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Activation of Jak2 Catalytic Activity Requires Phosphorylation of Y^{1007} in the Kinase Activation Loop

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The Janus protein tyrosine kinases (Jaks) play critical roles in transducing growth and differentiation signals emanating from ligand-activated cytokine receptor complexes. The activation of the Jaks is hypothesized to occur as a consequence of auto- or transphosphorylation on tyrosine residues associated with ligand-induced aggregation of the receptor chains and the associated Jaks. In many kinases, regulation of catalytic activity by phosphorylation occurs on residues within the activation loop of the kinase domain. Within the Jak2 kinase domain, there is a region that has considerable sequence homology to the regulatory region of the insulin receptor and contains two tyrosines, Y^{1007} and Y^{1008}, that are potential regulatory sites. In the studies presented here, we demonstrate that among a variety of sites, Y^{1007} and Y^{1008} are sites of trans- or autophosphorylation in vivo and in in vitro kinase reactions. Mutation of Y^{1007}, or both Y^{1007} and Y^{1008}, to phenylalanine essentially eliminated kinase activity, whereas mutation of Y^{1008} to phenylalanine had no detectable effect on kinase activity. The mutants were also examined for the ability to reconstitute erythropoietin signaling in γ2 cells, which lack Jak2. Consistent with the kinase activity, mutation of Y^{1007} to phenylalanine eliminated the ability to restore signaling. Moreover, phosphorylation of a kinase-inactive mutant ($K^{882}E$) was not detected, indicating that Jak2 activation during receptor aggregation is dependent on Jak2 and not another receptor-associated kinase. The results demonstrate the critical role of phosphorylation of Y^{1007} in Jak2 regulation and function.

The Janus tyrosine kinase (Jak) family consists of four members, Jak1, Jak2, Jak3, and Tyk2. Over the past several years, it has become evident that these kinases play a critical role in the functions of receptors of the cytokine receptor superfamily (4, 7, 8, 15, 19). In general, the Jaks associate with the membrane-proximal region of one or more of the receptor chains constitutively or following ligand-induced receptor aggregation. Following ligand binding, there is a rapid induction of tyrosine phosphorylation of the receptor-associated Jaks and a striking increase in their catalytic activity in in vitro kinase assays of the immunoprecipitated Jaks. It is hypothesized that ligand-induced receptor aggregation results in the aggregation of the associated Jaks, which allows Jak auto- or transphosphorylation at sites that regulate catalytic activity. The activated Jaks subsequently tyrosine phosphorylate the receptor chains as well as a variety of substrates that are recruited to the activated receptor complex.

The role of tyrosine phosphorylation of Jaks in regulation of their intrinsic kinase activity, although hypothesized, has only recently been addressed. The regulation of tyrosine kinase activity has been shown to occur in two distinct manners. The family of Src kinases are regulated through the tyrosine phosphorylation of a carboxyl-terminal site (2, 14, 16). It is speculated that this site interacts with the SH2 domain to induce an inactive conformation of the kinase domain. Activation of kinase activity is therefore associated with dephosphorylation of the carboxyl-terminal site. Alternatively, a number of receptor tyrosine kinases are catalytically activated by tyrosine phosphorylation of residues within the activation loop of the kinase domain. In the case of the insulin receptor (6), for which a crystal structure has been determined, it is hypothesized that Y^{1162} in the activation loop lies within the catalytic center in a manner that precludes access to the ATP binding loop as well as substrate. Phosphorylation of Y^{1162} is hypothesized to induce a conformation change that would move Y^{1162} out of the catalytic center and allow kinase activity. The elucidation of the structure of the fibroblast growth factor receptor kinase domain (11) suggests an alternative model in which Y^{653} in the activation loop interferes with substrate binding but not with ATP binding. Again, phosphorylation of Y^{653} is hypothesized to induce a conformation change that would allow substrate access to the catalytic center.

Regarding the Jaks, the lack of an SH2 domain would suggest that their activity is not regulated similarly to the Src family of kinases. In contrast, sequence alignment of the kinase domains of the Jaks with the receptor tyrosine kinases indicates considerable similarity within the activation loop. In particular, the (E/D)YY motif in the activation loop, which is implicated in the regulation of kinase activity, is found conserved among the Jaks. Consistent with a potential role in regulation of kinase activity, recent studies (5) demonstrated that mutation of the two tyrosines in this motif in Tyk2 prevented ligand-dependent activation of kinase activity. We therefore wanted to determine whether this site is phosphorylated in activated Jak2 and whether mutations in this motif would affect activation of Jak2 kinase activity. Our results demonstrate that this motif is tyrosine phosphorylated by Jaks and that mutations of the first tyrosine in the motif dramatically affect kinase activity. Moreover, these mutants are unable to reconstitute the cellular responses to erythropoietin (Epo) in cells lacking an endogenous Jak2.

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MATERIALS AND METHODS

Site-directed mutagenesis of Jak2. Mutation changing Y^{1007} or Y^{1008} of murine Jak2 to phenylalanine was generated by standard PCR methods. The primer sets for $Y^{1007}F$ mutation were as follows: 1, TTA*CCATAT*GAAGTTTACGAG AC; 2, CCTTTACTTTGTAGAATTCTTTGTCCTGCGG; 3, CCGCAGGACA AAGAATTCTACAAAGTAAAGG; and 4, CCCTTGTTTAT*CATTG*CAATC AT. Underlined nucleotides indicate the mutation from Y into F; italicized nucleotides in primers 1 and 4 represent the unique *Nde*I and *Bsr*DI sites in murine Jak2 cDNA, respectively. Two separate PCRs were performed, one with primers 1 and 2 and one with primers 3 and 4. Products were gel purified, mixed, annealed, and subjected to another PCR with primers 1 and 4. The final products were cloned in pCRII (Invitrogen) and fully sequenced to verify the desired mutation. The *Nde*I-*Bsr*DI fragment was then swapped into Jak2 cDNA in pBlueScript SK (Stratagene). $Y^{1008}F$ mutation was generated in the same manner, using the primers 1 and 5 (TGGCTCCTTTACTTTGAAGTATTCTTTGT CCTG) and primers 6 (CAGGACAAAGAATACTTCAAAGTAAAGGAGC CA) and 4. $K^{882}E$ mutation was generated as previously described (19).

Protein expression in insect cells. Jak2 wild-type or mutant cDNA and Stat5a cDNA were subcloned into pVL1392, and recombinant baculoviruses were generated by using the BaculoGold system (Pharmingen) as previously described (12). Insect (*Spodoptera frugiperda* Sf9) cells were cultured in Grace's medium (Life Technologies) and infected or not with pertinent high-titer viruses (multiplicity of infection of 5 to 10). After 60 h, cells were collected and lysed in ice-cold buffer containing 0.5% Nonidet P-40, 50 mM Tris (pH 8.0), 150 mM NaCl, 0.1 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 3 μg of aprotinin per ml, 2 μg of pepstatin A per ml, 2 μg of leupeptin per ml, and 10% glycerol. Lysates were centrifuged at 4°C at 10,000 × *g* for 15 min. The supernatant was used for immunoprecipitation, gel mobility shift assay, in vitro kinase assay, or phosphopeptide mapping.

Generation of stable cell lines expressing Epo receptor and wild-type or mutant Jak2. Epo receptor and Jak2 wild-type or mutant cDNA were subcloned in mammalian expression vector pRK5. Five micrograms each of pRK5/EpoR, pRK5/Jak2WT(or pRK5/JakFY, -YF, or -KE), and 0.5 μg of pGK/Hygromycin were linearized and transfected into γ2 cells (19), using LipofectAmine (Life Technologies). Cells were selected in Dulbecco modified Eagle medium containing 0.2 mg of hygromycin B (Boehringer Mannheim) per ml. Individual clones were picked with cloning rings and tested for expression of Epo receptor and Jak2 by immunoprecipitation and Western blotting. Positive clones were cultured in 10-cm-diameter dishes to 80 to 90% confluency and starved in Dulbecco modified Eagle medium without serum for 12 h before being stimulated or not with 10 U of recombinant human Epo (Amgen) per ml for 15 min and lysed in ice-cold lysis buffer as describe above. Lysates cleared of insoluble materials were used in immunoprecipitation and gel shift assays.

Immunoprecipitation and Western blotting. Antisera specific for Epo receptor and Jak2 have been described elsewhere (21). Antibodies against Stat1, Stat5a/b, and Jak2 were obtained from Santa Cruz Biotechnology. Antiphosphotyrosine was purchased from Upstate Biotechnology. Cell lysates were incubated with the pertinent antibodies for 1 h at 4°C and then with protein A-Sepharose (Pharmacia) for another hour. Sepharose beads were washed three times in lysis buffer and boiled in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer. The supernatants were run on SDS–7.5% polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane (Life Technologies), and the filter was blotted with relevant antibody and visualized with the Amersham ECL detection system.

In vitro kinase assays. Cell lysates were immunoprecipitated with anti-Jak2. The Sepharose beads coupled with Jak2 immune complex were washed three times in lysis buffer and then three times in kinase buffer (10 mM HEPES [pH 7.6], 50 mM NaCl, 0.1 mM Na_3VO_4, 5 mM $MnCl_2$, 5 mM $MgCl_2$). The beads were resuspended in 40 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP (Dupont NEN) and incubated at room temperature for 30 min. Reactions were terminated by addition of 1 ml of cold lysis buffer, and the beads were washed three times in lysis buffer. The proteins were separated on an SDS–7.5% polyacrylamide gel and visualized by autoradiography. For reactions using peptide DFGLTKVLPQDKEYYKVKEPGESPIF, which corresponds to Jak2 residues 994 to 1019, 1 mg of the peptide per ml was added to the reaction mixture as described above. The products were separated on an SDS–20% polyacrylamide gel and visualized by autoradiography.

Phosphopeptide mapping. Analysis of tryptic phosphopeptides was performed as described by Boyle et al. (1). Briefly, Sf9 cells were infected with relevant viruses and cultured for 60 h before being transferred to phosphate-free Grace's medium and incubated with 1 mCi of [^{32}P]orthophosphate per ml for 3 h. Cells were lysed as described above, and the lysates were immunoprecipitated with antiserum against Jak2. After the proteins were separated on SDS–7.5% polyacrylamide gels and visualized by autoradiography, the gel slices corresponding to the position of Jak2 were cut from the gel, and the protein was eluted and digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) and lysyl endopeptidase *Achromobacter* protease I (Wako Chemicals). The digest was mixed with relevant synthetic phosphopeptides and separated in two dimensions on thin-layer cellulose plates. ^{32}P-labeled peptides were visualized by autoradiography. The positions of synthetic phosphopeptides were visualized by ninhydrin staining.

Gel mobility shift assays. Cell lysates containing 10 μg of total proteins were incubated with 2 μg of poly(dI-dC) for 15 min on ice, followed by a 30-min incubation with or without antiserum against Stat5a at room temperature. ^{32}P-, end-labeled GRR probe (GATCAGCATGTTTCAAGGATTTGAGATGTAT TTCCCAGAAAAG) with or without a 100-fold excess of unlabeled probe was added, and the mixture was incubated for 15 min on ice. Samples were separated on 4.5% nondenaturing polyacrylamide gels in 200 mM Tris borate–2.2 mM EDTA. Gels were dried and subjected to autoradiography.

RESULTS

Multiple autophosphorylation sites on Jak2, including Y^{1007} and Y^{1008}. The transphosphorylation of several receptor tyrosine kinases has been shown to be required for activation of catalytic activity (17). Based on the crystal structure of the insulin receptor, it has been hypothesized that phosphorylation of Y^{1162} in the activation loop results in displacement of the residue from the catalytic pocket, thereby allowing activation of kinase activity (6). A comparison of the sequences of the Jak kinase domains with that of the insulin receptor demonstrates significant similarity in the sequences surrounding the insulin receptor Y^{1162} with that of Y^{1007} of Jak2. This finding suggested the possibility that phosphorylation of Y^{1007} in Jak2 plays a similar role in regulating catalytic activity. To assess this possibility, we wished to determine if Y^{1007} was phosphorylated in catalytically active Jak2 and whether this phosphorylation was required for optimal activity.

To identify sites of potential tyrosine autophosphorylation of Jak2, the protein was expressed in Sf9 insect cells with recombinant baculovirus expression constructs. Expression of Jak2 under these conditions results in its tyrosine phosphorylation and the activation of tyrosine kinase activity (13). Similar results were also obtained when Jak2 was expressed in COS-7 cells with mammalian expression vectors (22). Phosphoamino acid analysis of Jak2, produced under either condition, indicated that >90% of the phosphorylation occurred on tyrosine residues (data not shown). A typical tryptic phosphopeptide map of Jak2, produced and labeled in vivo in insect cells, is shown in Fig. 1A. As illustrated, the maps are complex, and approximately 15 major phosphopeptides are typically seen. Comparable maps were obtained from Jak2 produced in insect cells and labeled in vitro, Jak2 produced in COS-7 cells, and endogenous Jak2 activated by cytokine stimulation (data not shown). No phosphorylation was seen with a kinase-inactive Jak2 mutant ($K^{882}E$) (data not shown). To determine if Y^{1007} was phosphorylated, the migration of synthetic phosphopeptides (EY^PY^PK and EY^PYK) was examined. As indicated in Fig. 1, labeled tryptic fragments comigrated with the synthetic peptides, indicating the possible occurrence of tyrosine phosphorylation at both Y^{1007} and Y^{1008}.

To further determine whether the tryptic fragments corresponded to those containing Y^{1008} and Y^{1007}, we produced a mutant Jak2 protein in which Y^{1008} was mutated to a phenylalanine. As indicated below, this mutation does not affect catalytic activity. The tryptic peptide map of this mutant is shown in Fig. 1B. As illustrated, a phosphopeptide corresponding to the doubly phosphorylated peptide was not observed, nor was a peptide detected at the position of migration of the singly phosphorylated peptide. However, a new phosphopeptide which comigrated with a synthetic peptide having the sequence EY^PFK was present. These results support the conclusion that both Y^{1007} and Y^{1008} are phosphorylated in a fraction of activated Jak2. In addition, a fraction is singly phosphorylated; however, it cannot be determined from the results whether this is phosphorylation of Y^{1007}, Y^{1008}, or a mixture of singly phosphorylated peptides.

To further determine whether Y^{1007} and/or Y^{1008} was a

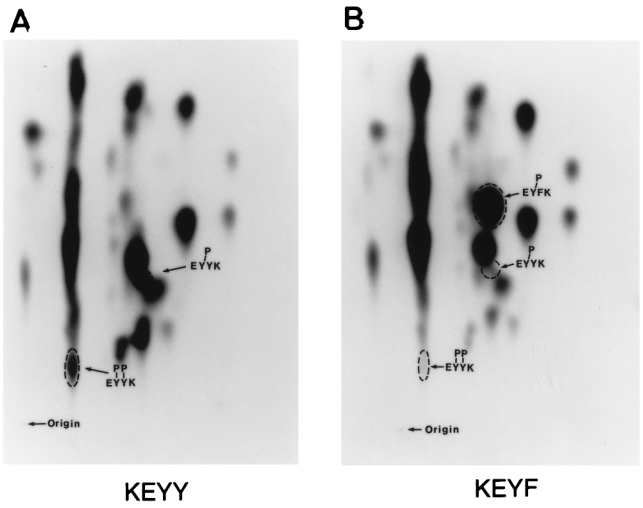

FIG. 1. Phosphopeptide mapping of Jak2. Sf9 cells were infected with baculoviruses that expressed either wild-type Jak2 (A) or the $Y^{1008}F$ mutant (B). Sixty hours after infection, cells were labeled with [^{32}P]orthophosphate. Subsequently, Jak2 was isolated by immunoprecipitation and SDS-PAGE separation. A trypsin and lysyl endopeptidase digest of the isolated Jak2 wild-type or $Y^{1008}F$ mutant protein was mixed with synthetic phosphopeptides EY^PY^PK and EY^PYK (A) or EY^PY^PK, EY^PYK and EY^PFK (B) and resolved in two dimensions on thin-layer cellulose plates. ^{32}P-labeled phosphopeptides were visualized by autoradiography. The positions of synthetic phosphopeptides were determined by ninhydrin staining and are indicated by broken circles. The origin of separation is indicated by an arrow.

potential substrate for Jak2, the ability of Jak2 to phosphorylate a synthetic peptide containing these tyrosines was examined. As illustrated in Fig. 2, Jak2 readily phosphorylated a peptide containing Y^{1007} and Y^{1008}. Tryptic peptide analysis of the phosphorylated peptide indicated that the majority consisted of singly phosphorylated peptides (data not shown). In addition to Jak2, the other Jak kinases (Jak1, Jak3, and Tyk2) were all capable of phosphorylating the Jak2 peptide to comparable levels (data not shown).

Jak2 tyrosine kinase activity is dependent on Y^{1007} but not Y^{1008}. To investigate the potential roles of Y^{1007} and/or Y^{1008}, we made mutants in which the tyrosines were mutated to

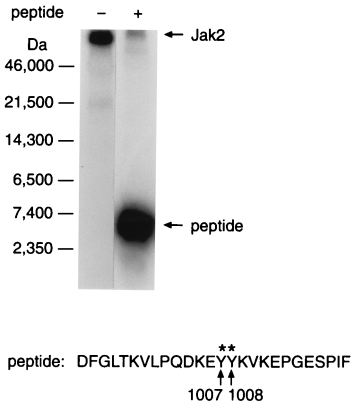

FIG. 2. Jak2 phosphorylation of a Jak2-Y^{1007} peptide. Jak2 immunoprecipitates were prepared from Sf9 cells infected with the wild-type Jak2-expressing virus. In vitro kinase reactions were performed on the immunoprecipitates without or with a synthetic peptide derived from the Y^{1007} region of Jak2. The reaction products were separated by SDS-PAGE and visualized by autoradiography. The positions of Jak2 and the peptide are indicated by arrows. The sequence of the peptide is given at the bottom.

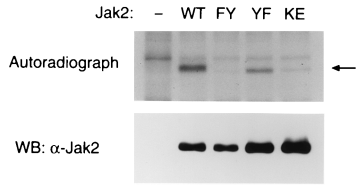

FIG. 3. In vitro kinase activities of Jak2 wild-type and mutants proteins. Sf9 cells were infected with wild-type Jak2 (WT) or the $Y^{1007}F$ (FY), $Y^{1008}F$ (YF), or $K^{882}E$ (KE) mutant. Sixty hours after infection, Jak2 immunoprecipitates were prepared from the cell lysates. Half of the precipitates were subjected to in vitro kinase reactions, separation by SDS-PAGE, and autoradiography (upper panel); the other half were separated by SDS-PAGE gel and subjected to Western blotting (WB) with anti-Jak2 (lower panel).

phenylalanines. For comparison, a kinase-inactive Jak2, containing a mutation in the ATP binding loop ($K^{882}E$), was used. The mutants were produced in insect cells, and catalytic activity was assessed in in vitro kinase assays (Fig. 3). When assayed at approximately comparable levels of protein, as indicated by the Western blots for Jak2 in the lower panel of Fig. 3, there were dramatic differences in phosphorylation of a protein of the expected size for Jak2 (upper panel, arrow). Importantly, like the $K^{882}E$ mutant, the $Y^{1007}F$ mutant had no detectable activity in the in vitro kinase assays. In contrast, the $Y^{1008}F$ mutant retained substantial kinase activity. Lastly, the $Y^{1007}F$/$Y^{1008}F$ double mutant had no detectable activity in this assay (data not shown).

The effects of these mutations on phosphorylations in vivo in insect cells were next examined (Fig. 4). For these experiments, the various mutants were coexpressed in insect cells with expression constructs for Stat5a, and tyrosine phosphorylation was assessed by immunoprecipitation and blotting with a

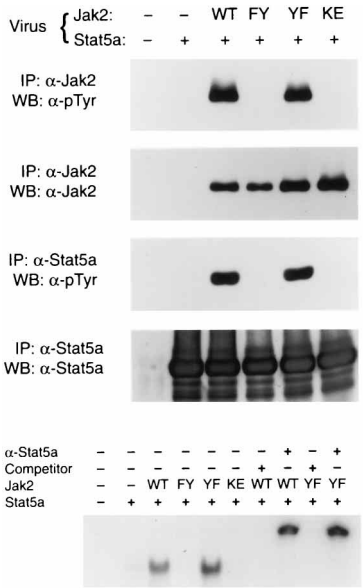

FIG. 4. Tyrosine phosphorylation and activation of Stat5a by Jak2 wild-type and mutant proteins. Sf9 cells were coinfected with Stat5a-expressing virus and wild-type Jak2 (WT) or the Y^{1007} (FY), $Y^{1008}F$ (YF), or $K^{882}E$ (KE) mutant. After 60 h, Jak2 and Stat5a were immunoprecipitated (IP) with specific antibodies and Western blotted (WB) with either antiphosphotyrosine (α-pTyr), anti-Jak2 (α-Jak2), or anti-Stat5a (α-Stat5a), as indicated. Cell lysates were also subjected to gel shift assay without or with unlabeled competitor DNA of the same sequence as the probe, without or with prior incubation with anti-Stat5a (bottom panel).

monoclonal antibody against phosphotyrosine. Expression of wild-type Jak2 with Stat5a results in tyrosine phosphorylation of Jak2 and Stat5a (Fig. 4). In contrast, phosphorylation of neither Jak2 nor Stat5a is seen in cell extracts from cells expressing the $K^{882}E$ mutant. Mutation of Y^{1007} to F eliminated tyrosine phosphorylation of either the Jak2 mutant or Stat5a, while mutation of Y^{1008} had no effect on the ability to phosphorylate the mutant protein in vivo or Stat5a. Somewhat surprisingly, expression of the Y^{1007}/Y^{1008} double mutant in insect cells resulted in a low level of tyrosine phosphorylation. However, this mutant did not significantly phosphorylate Stat5a (data not shown). The cell lysates were also used to assess Stat5a DNA binding activity as a measure of the specificity of tyrosine phosphorylation. No DNA binding activity against a gamma interferon (IFN-γ) activation site-containing probe was seen in the absence of Jak2, while coexpression with wild-type Jak2 and Stat5a resulted in DNA binding activity. Although not shown, expression of Jak2 alone did not give rise to detectable DNA binding activity against a probe containing an IFN-γ-activating sequence. Consistent with the previous results, Stat5a DNA binding activity was present in extracts from cells expressing the $Y^{1008}F$ mutant but not with the $Y^{1007}F$ or $K^{882}E$ mutant. Comparable results were obtained when the various Jak2 constructs were coexpressed with Stat1 (data not shown).

Y^{1007} is required for Epo signaling. To assess the role of Y^{1007} in Jak2-dependent signaling, various mutants were introduced into the γ2 human fibrosarcoma cell line. These cells were selected for the inability to respond to IFN-γ and have been shown to lack Jak2 (10, 19). The cells were transfected with the various Jak2 mutants and were cotransfected with the Epo receptor or with just the Epo receptor as a control. For a control, the Epo receptor was also transfected into the parental 2C4 cells, which contain Jak2. Stable cell lines were derived from the transfections, and lines expressing comparable levels of receptor and Jaks, as assessed by immunoprecipitation and Western blotting, were used for the studies (Fig. 5).

Stimulation of the parental γ2 cells with Epo or the parental cells expressing only the Epo receptor resulted in no detectable tyrosine phosphorylation of the Epo receptor or of Stat5. However, Epo stimulation of cells that expressed both the Epo receptor and wild-type Jak2 resulted in the tyrosine phosphorylation of Jak2, Stat5, and the Epo receptor, demonstrating the critical role that Jak2 plays in Epo signaling. It should be noted that the antiserum used in these experiments detects both Stat5a and Stat5b. We have previously shown that in hematopoietic cells, Epo induces the equivalent phosphorylation of Stat5a and Stat5b (18). Epo stimulation of cells expressing a kinase-inactivate Jak2 resulted in no detectable induction of tyrosine phosphorylation of the Jak2 mutant, the Epo receptor, or Stat5. Cells expressing the $Y^{1007}F$ mutant similarly failed to respond to Epo stimulation by tyrosine phosphorylation of the Jak2 mutant, the Epo receptor, or Stat5. However, Epo stimulation of cells expressing the Y^{1008} mutation responded comparably to cells expressing a wild-type Jak2 with regard to Jak2, Epo receptor, or Stat5 phosphorylation.

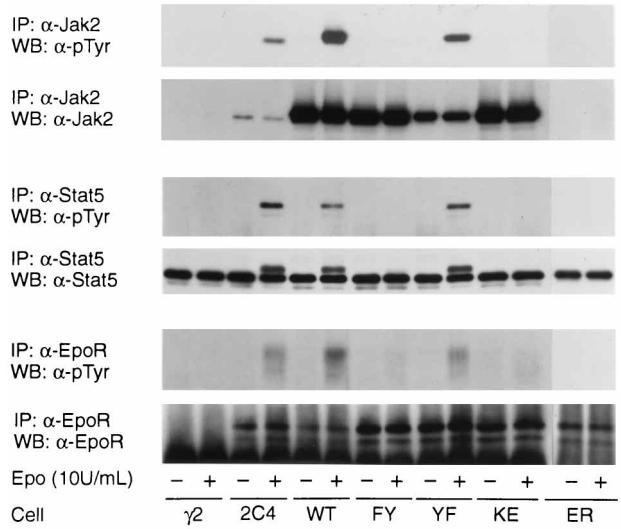

FIG. 5. Epo-induced signaling through wild-type or mutant Jak2 in a fibrosarcoma cell line. Human fibrosarcoma cell line γ2, which does not have endogenous Jak2 or Epo receptor, was cotransfected with Epo receptor and Jak2 wild-type or mutant expression constructs. For a control, the Epo receptor was also transfected into the parental 2C4 cells, which contain Jak2. Stable clones were selected for expression of Epo receptor and wild-type Jak2 (WT) or the $Y^{1007}F$ (FY), $Y^{1008}F$ (YF), or $K^{882}E$ (KE) mutant. 2C4 is the parental line of γ2 and expresses exogenously transfected Epo receptor. ER is γ2 transfected with the Epo receptor alone, without Jak2. Cells were stimulated with recombinant human Epo (10 U/ml) for 15 min or not stimulated after starvation for 12 h in medium without serum. Cell lysates were subjected to immunoprecipitation (IP) with a specific antibody against Jak2, Stat5, or Epo receptor (α-Jak2, α-Stat5, or α-EpoR, respectively) and Western blotting (WB) with antiphosphotyrosine (α-pTyr) or the relevant antibody.

DISCUSSION

Jaks play a critical role in the functions of receptors of the cytokine receptor superfamily (7, 9). However, the mechanism by which the catalytic activity of the Jaks is regulated has only recently been addressed. Our studies demonstrate that phosphorylation of Y^{1007}, within the activation loop of subdomain VII in Jak2, plays a critical role in the regulation of the kinase domain. In addition, recent studies with Tyk2 (5) demonstrated that mutation of the comparably positioned tyrosine pair, $Y^{1054}F/Y^{1055}F$, had a similar effect on Tyk2 catalytic activity. In these studies, however, the role of the individual tyrosines was not examined.

The regulation of Jak catalytic activity by phosphorylation within the activation loop was predicted from studies with a variety of kinases. However, the extent of the regulation can vary significantly among kinases. For example, the kinase activities of the insulin receptor or the fibroblast growth factor receptors are highly dependent on phosphorylations within the activation loop, whereas the catalytic activity of the epidermal growth factor receptor is much less dependent on phosphorylations within the activation loop. The crystal structure of the insulin receptor has shown that Y^{1162}, but not Y^{1163}, sits in the active site and precludes the access of ATP (6). It is hypothesized that phosphorylation of Y^{1162} causes a conformation change the allows access of the ATP binding loop to the catalytic site. The structure further predicts that activation could occur optimally only by transphosphorylation. More recently, the structure of the fibroblast growth factor receptor tyrosine kinase domain has been solved (11). In this case, residues in the activation loop appear to interfere with substrate binding, and not ATP binding, revealing a second potential autoinhibitory mechanism.

An essential question regarding the activation of Jak catalytic activity is, what kinases are required for phosphorylation of the critical sites within the activation loop? The simplest model is one in which individual Jaks can transphosphorylate themselves. Alternatively, certain Jaks may inefficiently transphosphorylate and thus require a second Jak for activation. This is a particularly intriguing model for cases in which two Jaks are recruited to the receptor complex by two different

receptor chains as in the case of the IFN receptors or the interleukin-2-related family of cytokine receptors. Lastly, it is possible that Jak phosphorylation requires other receptor-associated kinases, as exemplified by the requirement of Src family kinases in activation of focal adhesion kinase (3). Our results demonstrate that Jak2 is capable of phosphorylating Y^{1007} when expressed in insect cells and in in vitro kinase reactions and when presented as a peptide. More importantly, however, no phosphorylation of a kinase-inactive mutant was seen in γ2 cells in response to Epo, strongly indicating that Jak2 itself is required for phosphorylation of Y^{1007}. Similarly, no Epo receptor phosphorylation was evident in cells expressing the kinase-inactive mutants. Thus, receptor phosphorylation is mediated either directly by Jak2 or by a kinase that requires activation by a catalytically active Jak2.

The data demonstrate that Y^{1007} and Y^{1008} are sites for phosphorylation by Jak2. In addition, however, the results demonstrate that there are a number of autophosphorylation sites on Jak2. It can be hypothesized that one or more of these sites are important in signal transduction by Jak2. For this reason, we are currently determining which sites are phosphorylated and preparing Y-to-F mutations of these sites. It should be noted, however, that mutations of conserved Y residues other than Y^{1007} and Y^{1008} have been examined (5, 10) and have not affected the receptor functions that could be examined.

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