Tyrosine Phosphorylation of the Janus Kinase 2 Activation Loop Is Essential for a High-Activity Catalytic State but Dispensable for a Basal Catalytic State†

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ABSTRACT: The phosphorylation of an "activation loop" within protein kinases is commonly associated with establishing catalytic competence, and phosphorylation of the Tyr^{1007} residue in the activation loop of Janus kinase 2 (JAK2) has been shown to be essential for intracellular propagation of cytokine-initiated signaling. We provide evidence for the presence of a basal activity state of JAK2, which was observed in the absence of activation loop phosphorylation. Phosphorylation of the JAK2 activation loop was essential for conversion to the high-activity state, characterized by high-efficiency ATP utilization during autophosphorylation. Mutagenesis of activation loop tyrosine residues $Tyr^{1007/1008}$ to phenylalanine residues impaired, but did not abolish, the enzyme's ability to autophosphorylate. The activation loop mutant JAK2 could also transphosphorylate an inactive JAK2 fragment coexpressed in Sf21 cells, providing evidence of exogenous substrate phosphorylation. The mutant enzyme remained in a basal activity state characterized by low-efficiency ATP utilization during autophosphorylation. Mutagenesis of a critical Lys^{882} residue to a glutamate residue abolished all evidence of kinase activity, confirming that the observed activity of Tyr-to-Phe mutants was not due to another kinase. Our data are consistent with the proposal that JAK2 is an inefficient but active enzyme in the absence of activation loop phosphorylation and is capable of conversion to a high-activity state by autophosphorylation under physiological ATP concentrations. This theoretically precludes the need for an upstream activating kinase. The activation process of JAK2 may be envisioned as a multistate process involving at least two kinetically distinct states of activity.

Janus kinases (JAKs)[1] are essential early mediators of intracellular signaling events initiated by the extracellular binding of various cytokines to their respective receptors. The clearest evidence for the essential roles of JAKs derives from experiments employing transgenic animals with targeted disruptions in each of the four mammalian Janus kinases, JAK1, JAK2, JAK3, and TYK2 (*1*−*8*); in these experiments, cells from the transgenic animals lost their sensitivity to specific cytokines. Time course studies support the notion that JAKs become active as protein−tyrosine kinases almost immediately after cytokines bind to their receptors (*9*). Progressive truncations and point mutations of the cytoplasmic tails of cytokine receptors have shown that cytokine signal propagation depends on the ability of JAKs to associate with the receptors (*10*−*12*). Finally, several reports of the ability of "dominant negative" forms of JAKs to inhibit the cascade of cytokine-initiated signaling events (*13*−*15*) also attest to the importance of JAK-mediated catalysis in these signaling pathways.

Despite the overwhelming evidence establishing JAKs as essential protein tyrosine kinases that respond to cytokine receptor stimulation, the details of the initial activation process of JAKs have remained elusive.

A key observation, found throughout the JAK literature, is that only the tyrosine-phosphorylated forms of JAKs are known to exhibit kinase activity. To our knowledge, no JAK variants have yet been reported to function in the absence of tyrosine phosphorylation. The region that appears to require phosphorylation is the conserved "activation loop" within the catalytic JH1 domain of the JAKs, which contains two tandem tyrosines. In TYK2, these duplicate tyrosines correspond to Tyr^{1054}/Tyr^{1055} (*15*, *16*). In JAK3 they are Tyr^{980}/Tyr^{981} (*17*, *18*), and phosphorylation of Tyr^{981} has been shown to be inhibitory. In JAK1 they are Tyr^{1033}/Tyr^{1034} (*18*), and in JAK2 the tandem tyrosines are Tyr^{1007}/Tyr^{1008} (*19*). Expression of mutated JAKs in mammalian cells shows that the substitution of phenylalanine for tyrosine in the activation

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[1] Abbreviations: JAK, Janus kinase; rJAK2, rat Janus kinase 2; Lck, lymphocyte-specific kinase; Bcr-Abl, breakpoint cluster region protein−Abelson tyrosine kinase; v-Src, avian sarcoma virus tyrosine kinase; v-Abl, Abelson murine leukemia virus tyrosine kinase; v-Fps, Fujinami avian sarcoma virus tyrosine kinase; VEGFR, vascular endothelial growth factor receptor; SYK, spleen tyrosine kinase; GST, glutathione *S*-transferase; DTT, dithiothreitol.

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loop prohibits the mutant enzymes from propagating intracellular responses to cytokine receptor stimulation, including the induction of autokinase activity (*15*, *16*, *18*, *19*). In the interferon-α system, the activation of TYK2 relies on transphosphorylation by another kinase, presumably JAK1, and does not proceed through TYK2 autophosphorylation (*16*). These data raise the possibility that activation loop phosphorylation occurs due to another transphosphorylating kinase, rather than intrinsic JAK activity.

Phosphorylation of comparable activation loops in a number of protein kinases is crucial for their activity (*20*−*23*). X-ray crystallographic and biochemical studies suggest that phosphorylation of the activation loop causes it to convert to an open conformation, which allows the access of donor substrate (ATP) and/or acceptor substrate (peptides or proteins) to the catalytic site (*24*, *25*). If the appropriate mechanistic archetype for the JAK activation loop is the insulin receptor kinase domain (*26*), then the nonphosphorylated activation loop impedes the binding of ATP and protein substrates at the active site (*27*). From a simplistic perspective, this suggests that JAKs lacking activation loop phosphorylation would be inactive, which in turn suggests that a transphosphorylating kinase is required for JAK activation. This proposition would be consistent with observations of constitutive activation and phosphorylation of JAKs in transformed mammalian cell lines overexpressing other kinases such as Lck (*28*), Bcr-Abl (*29*), v-Src (*30*), and v-Abl (*31*). However, the absence of activation loop phosphorylation does not necessarily preclude ATP from entering the catalytic pocket, and autokinase activity has been observed in the absence of activation loop phosphorylation in protein−tyrosine kinases such as FGFR (*32*), v-Fps (*33*, *34*), VEGFR (*35*), and SYK (*36*).

In this paper we addressed the question of whether phosphorylation of the activation loop tyrosines is essential for JAK2's catalytic activity. An answer of "yes" would suggest that the activation process of JAK2 may require another kinase. Alternatively, an answer of "no" supports the hypothesis that JAK2 can be activated by autophosphorylation and that JAK2 autoactivation can be the first step in cytokine-initiated signaling pathways. We elected to conduct these experiments with JAK2 because it is essential for transducing the responses of many different hematopoietic cytokines, because it can be activated simply by overexpression, and because JAK2 is typically observed as a homooligomer, whereas JAK1, JAK3, and TYK2 are usually found in heterooligomeric complexes.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Baculoviruses. The construction of a recombinant baculovirus which produced the GST/rJAK2 chimeric protein and the corresponding baculoviral transfer vector, pAcGHLT:rJAK2, was previously described (*37*). The DNA fragment encoding the region of rat JAK2 which spans amino acid residues 661−1132 was generated via PCR using the plasmid pRA3.17 (*38*) as template and 5′ATATAACATATGCATGCACTTCCTCGAAGAAAAA3′ and 5′TTTTGAATTCAAGTCTACTTGGTCTCTGAGTAAA3′ as reaction primers. The resultant 1.45 kb PCR product was subcloned into the *Nde*I/*Eco*RI site of the baculoviral transfer vector pAcGHLT-A (Pharmingen), creating the pAcGHLT:(NΔ661)rJAK2 transfer vector, which was used to generate a recombinant baculovirus that produced the chimeric GST/(NΔ661)rJAK2 protein. The pAcGHLT:rJAK2 and pAcGHLT:(NΔ661)rJAK2 transfer vectors were then each mutated using the QuikChange mutagenesis kit (Stratagene) with the primers 5′CTGTGCTGGAGCTTCTCAACAGCTACCACCTC3′ and 5′GAGGTGGTAGCTGTTGAGAAGCTCCAGCACAG3′ to create the pAcGHLT:rJAK2(K882E) and pAcGHLT:(NΔ661)-rJAK2(K882E) transfer vectors, which were used to generate baculoviruses that produced GST/rJAK2(K:E) and GST/(NΔ661)rJAK2(K:E) chimeric proteins, respectively. Similarly, the primers 5′CACCTGGTTCCTTTACTTTGAAGAATTCTTTGTCCTGTGGCAAG3′ and 5′CTTGCCACAGGACAAAGAATTCTTCAAAGTAAAGGAACCAGGTG3′ were used to create the pAcGHLT:rJAK2(Y1007F,-Y1008) and pAcGHLT:(NΔ661)rJAK2(Y1007F,Y1008F) transfer vectors, which were used for the production of the GST/rJAK2(YY:FF) and GST/(NΔ661)rJAK2(YY:FF) chimeric proteins, respectively. The vector pAcGHLT:(NΔ661)-rJAK2(Y1007F,Y1008F) was used as a template for site-directed mutagenesis to introduce the inactivating K882E mutation, and this transfer vector was used to create a baculovirus that produced the protein GST/(NΔ661)rJAK2-(KYY:EFF). Following sequence verification, recombinant baculoviruses were generated, isolated, and amplified, and the infectious titers were ascertained essentially as previously described (*39*).

Production of Recombinant 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 producing GST/rJAK2, GST/(NΔ661)rJAK2, GST/rJAK2(YY:FF), GST/(NΔ661)rJAK2-(YY:FF), GST/rJAK2(K:E), GST/(NΔ661)rJAK2(K:E), or GST/(NΔ661)rJAK2(KYY:EFF) proteins 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/mL of insect cell lysis buffer (*37*). Unfractionated cellular proteins were used in some experiments; in other experiments, GST/rJAK2 and its variants were recovered from the clarified cell lysates by immunoprecipitation with polyclonal antibodies recognizing JAK2 (Upstate Biotechnology item no. 06-255) and protein A−Sepharose CL-4B, as before (*39*).

Western Blotting. Aliquots of clarified cell lysates equivalent to 5×10^5 cells per lane were boiled with SDS−PAGE sample buffer for 3 min and resolved by 7.5% SDS−PAGE, transferred to PVDF membrane, and probed with monoclonal anti-GST (BD Bioscience no. 554805), monoclonal anti-phosphotyrosine (Upstate Biotechnology no. 05-321), polyclonal anti-JAK2 (Upstate Biotechnology no. 06-255), or polyclonal anti-phosphoJAK2 antisera (Upstate Biotechnology no. 07-123). Protein A−Sepharose pellets containing forms of ratJAK2 immunoprecipitated from 1×10^7 cells with anti-JAK2 antisera (Upstate Biotechnology no. 06-255), were washed three times with insect cell lysis buffer and split into two equal aliquots. One aliquot was boiled with 30 μL of SDS−PAGE sample buffer, resolved on a 7.5% SDS gel, transferred to PVDF membrane, and probed with anti-JAK2. The membrane was then stripped and reprobed with anti-phosphotyrosine. The second immunoprecipitation aliquot was used in autokinase assays as described below.

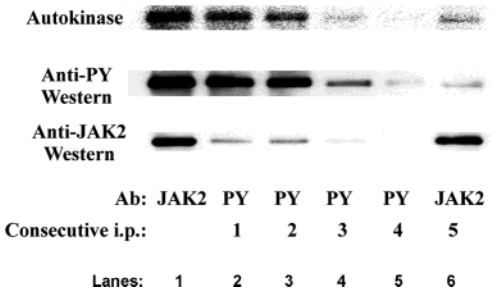

FIGURE 1: Tyrosine-phosphorylated subpopulation of purified GST/rJAK2 is an active autokinase. An equal volume (0.5 mL) of purified GST/rJAK2 was subjected either to a single round of immunoprecipitation with antisera directed against JAK2 (lane 1) or to four consecutive rounds of immunoprecipitation using monoclonal antibody recognizing phosphotyrosine (lanes 2−5) followed by anti-JAK2 immunoprecipitation (lane 6). The washed immunoprecipitates were then divided into two equal aliquots, one set of which was used in Western immunoblots to assess the relative JAK2 (bottom panel) and phosphotyrosine content (middle panel). The second aliquot set was preincubated for 1 h at 4 °C in the presence of 10 mM DTT and then assayed for autokinase activity (top panel).

Autokinase Assays. The above-described immunoprecipitation samples were incubated with 10 mM DTT for 1 h at 4 °C; the samples were then centrifuged, the supernatant was discarded, and the pellets were then resuspended with 100 μL of one of two kinase assay cocktails. The radioactive autokinase assay 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 7.4; 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. The gel was transblotted onto PVDF membrane, followed by autoradiography. The nonradioactive autokinase assay was performed on eight equal aliquots of anti-JAK2 immunoprecipitates from 1×10^7 Sf21 cells. This assay was performed essentially as the radioactive assay, except that the [γ-^{32}P]ATP was replaced with varying amounts of ATP, and anti-phosphotyrosine Western blotting, rather than autoradiography, was used as the detection method.

RESULTS

We recently engineered a recombinant baculovirus that produced a glutathione *S*-transferase-tagged rat JAK2 (GST/rJAK2) in infected insect cells. This GST/rJAK2 protein was purified through glutathione−Sepharose affinity chromatography (*37*). The partially purified GST/rJAK2 protein was a heterogeneous mixture of tyrosine-phosphorylated and non-tyrosine-phosphorylated forms of the enzyme, so immunoprecipitation provided a convenient approach for segregating the fraction of GST/rJAK2 containing surface-accessible phosphotyrosines from the superficially nonphosphorylated GST/rJAK2 fraction (Figure 1). The autokinase activities of these two subpopulations were then compared to the unfractionated GST/rJAK2 population. For this purpose, an aliquot of purified GST/rJAK2 was immunoprecipitated with antisera directed against JAK2 and then either immediately resolved via SDS−PAGE and probed with anti-JAK2 or with anti-phosphotyrosine antibodies or subjected to a radiolabeled autokinase activity assay prior to SDS−PAGE analysis. The recovered 140 kDa protein had an intense autokinase activity and demonstrated robust anti-JAK2 and anti-phosphotyrosine signals (Figure 1, lane 1). When an equal aliquot of purified GST/rJAK2 was immunoprecipitated with monoclonal antibody directed against phosphotyrosine, the 140 kDa GST/rJAK2 possessed comparable autokinase signals and anti-phosphotyrosine immunoblot intensities, but the anti-JAK2 immunoblot intensity was greatly diminished, as shown in lane 2. The same pattern was observed when the supernatant was subjected to a second round of anti-phosphotyrosine immunoprecipitation (lane 3). The signal intensities of all three assays were lowered when the supernatant was again immunoprecipitated with anti-phosphotyrosine (lane 4), and by the fourth round of anti-phosphotyrosine immunoprecipitation, the only signal which could be observed was a very weak signal in the anti-phosphotyrosine immunoblot (lane 5). The supernatant which had undergone four successive anti-phosphotyrosine immunoprecipitations was then immunoprecipitated with antisera recognizing JAK2 (lane 6). The recovered 140 kDa GST/rJAK2 protein had an anti-JAK2 immunoblot signal intensity which was comparable to that observed for the unfractionated anti-JAK2 immunoprecipitate. In contrast, only an exceedingly faint signal was observed in the anti-phosphotyrosine immunoblot and autokinase assays following extensive depletion of the phosphotyrosine subpopulation of GST/rJAK2.

These data clearly show that the tyrosine-phosphorylated GST/rJAK2 is the subpopulation which accounted for nearly all of the observed autokinase activity in the unfractionated sample, although it represented only a minor proportion of the total GST/rJAK2 in solution (lane 1 vs lanes 2 and 3). However, the faint autokinase activity that remained with the phosphotyrosine-depleted GST/rJAK2 population raised the possibility that the non-tyrosine-phosphorylated forms of GST/rJAK2 have extremely low activity.

To address these questions, we reengineered another series of baculoviruses to produce forms of JAK2 that eliminated the possibility of tyrosine phosphorylation within the activation loop. We created a recombinant baculovirus in which the tandem tyrosine residues of the activation loop (Y^{1007} and Y^{1008}) were converted to phenylalanine residues [GST/rJAK2(YY:FF)]. A third recombinant baculovirus was created to overproduce a catalytically inactive JAK2 enzyme in which a critical lysine residue (K^{882}) was converted to a glutamate residue [GST/rJAK2(K:E)]. This critical lysine mutation has been used as an inactivating mutation with the Janus kinases (*19*, *41*) and other protein kinases (*42*−*44*). Clarified detergent lysates from infected Sf21 cells were subjected to Western blot analysis for glutathione *S*-transferase, phosphotyrosine, and JAK2 content (panels A, B, and C of Figure 2, respectively). Infection of Sf21 insect cells with each of these baculoviruses resulted in the production of a protein that bound both antibodies recognizing glutathione *S*-transferase (Figure 2, lanes 1−3) and antibodies recognizing JAK2 (Figure 2, lanes 7−9). However, the phosphotyrosine content of these infected cell lysates varied greatly (Figure 2B). In addition to the intense phosphotyrosine signal associated with the GST/rJAK2 protein, the cell lysate shown in lane 4 also contained a broad spectrum of tyrosine-phosphorylated proteins. The results shown in lane 5 are in marked contrast. Only the GST/rJAK2(YY:FF)

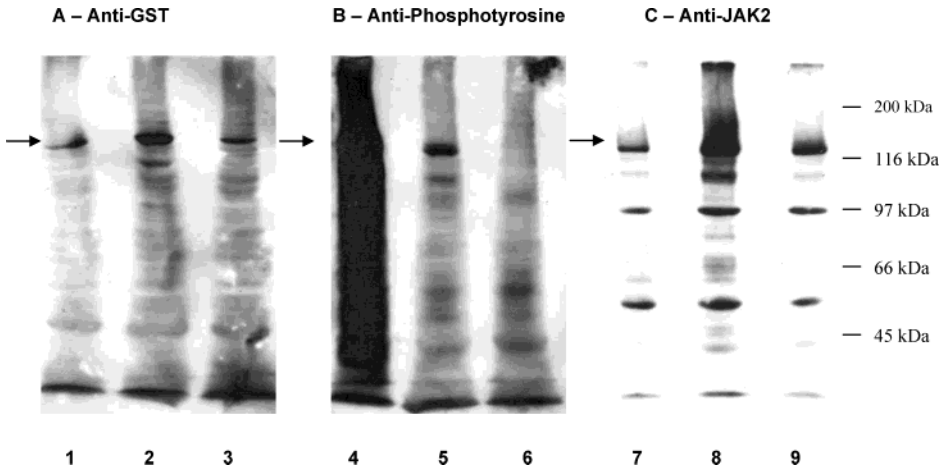

FIGURE 2: Tyrosine-phosphorylation profiles of Sf21 cells infected with baculoviruses encoding variants of GST/rJAK2 protein. 1×10^7 Sf21 cells were infected separately with recombinant baculoviruses expressing GST/rJAK2, GST/rJAK2(YY:FF), or GST/rJAK2(K:E). 72 h postinfection, the cells were lysed, and the lysates were resolved on 7.5% SDS−PAGE as follows: lanes 1, 4, and 7, GST/rJAK2; lanes 2, 5, and 8, GST/rJAK2(YY:FF); lanes 3, 6, and 9, GST/rJAK2(K:E). Western blots were then probed with anti-GST (panel A), anti-phosphotyrosine (panel B), and anti-JAK2 (panel C). Arrows point to GST/rJAK2 variants.

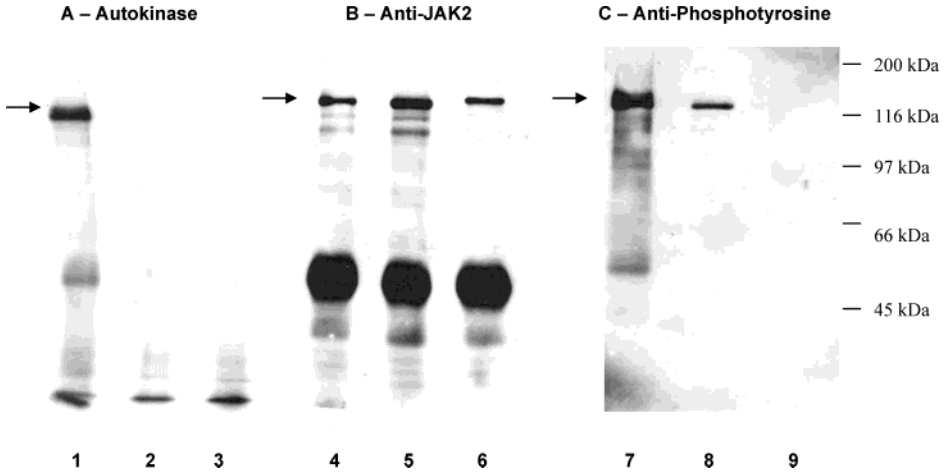

FIGURE 3: Autokinase activity and tyrosine-phosphorylation status of immunoprecipitated variants of GST/rJAK2 protein. Sf21 cells were infected with recombinant baculoviruses as in Figure 2. 72 h postinfection, the cells were lysed, and the cleared lysates were immunoprecipitated with anti-JAK2. Autokinase assays with one-half of the immunoprecipitated enzymes were performed as described in Experimental Procedures. The reaction mixtures were resolved by 7.5% SDS−PAGE, followed by transblotting onto PVDF and autoradiography (panel A). The other half of the immunoprecipitated enzymes were resolved by 7.5% SDS−PAGE, followed by Western blotting with anti-JAK2 (panel B). The blot was then stripped and reprobed with anti-phosphotyrosine (panel C). Samples were loaded as follows: lanes 1, 4, and 7, GST/rJAK2; lanes 2, 5, and 8, GST/rJAK2(YY:FF); lanes 3, 6, and 9, GST/rJAK2(K:E). Arrows point to GST/rJAK2 variants.

protein itself appeared to contain a significant level of phosphotyrosine, but its phosphotyrosine signal intensity was much lower than that of the GST/rJAK2 band in lane 4. The lack of detectable phosphotyrosine content in the GST/rJAK2(K:E) protein (lane 6) can be interpreted as evidence that the tyrosine phosphorylation of the GST/rJAK2(YY:FF) protein arose from an autophosphorylation event, rather than from a transphosphorylation by another kinase present in the infected insect cells. Moreover, the cell lysate which contained the GST/rJAK2(K:E) protein showed no significant increase in tyrosine-phosphorylated protein content when compared to uninfected cell lysates (data not shown).

These data show that although the phosphorylation of the tyrosines in the activation loop of JAK2 has a profound effect on the enzyme's catalytic competency, these tyrosines are not essential for catalytic activity, in contrast to the invariant lysine residue of subdomain II of the kinase domain (*44*). To demonstrate this more clearly, the engineered JAK2 proteins were recovered from infected Sf21 cells by immunoprecipitation using antibodies directed against JAK2. An aliquot of the immunoprecipitated protein was directly analyzed for JAK2 and phosphotyrosine content by Western blot, and an equal aliquot was subjected to an autokinase assay using carrier-free [γ-^{32}P]ATP. The data obtained are shown in Figure 3. All immunoprecipitated samples contained equivalent amounts of recombinant protein, based on the signal intensities observed in the anti-JAK2 immunoblot (panel B). The anti-phosphotyrosine immunoblot data corroborated the observations from Figure 2, in that GST/rJAK2 was the most heavily tyrosine-phosphorylated of the three forms (Figure 3, lane 7), GST/rJAK2(YY:FF) was clearly tyrosine-phosphorylated but notably less so than GST/rJAK2 (Figure 3, lane 8), and the GST/rJAK2(K:E) protein has no detectable phosphotyrosine content (Figure 3, lane 9). These data provide evidence that the phosphorylation of tyrosine residues within GST/rJAK2 and GST/rJAK2(YY:FF) was almost certainly due to autophosphorylation events, since GST/rJAK2(K:E), which should have been a suitable can-

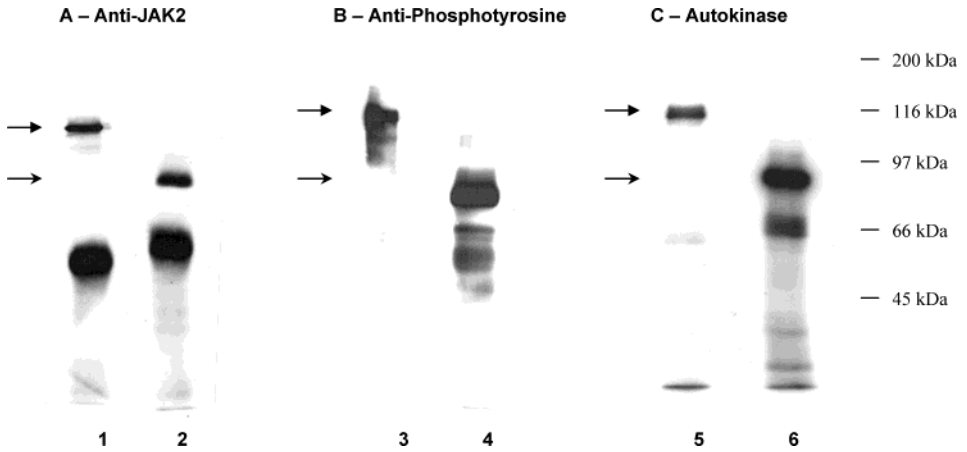

FIGURE 4: Comparison of tyrosine-phosphorylation and autokinase activity of GST/rJAK2 and GST/(NΔ661)rJAK2 proteins. 1×10^7 Sf21 cells were infected separately with recombinant baculoviruses expressing GST/rJAK2 or GST/(NΔ661)rJAK2, harvested 72 h postinfection, and lysed, and the clarified lysates were immunoprecipitated with anti-JAK2. Autokinase assays were performed using one-third of the immunoprecipitated enzymes, followed by SDS−PAGE and autoradiography as described in Experimental Procedures (panel C). The other two parts of the immunoprecipitated enzymes were resolved by SDS−PAGE and probed with anti-JAK2 (panel A) or with anti-phosphotyrosine (panel B). Samples were loaded as follows: lanes 1, 3, and 5, GST/rJAK2; lanes 2, 4, and 6, GST/(NΔ661)rJAK2.

didate for transphosphorylation by another endogenous kinase, exhibited no evidence of tyrosine phosphorylation. The GST/rJAK2 protein exhibited robust autokinase activity (Figure 3, lane 1), and as expected, GST/rJAK2(K:E) (Figure 3, lane 3) lacked detectable autokinase activity. However, it was remarkable to observe no detectable autokinase activity associated with the immunoprecipitated GST/rJAK2(YY:FF) (Figure 3, lane 2), because the presence of phosphotyrosine implied that this enzyme had intrinsic catalytic competency.

It has been speculated that the amino-terminal half of JAK2 might contain autoinhibitory properties, based on the observation that amino-terminally truncated forms of JAKs appear to be "hyperactive" in comparison to full-length forms of JAKs (*39*, *40*). This raised the suspicion that the unexpected lack of demonstrable autokinase activity of GST/rJAK2(YY:FF) reflected an inability to relieve autoinhibition. We reasoned that removal of the putative autoinhibitory domain might dramatically enhance the apparent activity of the activation loop mutant. So we created recombinant baculoviruses which produced a glutathione *S*-transferase-tagged rJAK2 that lacked the first 661 amino acids of the rJAK2 sequence but that contained all of the catalytic JH1 domain and approximately 60% of the pseudo-kinase JH2 domain [GST/(NΔ661)rJAK2]. We compared the autokinase activity and tyrosine phosphorylation status of these two forms of rJAK2. The GST/(NΔ661)rJAK2 form was more heavily tyrosine-phosphorylated than an equivalent amount of the full-length GST/rJAK2 form (Figure 4, panel B). In vitro autokinase assays (Figure 4C) clearly indicated that GST/(NΔ661)rJAK2 possessed higher autokinase activity than GST/rJAK2. We then created a recombinant baculovirus to produce a protein, GST/(NΔ661)rJAK2(YY:FF), in which the rJAK2 activation loop tyrosine residues (Y^{1007} and Y^{1008}) were converted to phenylalanine residues. Another baculovirus was generated to produce a kinase-inactive mutant, GST/(NΔ661)rJAK2(K:E), by converting the critical lysine residue (K^{882}) to a glutamate residue. As before, clarified detergent lysates from infected Sf21 cells were subjected to Western blot analysis for glutathione *S*-transferase, phosphotyrosine, and JAK2 content (panels A, B, and C of Figure 5, respectively). Infection of Sf21 insect cells with these baculoviruses resulted in the generation of an 84 kDa protein detected by antibodies capable of recognizing glutathione *S*-transferase, phosphotyrosine, and JAK2 (Figure 5, lanes 1, 4, and 7, respectively). The production of GST/(NΔ661)-rJAK2(YY:FF) was confirmed by anti-GST and anti-JAK2 immunoblotting (Figure 5, lanes 2 and 8, respectively), and the production of the GST/(NΔ661)rJAK2(K:E) protein was likewise confirmed by anti-GST and anti-JAK2 immunoblotting (Figure 5, lanes 3 and 9, respectively). As was the case with the GST/rJAK2-producing cells, the crude cell lysate from the GST/(NΔ661)rJAK2-producing cells contained a high level of tyrosine phosphorylation in a variety of undefined proteins possessing a broad range of apparent molecular masses (Figure 5, lane 4). There was a significant phosphotyrosine signal associated with the GST/(NΔ661)-rJAK2(YY:FF) protein in the crude cell lysate, but unlike the GST/(NΔ661)rJAK2-producing cell lysate, no other cellular proteins appeared to be heavily tyrosine-phosphorylated (Figure 5, lane 5). Further, there was no evidence of significant tyrosine phosphorylation in the GST/(NΔ661)-rJAK2(K:E) protein or in other proteins from that cell lysate (Figure 5, lane 6).

We then attempted to directly demonstrate that the GST/(NΔ661)rJAK2(YY:FF) protein possessed autokinase activity. The three GST/(NΔ661)rJAK2 variant proteins were recovered from infected Sf21 cells by immunoprecipitation using antibodies directed against JAK2. An aliquot of the immunoprecipitated protein was directly analyzed for JAK2 and phosphotyrosine content by Western blot, and an equal aliquot was subjected to an autokinase assay using carrier-free [γ-^{32}P]ATP. The data obtained are shown in Figure 6. All immunoprecipitated samples contained equivalent amounts of recombinant protein, based on the signal intensities observed in the anti-JAK2 immunoblot. Anti-JAK2 immunoblotting confirmed the presence of GST/(NΔ661)rJAK2, GST/(NΔ661)rJAK2(YY:FF), and GST/(NΔ661)rJAK2(K:E) (Figure 6B). The anti-phosphotyrosine immunoblot data are consistent with the data from preceding figures in that GST/(NΔ661)rJAK2 is the most heavily tyrosine-phosphorylated of the three forms (Figure 6, lane 7), GST/(NΔ661)-rJAK2(YY:FF) is clearly tyrosine-phosphorylated but to a

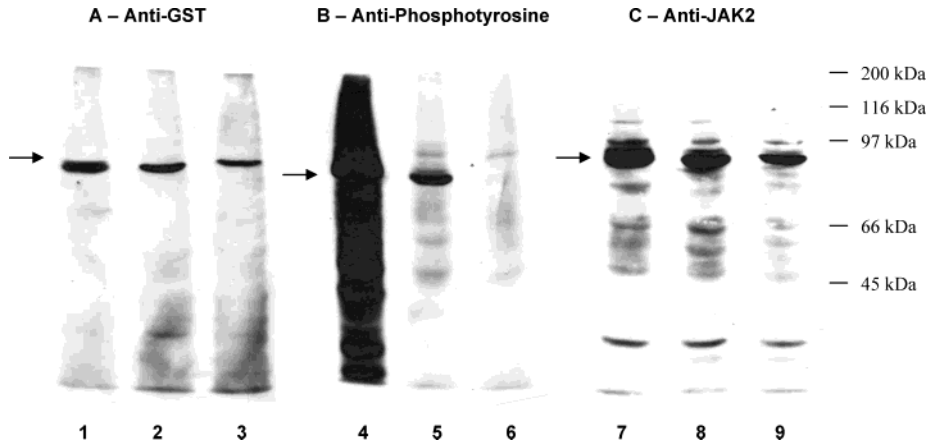

FIGURE 5: Tyrosine-phosphorylation profiles of Sf21 cells infected with baculoviruses encoding variants of GST/(NΔ661)rJAK2 protein. 1×10^7 Sf21 cells were infected separately with recombinant baculoviruses expressing GST/(NΔ661)rJAK2, GST/(NΔ661)rJAK2(YY:FF), or GST/NΔ661rJAK2(K:E). 72 h postinfection, the cells were lysed, and the lysates were resolved on 7.5% SDS−PAGE as follows: lanes 1, 4, and 7, GST/(NΔ661)rJAK2; lanes 2, 5, and 8, GST/(NΔ661)rJAK2(YY:FF); lanes 3, 6, and 9, GST/NΔ661rJAK2(K:E). Western blots were then probed with anti-GST (panel A), anti-phosphotyrosine (panel B), and anti-JAK2 (panel C). Arrows point to GST/(NΔ661)-rJAK2 variants.

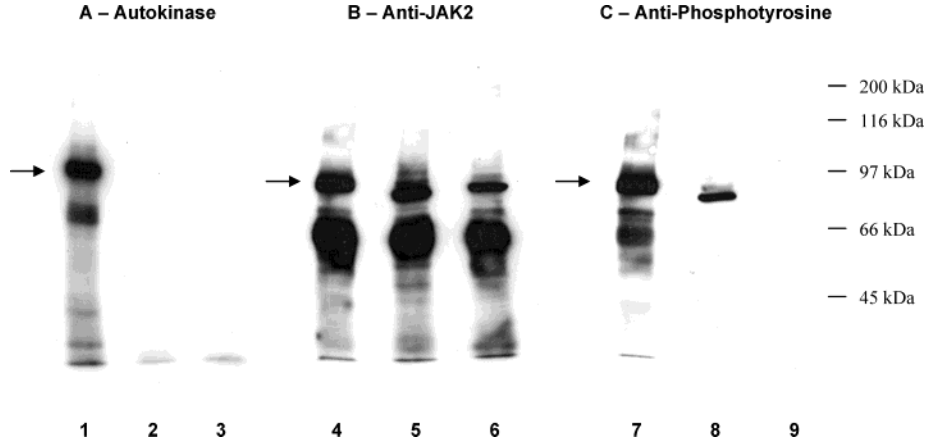

FIGURE 6: Autokinase activity and tyrosine-phosphorylation status of immunoprecipitated variants of GST/(NΔ661)rJAK2. Sf21 cells were infected with recombinant baculoviruses as in Figure 5. The cells were lysed 72 h postinfection, and the cleared lysates were immunoprecipitated with anti-JAK2. Autokinase assays using one-half of the immunoprecipitated enzymes, followed by SDS−PAGE and autoradiography, were performed as described in Experimental Procedures (panel A). The other half of the immunoprecipitated enzymes was resolved by SDS−PAGE and probed with anti-JAK2 (panel B). The blot was then stripped and reprobed with anti-phosphotyrosine (panel C). Samples were loaded as follows: lanes 1, 4, and 7, GST/(NΔ661)rJAK2; lanes 2, 5, and 8, GST/(NΔ661)rJAK2(YY:FF); lanes 3, 6, and 9, GST/(NΔ661)rJAK2(K:E). Arrows point to GST/(NΔ661)rJAK2 variants. The GST/(NΔ661)rJAK2(YY:FF) form migrates slightly faster than the other two variants.

lesser extent (Figure 6, lane 8), and the GST/(NΔ661)rJAK2-(K:E) protein has no detectable phosphotyrosine content (Figure 6, lane 9). While these data hinted that the phosphorylation of tyrosine residues within GST/(NΔ661)rJAK2 and the GST/(NΔ661)rJAK2(YY:FF) forms almost certainly arose from an autophosphorylation event, rather than from a transphosphorylation event catalyzed by another kinase, the autokinase assay again failed to provide direct evidence that the immunoprecipitated GST/(NΔ661)rJAK2(YY:FF) protein was a catalytically competent enzyme. Autokinase assays showed that GST/(NΔ661)rJAK2 was an active enzyme (Figure 6, lane 1), but neither the immunoprecipitated GST/(NΔ661)rJAK2(YY:FF) nor the immunoprecipitated GST/(NΔ661)rJAK2(K:E) exhibited any detectable autokinase activity (Figure 6, lanes 2 and 3, respectively).

As we sought to explain this apparent paradox, we considered that the concentration of ATP in the autokinase assay is far lower than typical intracellular ATP concentrations. As shown thus far, anti-phosphotyrosine immunoblots detected JAK2 tyrosine phosphorylation which had occurred within the cell and had therefore occurred when the ATP concentration was much higher than in the radioactive autokinase assays which were conducted "carrier-free", i.e., without the addition of nonradiolabeled ATP (Figures 3 and 6, panel A). On the basis of the vendor's description of the [γ-^{32}P]ATP used in these experiments, the ATP concentration was approximately only 83 nM. Additional nonradiolabeled ATP can be added but only to a maximum practical ATP concentration of approximately 1 μM (*37*) because further isotopic dilution results in an unacceptably low signal-to-noise ratio under most circumstances. Such experimental ATP concentrations would still be far below the reported ATP concentrations observed in eukaryotic cells. Using an estimated cellular volume of approximately 4 pL, ATP concentrations in Sf9 insect cells range from 8 to 12 mM, based on ^{31}P NMR spectroscopy measurements (*45*). This range is comparable to that reported for other eukaryotic cellular sources. Intact animal muscles contain from 3 to 6 mM ATP (*46*), although it has been noted that ATP is not uniformly distributed throughout the cell (*47*). Thus, the

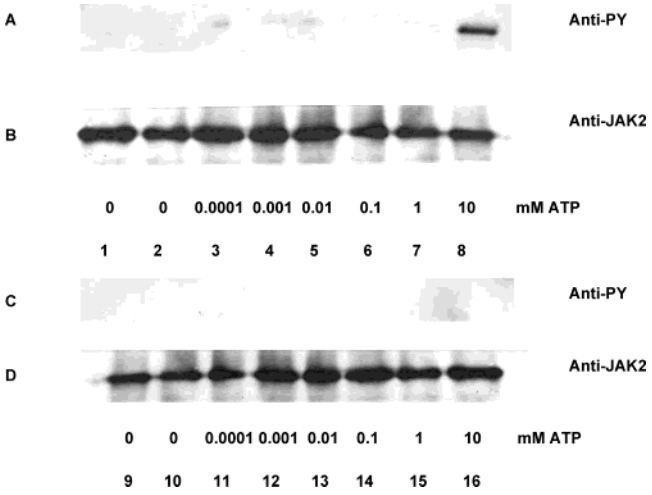

FIGURE 7: ATP dependence of autokinase activity of GST/rJAK2-(YY:FF). 1×10^7 Sf21 cells were infected with recombinant baculovirus producing GST/rJAK2(YY:FF) (panels A and B, lanes 1−8) or GST/rJAK2(K:E) (panels C and D, lanes 9−16). 72 h postinfection, the cells were lysed, and the cleared lysates were immunoprecipitated with anti-JAK2. The immunoprecipitated JAK2 was split into eight equal aliquots, and autokinase activity was assayed by incubating with nonradioactive kinase cocktail containing the indicated concentration of ATP, as described in Experimental Procedures. The reaction mixture was resolved by 7.5% SDS−PAGE, transblotted onto PVDF membranes, and probed with anti-phosphotyrosine (panels A and C). The blots were then stripped and reprobed with anti-JAK2 (panels B and D).

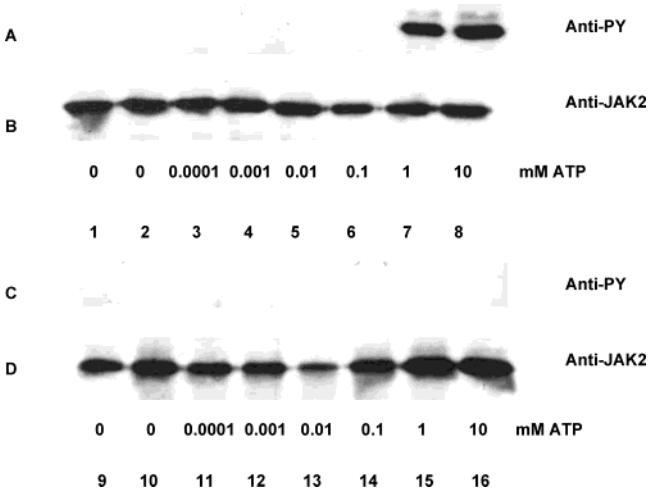

FIGURE 8: ATP dependence of autokinase activity of GST/(NΔ661)rJAK2(YY:FF). 1×10^7 Sf21 cells were infected with recombinant baculovirus producing GST/(NΔ661)rJAK2(YY:FF) (panels A and B, lanes 1−8) or GST/(NΔ661)rJAK2(K:E) (panels C and D, lanes 9−16). 72 h postinfection, the cells were lysed, and the cleared lysates were immunoprecipitated with anti-JAK2. The immunoprecipitated JAK2 was split into eight equal aliquots, and autokinase activity was assayed by incubating with nonradioactive kinase cocktail containing the indicated concentration of ATP, as described in Experimental Procedures. The reaction mixtures were resolved by 7.5% SDS−PAGE, transblotted onto PVDF membranes, and probed with anti-phosphotyrosine (panels A and C). The blots were then stripped and reprobed with anti-JAK2 (panels B and D).

radiolabeled autokinase assay might have provided a false negative result if the GST/(NΔ661)rJAK2(YY:FF) and GST/rJAK2(YY:FF) enzymes were inefficient (*48*) with respect to ATP, even if these enzymes were able to autophosphorylate in the Sf21 cells, where the ATP concentration was approximately 10000-fold higher.

The autokinase assay was then attempted without the use of radioisotope. The final concentration of ATP in these assays was varied from 100 nM, which was close to the concentration used in the radioisotopic autokinase assay, to 10 mM, which is near the upper range of the physiologically relevant ATP concentrations. The amount of tyrosine autophosphorylation in the absence and presence of added ATP was then determined via SDS−PAGE and anti-phosphotyrosine Western blots. Protein samples with extensive preexisting tyrosine phosphorylation, such as GST/rJAK2 and GST/(NΔ661)rJAK2, proved not to be well-suited for this assay. However, the phosphotyrosine content of GST/rJAK2-(YY:FF) and GST/(NΔ661)rJAK2(YY:FF) was sufficiently low (cf. Figures 2 and 5, panel B) that they could be used in this assay.

We evaluated the phosphotyrosine content of GST/rJAK2-(YY:FF) following incubation with various concentrations of ATP and then stripped and reprobed the membrane to ensure that equivalent amounts of GST/rJAK2(YY:FF) were being compared. As shown in panel A of Figure 7, there was no significant increase in the phosphotyrosine content when the enzyme was incubated in the presence of 100 nM, 1 μM, 10 μM, 100 μM, or 1 mM ATP, but there was a distinct increase in phosphotyrosine content when immunoprecipitated GST/rJAK2(YY:FF) was incubated with 10 mM ATP. In contrast, there was no detectable change in the phosphotyrosine content of GST/rJAK2(K:E) (Figure 7, panel C), which supported the notion that the tyrosine phosphorylation of the Tyr-to-Phe mutant reflected its autokinase activity. These experiments were repeated with the GST/(NΔ661)rJAK2(YY:FF) (Figure 8A,B) and GST/(NΔ661)-rJAK2(K:E) (Figure 8C,D) proteins. The GST/(NΔ661)-rJAK2(YY:FF) protein showed no evidence of autokinase activity when incubated in the presence of 100 nM, 1 μM, 10 μM, or 100 μM ATP, but there was a clear increase in the phosphotyrosine content when GST/(NΔ661)rJAK2(YY:FF) was incubated with 1 or 10 mM ATP. No such increase in phosphotyrosine content was observed under any conditions using the kinase-inactive form of the enzyme, as anticipated. It was interesting to note that the GST/rJAK2-(YY:FF) consistently required a higher ATP concentration to exhibit autokinase activity than did the GST/(NΔ661)-rJAK2(YY:FF) form, which suggests that the nature of the "hyperactivity" induced by amino-terminal deletions may reflect an increase in enzyme efficiency (compare Figure 7A to Figure 8A). Our data are in agreement with more recent data using progressively truncated mouse JAK2 mutants (*49*) in which a form of the enzyme lacking amino acids 1−671, which most closely approximates our GST/(NΔ661)rJAK2 constructs, was shown to possess more activity than the full-length enzyme.

While autophosphorylation provides one measure of JAK2 activity, direct measurements of rates of exogenous substrate phosphorylation would provide more useful characteristics (e.g., V_{max}, k_{cat}, and K_M) by which the activity states could be described. Phosphorylation of an exogenous substrate would also conclusively demonstrate the catalytic competency of the activation loop mutant, GST/rJAK2(YY:FF). Despite repeated attempts to obtain such measurements using a variety of phosphorylation substrates and several assay methodologies, we have been unable to obtain reaction rates

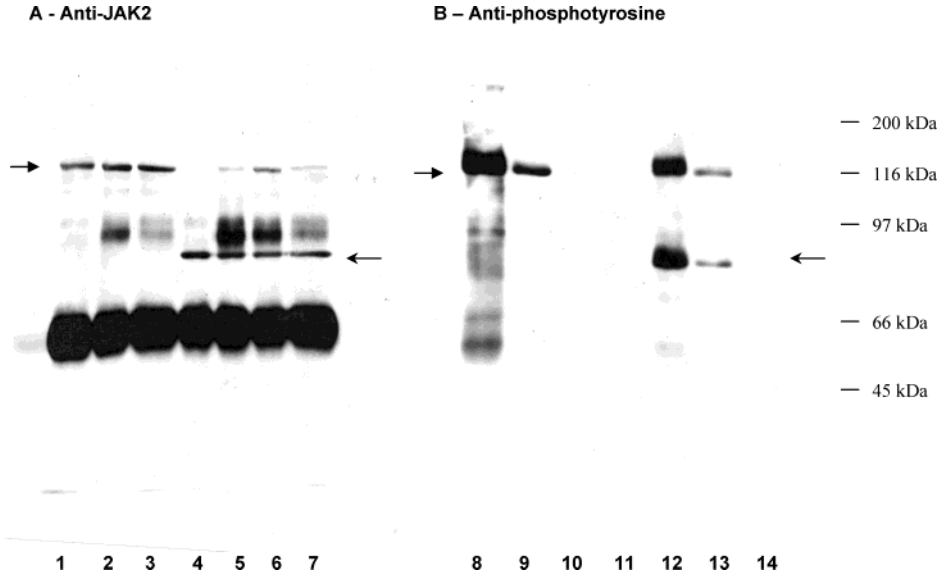

FIGURE 9: Tyrosine-phosphorylation status of immunoprecipitated GST/rJAK2 and GST/(NΔ661)rJAK2 variants from co-infected Sf21 cells. 1×10^7 Sf21 cells were infected separately with recombinant baculoviruses expressing variants of GST/rJAK2 individually or in the combinations described below. 72 h postinfection, the cells were lysed, and the lysates were immunoprecipitated with anti-JAK2 and resolved on 7.5% SDS−PAGE as follows: lanes 1 and 8, GST/rJAK2; lanes 2 and 9, GST/rJAK2(YY:FF); lanes 3 and 10, GST/rJAK2-(K:E); lanes 4 and 11, GST/(NΔ661)rJAK2(K:E); lanes 5 and 12, GST/rJAK2 + GST/(NΔ661)rJAK2(K:E); lanes 6 and 13, GST/rJAK2-(YY:FF) + GST/(NΔ661)rJAK2(K:E); lanes 7 and 14, GST/rJAK2(K:E) + GST/(NΔ661)rJAK2(K:E). Following transblotting onto PVDF, the membranes were probed with anti-JAK2 (panel A) or anti-phosphotyrosine (panel B). Arrows point to GST/rJAK2 variants.

for the tyrosine kinase activity of the purified GST/rJAK2 variants that significantly exceed those of the purified inactive GST/rJAK2(K:E) variants.

On the other hand, we found that we could exploit simultaneous production of two different proteins via the baculoviral expression system to obtain evidence of exogenous substrate phosphorylation (Figure 9). We used the 84 kDa inactive GST/(NΔ661)rJAK2(K:E) form of the enzyme as a substrate for the 140 kDa GST/rJAK2 variants. Sf21 insect cells were infected with the GST/rJAK2 variants alone or in co-infection with GST/(NΔ661)rJAK2(K:E). The cells were lysed 72 h postinfection, and the lysates were subjected to immunoprecipitation with anti-JAK2. The immunoprecipitates were resolved by SDS−PAGE and probed with anti-JAK2 or anti-phosphotyrosine antibodies. As shown in Figure 9, coexpression of GST/rJAK2 and GST/(NΔ661)rJAK2-(K:E) results in the transphosphorylation of the shorter inactive variant (Figure 9, lanes 5 and 12), which was incapable of autophosphorylation when expressed alone under identical conditions (Figure 9, lanes 4 and 11). As an additional negative control, coexpression of GST/rJAK2(K:E) and GST/(NΔ661)rJAK2(K:E) did not result in any detectable phosphorylation of either protein (Figure 9, lanes 7 and 14). Evidence of the catalytic activity of the activation loop mutant, GST/rJAK2(YY:FF), is presented in lanes 6 and 13 of Figure 9. It was clear that coexpression of GST/rJAK2(YY:FF) and GST/(NΔ661)/rJAK2(K:E) resulted in transphosphorylation of the shorter inactive variant by the full-length activation loop mutant. The transphosphorylation activity of the activation loop mutant was apparently lower than that of GST/rJAK2, which was consistent with the cellular autophosphorylation data presented in Figures 2, 3, 5, and 6.

One of the functional consequences of JAK2's basal state activity might be to raise the enzyme's catalytic efficiency via phosphorylation of the activation loop tyrosines. However, the data obtained thus far do not provide specific clues about the identity of the transphosphorylated residues. We therefore investigated whether GST/rJAK2(YY:FF) was able to transphosphorylate the activation loop tyrosines on the substrate GST/(NΔ661)rJAK2(K:E). Sf21 cells were infected with variants of GST/rJAK2 in combination with the inactive GST/(NΔ661)rJAK2(K:E). The cells were harvested 72 h postinfection, lysed, and immunoprecipitated with anti-JAK2 antibodies. SDS−PAGE and Western blotting with anti-JAK2 antibodies (Figure 10A) confirmed that all GST/rJAK2 variants were produced in equivalent amounts. Western blotting with antibodies that specifically recognized JAK2's phosphorylated activation loop tyrosines (Figure 10B) demonstrated that GST/rJAK2(YY:FF) transphosphorylated the activation loop tyrosine residues (Figure 10B, lane 7), as did the wild-type GST/rJAK2 (Figure 10B, lane 6). No phosphorylation was detected on the inactive substrate when it was expressed alone (Figure 10, lanes 1 and 5) or in combination with the inactive GST/rJAK2(K:E) (Figure 10, lanes 4 and 8). We further explored this question with the use of an inactive mutant, GST/(NΔ661)rJAK2(KYY:EFF), as a substrate. Sf21 cells were infected with recombinant baculoviruses producing GST/rJAK2 variants singly or in combination with the kinase-inactive mutant lacking the activation loop tyrosines. The cells were lysed and immunoprecipitated with anti-JAK2 antibodies, as before. Equivalent production of all protein variants was confirmed by SDS−PAGE followed by anti-JAK2 Western blotting (Figure 11A). Anti-phosphotyrosine Western blotting was performed, and the results are shown in Figure 11B. The activation loop mutant was unable to transphosphorylate GST/(NΔ661)rJAK2(KYY:EFF) (Figure 11B, lane 13), even though it was capable of autophosphorylation (Figure 11B, lanes 9 and 13). The wild-type GST/rJAK2, in contrast, was able to transphosphorylate GST/(NΔ661)rJAK2(KYY:EFF) (Figure 11B, lane 12). No phosphorylation was detected on

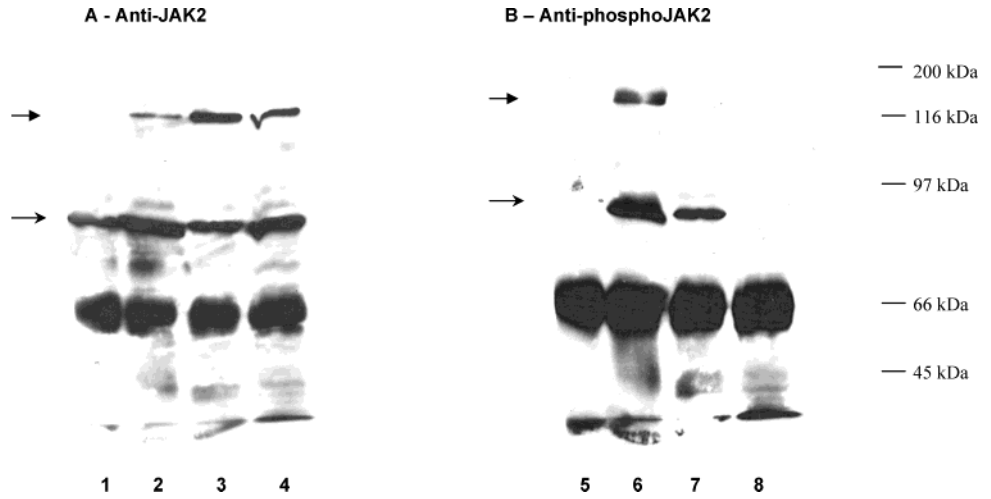

FIGURE 10: Activation loop-specific tyrosine-phosphorylation status of immunoprecipitated GST/rJAK2 and GST/(NΔ661)rJAK2 variants from co-infected Sf21 cells. 1×10^7 Sf21 cells were infected separately with recombinant baculoviruses expressing variants of GST/rJAK2 in the combinations described below. 72 h postinfection, the cells were lysed, and the lysates were immunoprecipitated with anti-JAK2 and resolved on 7.5% SDS−PAGE as follows: lanes 1 and 5, GST/(NΔ661)rJAK2(K:E); lanes 2 and 6, GST/rJAK2 + GST/(NΔ661)rJAK2-(K:E); lanes 3 and 7, GST/rJAK2(YY:FF) + GST/(NΔ661)rJAK2(K:E); lanes 4 and 8, GST/rJAK2(K:E) + GST/(NΔ661)rJAK2(K:E). Following transblotting onto PVDF, the membranes were probed with anti-JAK2 (panel A) or anti-phosphoJAK2 (panel B). Arrows point to GST/rJAK2 variants.

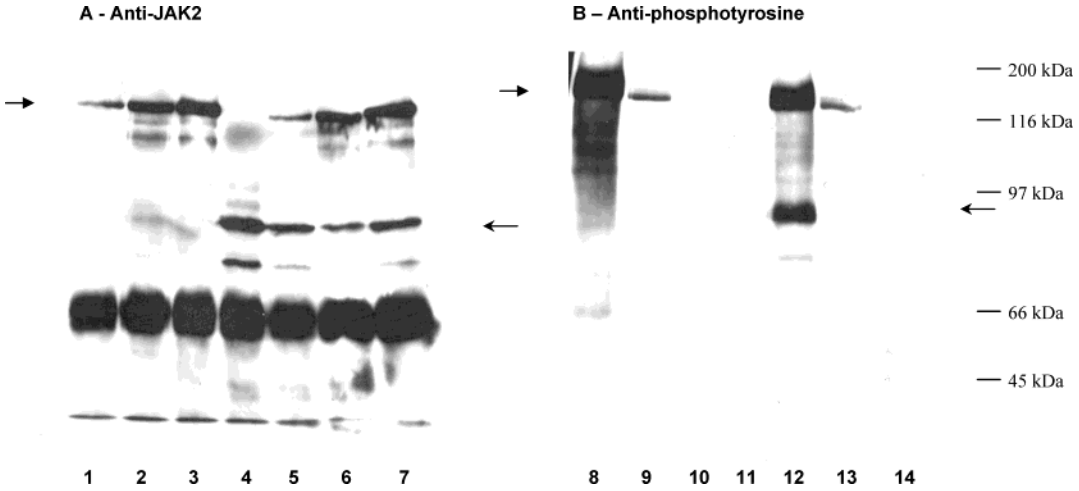

FIGURE 11: Tyrosine-phosphorylation status of immunoprecipitated GST/rJAK2 variants and GST/(NΔ661)rJAK2(KYY:EFF) substrates from co-infected Sf21 cells. 1×10^7 Sf21 cells were infected separately with recombinant baculoviruses expressing variants of GST/rJAK2 individually or in the combinations described below. 72 h postinfection, the cells were lysed, and the lysates were immunoprecipitated with anti-JAK2 and resolved on 7.5% SDS−PAGE as follows: lanes 1 and 8, GST/rJAK2; lanes 2 and 9, GST/rJAK2(YY:FF); lanes 3 and 10, GST/rJAK2(K:E); lanes 4 and 11, GST/(NΔ661)rJAK2(KYY:EFF); lanes 5 and 12, GST/rJAK2 + GST/(NΔ661)rJAK2(KYY:EFF); lanes 6 and 13, GST/rJAK2(YY:FF) + GST/(NΔ661)rJAK2(KYY:EFF); lanes 7 and 14, GST/rJAK2(K:E) + GST/(NΔ661)rJAK2(KYY:EFF). Following transblotting onto PVDF, the membranes were probed with anti-JAK2 (panel A) or anti-phosphotyrosine (panel B). Arrows point to GST/rJAK2 variants.

this kinase-inactive mutant lacking activation loop tyrosines when expressed alone (Figure 11, lanes 4 and 11) or in combination with inactive GST/rJAK2(K:E) (Figure 11, lanes 7 and 14).

On the basis of the data shown, the kinase activity of JAK2 does not have an absolute requirement for the phosphorylation of the conserved Tyr^{1007}/Tyr^{1008} residues found in the activation loop. Rather, the nonphosphorylated enzyme can exist in a basally active state characterized by inefficient ATP use. The basally active enzyme is capable of tyrosine autophosphorylation and transphosphorylation under cellular conditions, where ATP concentrations are in the millimolar range. Phosphorylation of the Tyr^{1007}/Tyr^{1008} residues within the activation loop converts the enzyme to an activated state characterized by highly efficient use of ATP.

DISCUSSION

The activity of Janus kinase in the cell is controlled by multiple regulatory factors, and dissection of the contributions of these factors can be a complex challenge. This study therefore used a simplified in vitro system to investigate a fundamental question of whether the phosphorylation of tyrosine residues within the activation loop of JAK2 is an obligate process for the manifestation of its catalytic activity. The data presented in this paper provide evidence for a simple multistate model of the JAK2 activation process, and this model suggests that the normal receptor-mediated activation of JAK2 can proceed without preexistent phosphorylation of the JAK2 activation loop. An initial "basal" state of activity leads to phosphorylation of the activation

loop tyrosines and a maximally active state. This model does not rely upon another kinase to activate JAK2. This has a significant bearing on our understanding of in vivo cytokine-mediated signal transduction, because if Janus kinase cannot become activated without first being phosphorylated by another kinase, then the currently accepted model of the JAK/STAT pathway fails to identify the primary signaling kinase in the pathway.

The data in this paper clearly show that JAK2 can function as an active tyrosine kinase, whether or not the tyrosines within its activation loop have been phosphorylated. Moreover, these data document the presence of at least one additional tyrosine residue in GST/rJAK2(YY:FF) and GST/(NΔ661)rJAK2(YY:FF) that is phosphorylated. The identity and significance of these tyrosines remain to be determined. Phosphorylation of these unidentified tyrosines may have originated due to the activity of nonphosphorylated JAK2. Given the limitations of the experiments shown here, one cannot completely rule out the possibility that another kinase was required for the initial phosphorylation of these unidentified tyrosine residue(s) in JAK2. This possibility must be formally considered because both the GST/rJAK2(YY:FF) and the GST/(NΔ661)rJAK2(YY:FF) were isolated from Sf21 cells in a tyrosine-phosphorylated state.

Additional experiments using completely unphosphorylated JAK2 forms will add to the validity of the results presented here. However, the findings in this paper do permit us to propose that the activity in the absence of activation loop phosphorylation represents a basal activity state of JAK2 and that the distinctly higher state of activity attained through activation loop phosphorylation represents cytokine-stimulated kinase activity. The basal activity state exhibited by GST/rJAK2(YY:FF) and GST/(NΔ661)rJAK2(YY:FF) apparently utilizes ATP with very low efficiency (*48*) and is capable of autophosphorylation and the transphosphorylation of an exogenous substrate protein. Once the critical tyrosines of the activation loop have been phosphorylated, the enzyme attains an efficient activated state that is capable of kinase activity at ATP concentrations approximately 10000-fold lower than the normal intracellular concentrations. It is possible that the activated JAK2 also becomes more promiscuous and/or more efficient in phosphorylating cellular substrates when in the activated state than when in the basal state. The multiple unidentified tyrosine-phosphorylated protein bands associated only with the GST/rJAK2 and GST/(NΔ661)rJAK2 forms, and not with their Tyr-to-Phe mutants, provide circumstantial evidence for this suggestion (lanes 4 vs 5 of Figures 2 and 5). We show that the basal state, as represented by the GST/rJAK2(YY:FF) mutant, is capable of phosphorylating an exogenous substrate protein. However, our current data do not allow us to comment on the differential behavior of the activated JAK2 versus the basal JAK2 state toward other physiologically relevant substrates, such as the STATs.

It is interesting to note that, rather than providing evidence for a transinhibitory effect of inactive GST/(NΔ661)rJAK2(K:E) on the in situ tyrosine phosphorylation of GST/rJAK2 and GST/rJAK2(YY:FF), which would be consistent with dominant negative behavior, the data in Figure 9 instead show that GST/rJAK2 and GST/rJAK2(YY:FF) transphosphorylate the inactive GST/(NΔ661)rJAK2(K:E). Similar data are shown in Figures 10 and 11. Previous reports (*13*, *39*, *50*) have shown that mutants containing specific inactivating deletions and point mutations in the kinase domain inhibit normal JAK2 function in a dominant negative fashion. It is important to note that the GST/(NΔ661)rJAK2(K:E) and GST/(NΔ661)rJAK2(KYY:EFF) proteins lack much of the IR1 region (amino acids 619−670) of the JH2 domain which has been implicated in the autoinhibitory phenomenon (*49*). Therefore, while our results should not be interpreted as a contradiction of earlier work, our data show that "kinase inactive JAK2" is not necessarily synonymous with "dominant negative JAK2".

The observations of increased activity in amino-terminally deleted mutants of JAK2 are consistent with a model in which the amino-terminal domains possess autoinhibitory properties (*39*, *40*, *49*). The results in Figures 6 and 7 of this paper show that the N-terminal deleted GST/(NΔ661)-rJAK2(YY:FF) is able to autophosphorylate at a lower ATP concentration than does the full-length GST/rJAK2(YY:FF). This observation not only adds credibility to the proposal that an amino-terminal autoinhibitory domain regulates JAK2 activity but also suggests that an increase in catalytic efficiency with respect to ATP is part of the mechanism of autoinhibitory relief.

In conclusion, this paper documents the presence of at least two states of JAK2 activity which are distinguishable by their kinetics of ATP use. The basal activity state is independent of, and the high-activity state is dependent on, the phosphotyrosine status of the activation loop.

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