) (lane 3), GST/(NΔ661)rJAK2(K882E) (lane 4), or GST/(NΔ661)rJAK2(9C:9S) (lane 5). Immunoprecipitated proteins were analyzed via Western immunoblot with anti-JAK2 (panel A), and then the PVDF membranes were "stripped" and reprobed with anti-phosphotyrosine antibodies (panels B and C). Short (2 s, panel B) and long (15 s, panel C) exposures were obtained for clarity. The upper arrow indicates GST/rJAK2(K882E), and the lower arrow indicates the GST/(NΔ661)rJAK2 forms. This experiment was performed three times.

inactive in situ (Figure 4C,D, lanes 5−8 and 17−20). In contrast, GST/(NΔ661)rJAK2 contains a significant amount of phosphotyrosine and is capable of robust autophosphorylation under in situ conditions (Figure 4C,D, lanes 9−12 and 21−24). The form which retained only the critical cysteine quartet of Cys866, Cys917, Cys1094, and Cys1105 (i.e., GST/(NΔ661)rJAK2(5C:5S)) contains approximately the same level of phosphotyrosine as does GST/(NΔ661)-rJAK2 (compare Figure 4C,D, lanes 13−16 versus lanes 21−24). However, when these four cysteines are converted to serines and the other five noncritical cysteines (Cys675, Cys723, Cys748, Cys787, and Cys961) are retained, the phosphotyrosine content dramatically diminishes (compare Figure 4C,D, lanes 1−4 versus lanes 9−12). While the total phosphotyrosine content of GST/(NΔ661)rJAK2(4C:4S) is very low, it is clearly higher than that of the inactive GST/(NΔ661)rJAK2(K882E) (compare Figure 4C, lanes 1−4 versus lanes 5−8). It is also noteworthy that phosphorylation of the activation loop tyrosines of GST/(NΔ661)rJAK2(4C:4S) was clearly detectable, because these particular sites of tyrosine phosphorylation are important for the attainment of a high activity state (*36*). No such tyrosine phosphorylation within the activation loop of the inactive GST/(NΔ661)-rJAK2(K882E) could be detected (see Figure 4D, lanes 5−8 and 17−20).

The results of the in vitro radiolabeling autokinase assay implied that the four critical cysteine residues might be involved in the redox sensitivity of the enzyme. The redox-reversible response of GST/(NΔ661)rJAK2(5C:5S) was indistinguishable from that of GST/(NΔ661)rJAK2 (Figure 4A, lanes 13−16 versus lanes 21−24). The redox reversibility of GST/(NΔ661)rJAK2(4C:4S)'s autokinase activity could not be assessed because insufficient activity remains once all four of these cysteines have been converted to serines (Figure 4A, lanes 1−4). These data strongly support the notion that Cys866, Cys917, Cys1094, and Cys1105 are important not only for catalytic competence per se, but also for the redox sensitivity of catalysis.

The in vitro radiolabeling autokinase activities of GST/(NΔ661)rJAK2(9C:9S) and GST/(NΔ661)rJAK2(4C:4S) were so low that we had to reexamine whether they indeed retained catalytic activity under cellular conditions. We used the in situ transphosphorylation assay (*38*) to coexpress two recombinant proteins in Sf21 cells such that the inactive 140 kDa GST/rJAK2(K882E) served as the phosphorylation

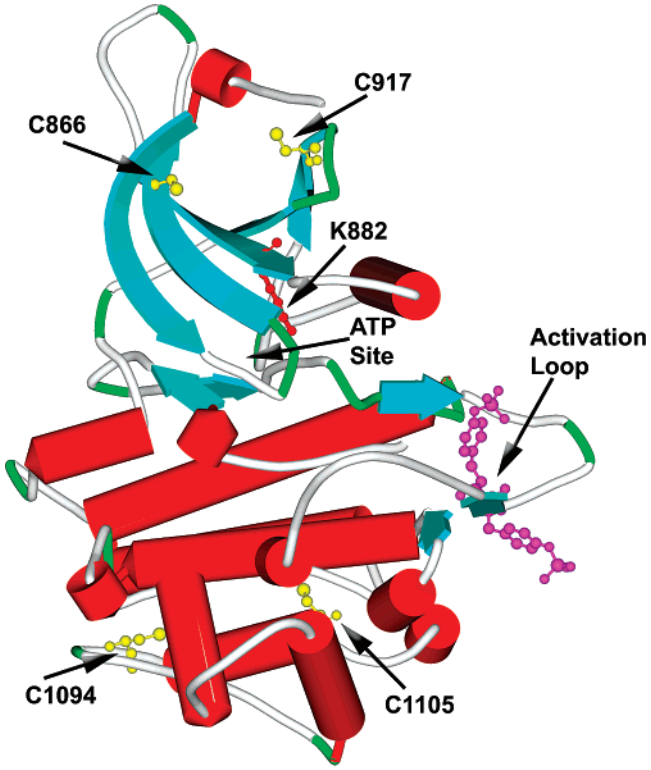

FIGURE 6: Location of critical cysteine residues in the JAK2 catalytic domain. Arrows indicate the positions of the critical cysteines (C866, C917, C1094, and C1105) in the three-dimensional structure of the JAK2 catalytic domain (*40*). Arrows also show the locations of the invariant lysine (K882), the activation loop in an "open conformation" with phosphorylated tandem tyrosines (pY1007 and pY1008), and the approximate location of the ATP binding site (buried deep between the two lobes).

substrate for 84 kDa GST/(NΔ661)rJAK2 variants. As shown in Figure 5A, comparable amounts of protein were present in each experiment. Coinfection of GST/rJAK2(K882E) with GST/(NΔ661)rJAK2 resulted not only in autophosphorylation of the shorter active variant but also in transphosphorylation of the longer inactive variant (Figure 5B,C, lane 1). The conversion of noncritical cysteines to serines did not perceptibly impair the in situ autokinase or exokinase activities of the enzyme, as demonstrated by the nearly identical assay results for GST/(NΔ661)rJAK2(5C:5S) and GST/(NΔ661)rJAK2 (Figure 5B,C, lane 2 versus lane 1, respectively). The simultaneous production of catalytically inactive GST/rJAK2(K882E) and GST/(NΔ661)rJAK2-

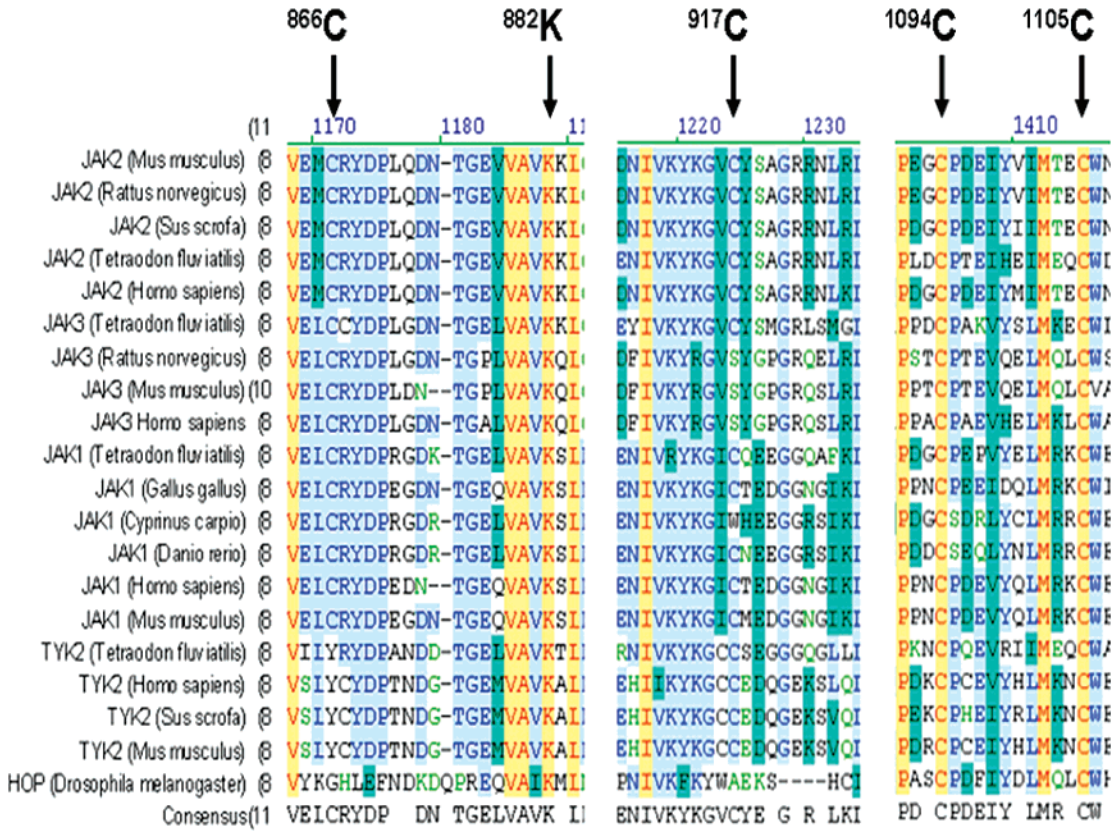

FIGURE 7: Conservation of 4 critical cysteines in 20 Janus kinase amino acid sequences. The predicted amino acid sequences of 20 Janus kinases in the NCBI Entrez Protein Sequence database were aligned using Vector NTI 6.0 AlignX software. Arrows indicate the location of rat JAK2's Cys866, Lys882, Cys917, Cys1094, and Cys1105. Note that Lys882, Cys1094, and Cys1105 are invariant.

(K882E) resulted in the phosphorylation of neither variant, as expected (Figure 5B,C, lane 4). In contrast, the GST/(NΔ661)rJAK2(4C:4S) and GST/(NΔ661)rJAK2(9C:9S) mutants themselves contained very low levels of phosphotyrosine, and even lower levels of phosphorylated tyrosine residues were detected in their substrates (Figure 5B,C, lane 3 versus lane 5, respectively). It should be emphasized that the in situ autokinase and exokinase activities of the GST/(NΔ661)rJAK2(9C:9S) and GST/(NΔ661)rJAK2(4C:4S) mutants are nearly indistinguishable from each other, but dramatically lower than those of GST/(NΔ661)rJAK2(5C:5S) and GST/(NΔ661)rJAK2.

DISCUSSION

The data shown in this paper provide novel evidence for a critical role of cysteine residues in JAK2-mediated catalysis, and we suggest that this role is intimately involved with the redox regulation of JAK2 activity. It is important to remember that the bulk of the evidence provided was derived from truncated GST/(NΔ661)rJAK2 variants overexpressed in Sf21 insect cells. While it is tempting to presume that our observations will apply to the full-length enzyme which contains additional autoinhibitory and receptor-association domains, such presumptions must be validated by additional experimental evidence. Nine cysteine residues within GST/(NΔ661)rJAK2 were evaluated for their role in the redox regulation of the catalytic activity of JAK2. Mutations of four cysteines were found to significantly impair enzyme activity. While we do not yet know the specific mechanistic role(s) of these residues, we have been able to exclude two possible regulatory mechanisms from further consideration. First, it is improbable that the oxidative inhibition of JAK2 arises from a disulfide-induced constraint which keeps JAK2's inhibitory domain in proximity to the catalytic domain. This explanation can be easily ruled out because the removal of the inhibitory domain did not abrogate the oxidative inhibitory phenomenon. Second, it is also improbable that oxidation induces a disulfide that triggers the collapse of a catalytically competent structure. In such a case, not only should the GST/(NΔ661)rJAK2(9C:9S) and GST/(NΔ661)rJAK2(4C:4S) variants exhibit a high level of activity but their activities should resist oxidative inhibition. In reality, the substitution of cysteines with serines diminished, rather than stabilized, activity.

We looked to the three-dimensional structure of the JAK2 kinase domain, recently determined at a resolution of 2.0 Å (*40*) (Protein Data Bank entry number 2B7A, Molecular Modeling Database entry number 37130), to envision how the four critical cysteines might interact with other components of the enzyme's catalytic pocket (Figure 6). Two of these critical cysteine residues, Cys866 and Cys917, may have a direct role in catalysis, on the basis of their positions in the JAK2 catalytic domain; the two sulfur centers are approximately 9 Å apart. Cys866, Lys882, and Cys917 are located in the β2, β3, and β4 sheets, respectively, of the N-terminal lobe of the kinase domain. This general region contains several other polar residues which might coordinate divalent cations. Cysteines are well-known for their ability to coordinate metals including iron, cadmium, copper, and most notably zinc (*41*). Magnesium or manganese, in complex with ATP, is generally believed to be the physiologically relevant divalent cation bound to JAKs. While

aspartate residues are known to coordinate manganese in some enzymes (*42*, *43*), the fact that cysteines can functionally substitute for aspartates in the coordination of Mn^{2+} (*44*) raises the possibility that Cys866 and/or Cys917 might functionally coordinate the metallo-ATP substrate complex in JAK2.

Two of the four cysteines, Cys1094 and Cys1105, affecting JAK2 activity are positionally conserved in 20 members of the Janus kinase family, including the insect orthologue HOP (Figure 7). The sulfur atoms of these two residues are approximately 12 Å apart in the JAK2 structure, and they are located at opposite ends of the H-helix in the C-lobe of the structure. While the roles of these two cysteines in catalysis are not obvious from inspection and will require further study, we note that the kinase-deficient B-form splice variant of human JAK3 (*45*) contains the equivalent of Cys1094 but lacks both the Cys1105 equivalent and the I-helix. Critical cysteines have been identified in other protein-tyrosine kinases, such as lymphocyte-specific tyrosine kinase, p56lck, v-Src kinase, and human platelet-derived growth factor receptor-β (*46*−*48*).

These data may be applicable to the controversy surrounding the physiological relevance of the oxidative regulation of JAK activity. The physiologically relevant redox mediator thioredoxin was capable of restoring autokinase activity to preoxidized JAK2, but was unable to do so when mutated to the redox-inactive form (Figure 1). Several laboratories have provided data suggesting that oxidative cellular conditions stimulate JAK activity (*22*−*27*). While the data in this manuscript certainly do not discredit these observations, they do inspire the search for a plausible explanation for the oxidative stimulation of JAKs. One might argue that the apparent JAK stimulation is indirect and occurs due to the effect of hydrogen peroxide on other biomolecules that regulate JAK activity. The recent report that H_2O_2 inhibits, rather than stimulates, the JAK/STAT pathway in nerve cells (*20*) implies that such indirect pathways may not apply to all cell types. One such indirect mechanism arises from the oxidative inhibition of protein tyrosine phosphatase; another indirect mechanism might involve transphosphorylation by another protein tyrosine kinase which is directly stimulated by oxidation. These two hypotheses are not exhaustive, and the true reconciliatory explanation may, of course, be unprecedented. The absence of a definitive mechanistic basis for the oxidative stimulation of JAK2 warrants further investigation into this phenomenon. On the basis of our observations that multiple cysteine residues are implicated in JAK2-mediated catalysis, we propose that there is a direct biochemical basis for the oxidative inhibition of JAK2-mediated signal propagation.

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