Identification of Molecular Switch Regulating Interactions of Janus Kinase 3 with Cytoskeletal Proteins

**Background:** Jak3 is a tyrosine kinase, and the mechanism of Jak3 interactions with cytoskeletal proteins is not known.

**Results:** Tyrosine autophosphorylation of SH2 domain of Jak3 facilitated the interactions between the Jak3-FERM domain and cytoskeletal proteins.

**Conclusion:** Results demonstrate the molecular mechanism of interactions between Jak3 and cytoskeletal proteins.

**Significance:** Understanding of Jak3 functions has important implications in transplant biology, epithelial wound repair, cancer metastasis, and immune cell migration.

Janus kinase 3 (Jak3) is a nonreceptor tyrosine kinase expressed in both hematopoietic and nonhematopoietic cells. Although mutations that abrogate Jak3 functions cause different immunological disorders, its constitutive activation leads to various types of cancer. Previously, we demonstrated that Jak3 interacted with actin-binding protein villin, thereby facilitating cytoskeletal remodeling and wound repair. In this study, we characterize the structural determinants that regulate the interactions between Jak3 and cytoskeletal proteins of the villin/gelsolin family. Functional reconstitution of kinase activity by recombinant full-length (wt) Jak3 using Jak3-wt or villin/gelsolin-wt as substrate showed that Jak3 autophosphorylation was the rate-limiting step during interactions between Jak3 and cytoskeletal proteins. Determination of kinetic parameters showed that phosphorylated (P) Jak3-wt binds to P-villin-wt with a dissociation constant ($K_d$) of 23 nM and a Hill’s coefficient of 3.7. Pairwise binding between Jak3 mutants and P-villin-wt showed that the FERM domain of Jak3 was sufficient for binding to P-villin-wt with a $K_d$ of 40.0 nM. However, the SH2 domain of Jak3 prevented P-villin-wt from binding to the FERM domain of nonphosphorylated protein. We demonstrate that the intramolecular interaction between the FERM and SH2 domains of nonphosphorylated Jak3 prevented Jak3 from binding to villin and that tyrosine autophosphorylation of Jak3 at the SH2 domain decreased these intramolecular interactions and facilitated binding of the FERM domain to villin. Thus we demonstrate the molecular mechanism of interactions between Jak3 and cytoskeletal proteins where tyrosine phosphorylation of the SH2 domain acted as an intramolecular switch for the interactions between Jak3 and cytoskeletal proteins.

Janus kinases (Jaks) are a family of nonreceptor tyrosine kinase with four members: Jak1, Jak2, Jak3, and Tyk2. Like other members, Jak3 mediates signals initiated by cytokine through interactions with receptors for IL-2, IL-5, IL-7, IL-9, and IL-15 via the common γ chain of these receptors (1). Jak3 protein contains seven Jak homology (JH)² domains common with other Jak proteins. The characteristic feature of the Jaks is the presence of a fully functional tyrosine kinase domain (JH1) and a catalytically inactive pseudokinase domain (JH2) (3). Apart from these two, Jaks also contain five other conserved regions. The recently described JH3-JH4 regions have homology with SH2 domains (5). Although the presence of the SH2 domain indicates interactions with other signaling molecule, the specific signaling partner(s) has not been identified. On the other hand, the JH6-JH7 domains have homologies with the FERM domain found in molecules such as Band 4.1, ezrin, radixin, and moesin. Although the FERM domains mediate intermolecular interactions with cytokine receptor (6), they are also involved in intramolecular binding to JH1 kinase domain, thereby enhancing the kinase activity (7). The crystal structure of the JH1 kinase domain has been solved (PDB ID:1 YVJ) but there is no report on the functions and/or crystal structure of other domains of Jak3. Previously, we reported that intestinal epithelial cells (IECs) express functional specific Jak3 (8, 9), whose biological functions had been presumed to be largely limited to lymphocyte and macrophage populations (1), and proposed mechanisms through which activated Jak3 regulated mucosal wound repair through its interactions with cytoskeletal protein villin (8) and mucosal homeostasis (9).

Villin is an actin-binding protein expressed in specific epithelial cells where it can nucleate, cap, sever, and bundle actin filaments (10). Villin knock-out mice showed compromised mucosal wound repair (11), and Jak3 mediated tyrosine phosphorylation of villin was necessary for its role in wound repair (8). However, the molecular mechanism of Jak3 interactions with villin is not known. In this study, we demonstrate a molecular switch on Jak3 that regulates its interactions with cytoskeletal proteins including epithelial-specific villin and ubiquitously expressed gelsolin.
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EXPERIMENTAL PROCEDURES

Cell Culture, IL-2 Treatment, Wound Closure, and Cell Proliferation Assays—HT-29 CI-19A is a human-derived differentiated IEC line. Methods for culture maintenance, IL-2 treatment, wound closure, and cell proliferations were reported before (8, 9).

Site-directed Mutagenesis—Mutations were created by introducing a stop codon (TGA) at different positions in full-length Jak3 cDNA as reported (12). Jak3-SH2 domain with N-terminal FLAG tag in p6X His-ET vector was synthesized by a commercial facility (Integrated DNA Technologies Inc.).

Expression and Purification of the Recombinant Proteins—Wild type or mutant Jak3 cDNAs cloned in pGEX-4T or p6X His-ET were expressed in Escherichia coli BL21 or TKX1 cells using protocols as reported before (12) and detailed in the supplemental Methods.

In Vitro Kinase Assay and Protein-Protein Interaction Study—In vitro kinase and pairwise binding assays were developed (available through the Texas A&M University System, Office of Technology Commercialization, Disclosure 3196HSC10). Kinetic parameters were determined as reported (12).

Stable Transfection—pcDNA-HA-Jak3-wt and pcDNA-HA-Jak3-V484* were stably transfected into the HT-29 CI.19 A cells using methods as reported before (9).

Immunoprecipitations (IP), Immunoblotting (IB), and Immunofluorescence Microscopy (IM)—Standard methods for IP, IB, and IM were used as reported before (8), using villin, HA (Santa Cruz Biotechnology Cruz), pY20 (MP Biomedicals), GST (Milipore), His (GenScript), and FLAG (Sigma) antibody.

RESULTS

Recombinant Jak3 Autophosphorylates Itself and Transphosphorylates Cytoskeletal Proteins of Villin/Gelsolin Family—The molecular mechanism and the structural determinants that regulate Jak3 interactions with villin are not known. As a first step to determine these, we expressed and purified the phosphorylated (P) and nonphosphorylated forms of Jak3-wt and villin-wt using the TKX1 and BL21 expression systems, respectively (Fig. 1A). To determine whether the nonphosphorylated form of Jak3-wt was functionally active, we reconstituted its in vitro kinase activity. Since immunoprecipitated Jak3 autophosphorylates itself (15), we determined the autophosphorylation of recombinant Jak3-wt. As shown in Fig. 1B, Jak3-wt autophosphorylated itself in a time-dependent manner with a $t_{1/2}$ (the time taken to reach half of the maximum phosphorylation) of 135 s. To further confirm these, we determined the dose effect of Jak3 inhibitor CP-690550. Using crystal structure studies, it was shown that CP-690550 directly bound to the kinase domain of nonphosphorylated Jak3 (14). Fig. 1C showed that CP-690550 inhibited Jak3 autophosphorylation in a dose-dependent manner with an inhibition constant ($IC_{50}$) of 128 nM. Because autophosphorylation of Jak3 led to the activation of Jak3 (13), we determined whether the autophosphorylated Jak3-wt could transphosphorylate cytoskeletal proteins of the villin/gelsolin family. As shown in Fig. 1D, incubation of villin-wt with activated Jak3-wt (from Fig. 1B) led to phosphorylation of villin-wt in a time-de-
induced wound repair (8). Here we showed that IL-2 treatment also led to tyrosine phosphorylation of Jak3-V484* that co-immunoprecipitated with villin only in the presence of IL-2 (Fig. 2B). To further confirm these, we used a pairwise binding assay using tyrosine-phosphorylated Jak3-V484* and P-villin-wt generated using TKX1 cells. As shown in Fig. 1J (sixth bar from the left), tyrosine phosphorylation of the SH2 domain in Jak3-V484* was necessary for the interactions of the FERM domain with P-villin-wt. Determination of IL-2 functions in IEC showed that deletion of the pseudokinase and kinase domain resulted in increased deposition of villin-HA-Jak3V484* complex at the cell margin with defective cell periphery (Fig. 2C) and F-actin turnover (data not shown), which were absent in HA-Jak3-wt-expressing cells. However, all untreated control cells showed no difference (supplemental Fig. S1C). Moreover, IL-2-induced wound closure and cell proliferation were decreased in these mutant cells compared to their wt counterparts (data not shown).

**Intramolecular Interactions between FERM and SH2 Domains of Jak3 Prevent Jak3 Interactions with Villin—**Because nonphosphorylated Jak3-FERM (Jak3-G257*) domain interacted with P-villin-wt but only the phosphorylated form of the Jak3-FERM plus SH2 (Jak3-V484*) domains interacted with P-villin-wt, we determined how the SH2 domain regulated the interaction between FERM and SH2 domains of Jak3.
interactions between Jak3 and villin. To do that, first we determined whether the Jak3-FERM domain interacted with the Jak3-SH2 domain and whether these interactions were affected by tyrosine phosphorylation of either of these domains. Phosphorylated and nonphosphorylated forms of GST-tagged Jak3-FERM and His-tagged Jak3-SH2 domain proteins were expressed and purified using the BL21 and TKX1 expression systems, respectively (Fig. 2D). Pairwise binding kinetics showed that the nonphosphorylated Jak3-FERM domain interacted with the Jak3-SH2 domain with a \( K_d \) of 72 nM (Fig. 2E); however, tyrosine phosphorylation of the Jak3-SH2 domain resulted in decreased affinity between the SH2 and FERM domain, which was reflected by an increase in \( K_d \) from 72 nM to 1.73 \( \mu \)M (Fig. 2F). On the other hand, tyrosine phosphorylation of the Jak3-FERM domain increased its affinity for the SH2 domain, and the affinity was highest when the FERM domain was phosphorylated and the SH2 domain was not phosphorylated (supplemental Fig. S2, A and B, and supplemental Table ST1).

Taken together these results showed that there were intramolecular interactions between the FERM and SH2 domains of Jak3 that prevented its interactions with phosphorylated villin. Moreover, tyrosine phosphorylation of Jak3 specifically at the SH2 domain was necessary to decrease the interactions between the SH2 and FERM domains, making the FERM domain available to interact with phosphorylated villin (Fig. 2G).

**DISCUSSION**

Jak3 is involved in regulation of different signal transduction pathways mainly through its association and activation of the common \( \gamma \) chain of diverse cytokine receptor including IL-2.
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(1). Inactivating mutation of Jak3 leads to childhood immuno-
deficiency (3, 15), and its abnormal activation is associated with
hematologic and epithelial malignancies (3, 4), indicating that
regulation of its activity is essential for normal hematopoietic
and epithelial functions. Although nonhematopoietic expres-
sion of this protein has been reported, the understandings of
Jak3 functions in these cells were very limited (1, 2). Recently,
we showed that Jak3 plays an essential role during mucosal
wound repair and homeostasis (8, 9). Interestingly, although
Jak3 interactions with cytoskeletal protein villin were essential
for its wound repair function (8), the mechanism of Jak3 inter-
actions with cytoskeletal proteins were not known. To achieve
this, we characterized the kinetics parameters of Jak3 auto-
phosphorylation and its transphosphorylation of villin. Con-
sistent with our previous study where we showed that Jak3
kinase domain phosphorylated villin (8), the current study
showed that full-length Jak3 also phosphorylated villin and
gelsolin where the $K_d$ of villin/gelsolin transphosphorylation
was lower than that of Jak3 autophosphorylation (Fig. 1).
This indicated that Jak3 autophosphorylation was rate-limit-
ing during Jak3-cytoskeletal protein interactions. We fur-
ther confirmed these interactions by inhibition studies where
CP-690550 inhibited both Jak3 autophosphorylation and
villin transphosphorylation by Jak3. Although CP-690550 had
been reported to bind directly to the kinase domain of truncated
Jak3 with IC$_{50}$ of 35 nM (14), we showed that full-length Jak3
also bound to this inhibitor, albeit with a higher IC$_{50}$ (128 nM).
These indicated that the presence of other domains in Jak3-wt
decreased the binding affinity of inhibitor to its kinase domain.

Information on the structure-function relationship of the
Jak3-cytoskeletal protein complex showed that P-Jak3-wt
interacted with P-villin-wt in a dose-dependent manner with a
$K_d$ of 23 nM and a Hill’s coefficient of 3.7, indicating a high
affinity and cooperative binding between Jak3 and villin (Fig. 1).
Due to lack of crystallographic data, the structure-function
relationship between Jaks and their interacting partners still
remains largely elusive. However, most of the Jak3 bind to their
cytokine receptor through the N-terminal FERM domain,
which encompasses from JH7 through a part of JH4 domain
(16). In the case of Jak3, the FERM domain also interacted with
the JH1 kinase domain, thereby activating it (14). Our data
showed that tyrosine phosphorylation of Jak3 was necessary for
its interactions with villin and that these interactions took place
directly between the FERM domain of Jak3 and villin
(Fig. 1). Tyrosine phosphorylation of the FERM domain
per se was not necessary for this interaction; however, the tyro-
sine phosphorylation of the SH2 domain was essential. This was
because under nonphosphorylated conditions, there were
intramolecular interactions between the FERM and SH2
domain of Jak3 that prevented villin from interacting with Jak3.
Tyrosine phosphorylation of the SH2 domain decreased its
affinity for the FERM domain (F1–F3), resulting in disruption
of the interactions between these two domains, and this facili-
tated the interactions between the (now free) FERM domain
of Jak3 and villin (Fig. 2G). Conversely and interestingly, our data
also showed that tyrosine phosphorylation of the FERM
domain increased its affinity for the SH2 domain. However,
there was substantial decrease in affinity for the FERM domain
when the SH2 domain was tyrosine-phosphorylated irrespec-
tive of the phosphorylation status of the FERM domain (sup-
plemental Table ST1). Moreover, pairwise binding showed that
nonphosphorylated Jak3-G257* directly bound to villin. These
data, combined with data using the IEC culture model, which
showed that IL-2 activation resulted in tyrosine phosphoryla-
tion-dependent interactions between Jak3-V484* and villin,
indicated that tyrosine phosphorylation of the SH2 domain of
Jak3 played a major role during interactions between Jak3 and
villin. It was reported that the clover-shaped FERM domain is
composed of three subdomains: F1 with a ubiquitin-like
$\beta$-grasp fold, F2 with an acyl-CoA-binding protein-like fold,
and F3 that shares the fold of the phosphotyrosine binding or
pleckstrin homology domains (3). We predict that the F3 sub-
domain may be responsible for the intermolecular interactions
between tyrosine-phosphorylated villin and the FERM domain
(Fig. 2G). Previously, we reported that Jak3 regulated IL-2-induced
wound closure and proliferation in IEC (8, 9). The pres-
ent study showed that although Jak3-V484* interacted with vil-
lin, lack of kinase and pseudokinase domains compromised
Jak3-mediated physiological functions. This could be due to
defective F-actin turnover and failed signal integration leading
to surface defects induced by Jak3 mutants (Fig. 2C) and loss of
cobblestone morphology as seen in HA-Jak3-wt-expressing
epithelial cells (Fig. S1B). Taken together, these results showed
for the first time that the molecular mechanism of the interac-
tions between Jak3 and cytoskeletal proteins of the villin/gelso-
lin family and the structural determinants of Jak3 are responsi-
ble for these interactions.

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