Biochemical and Biophysical Research Communications **278,** 175–182 (2000)
doi:10.1006/bbrc.2000.3757, available online at http://www.idealibrary.com on IDEAL®

Computational and Functional Analysis of the Putative SH2 Domain in Janus Kinases

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Received September 25, 2000

Src homology 2 (SH2) domains interact in a highly specific manner with phosphorylated tyrosine residues on other signaling molecules. Protein tyrosine kinases (PTK) frequently contain SH2 domains, which often control signaling specificity. The Janus Kinases (JAKs) are a family of PTKs involved in signal transduction pathways mediated by various cytokines. Initial characterization of JAKs showed no identifiable SH2 domain. However, we have found substantial evidence supporting the existence of an SH2 domain in JAKs through the use of various web-based computational analysis programs. Predictive secondary and tertiary structures recognize an SH2 domain in JAKs. In addition, a three-dimensional homology model was constructed using the SH2 domains of Src tyrosine kinase and Syp tyrosine phosphatase as templates. These results, in conjunction with preliminary binding studies showing interactions with tyrosine phosphorylated proteins in activated splenocytes, suggest a functional role for this domain in JAKs. © 2000 Academic Press

Key Words: **Janus tyrosine kinase; SH2 domain; structure determination; web-based computational analysis.**

SH2 domains are non-catalytic domains capable of binding tyrosine phosphorylated polypeptides and are involved in protein–protein interactions and cellular signaling. These domains interact in a highly specific manner with phosphorylated tyrosine residues of other signaling molecules and in doing so form highly integrated pathways for transmission of signals. SH2 domains are characteristic of many different cytoplasmic protein tyrosine kinases (PTKs) involved in intracellular signaling.

The Janus Kinases (JAKs) are a family of PTKs that play critical roles in the signal transduction pathway mediated by cytokines, interferons, and growth factors. In the initial characterization of Tyk2, the first JAK family member identified, it was argued that a new class of PTKs was revealed because they contained two kinase domains found in tandem in the C-terminus and they lacked both a transmembrane domain and an SH2 domain. In the cloning of murine JAK2, it was noted that the sequence, GLYVLRWS, has a weak homology to the core sequence of SH2 domains (FLVRES), however no additional homology was noted (1). Therefore, JAKs were structurally categorized based upon sequence homology between family members into seven homology domains (JH7-1) (2). Current research and review articles consistently point to the absence of an SH2 domain in the JAKs (3–7). We and others (8–12) suggest that JAKs in fact contain a divergent SH2 domain, which spans JH4-3 domains. A comparison of the region upstream of the kinase domains (JH2-1) against the SWISSPROT database, using the FASTA program, revealed a significant similarity to bovine GTPase stimulatory protein SH2 domain (13). More contemporary pattern searching tools have pointed to the presence of an SH2 domain in JAKs as well as several other known domains including an Band 4.1/ezrin-radixin-moesin (ERM) domain (JH7-6) (9–12). The presence or absence of an SH2 domain is of particular significance, since signaling specificity could depend on proteins that interact with JAKs through this domain.

Structure prediction, computational analysis and homology modeling have recently become a widely used technique for many applications such as the comparison of proteins between species, the investigation of binding sites of proteins, and many other applications (14–18). We examined the putative SH2 domain in JAKs and found based on predictive primary, secondary, and tertiary structure analysis that it is likely that this region is an SH2 domain. Furthermore, through the use of a T cell lysate binding assay, a functional role for this domain is examined.

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0006-291X/00 $35.00

FIG. 1. Alignment of the putative SH2 domain of JAK proteins with SH2 domains of known proteins. The sequences of human Tyk2 (hTyk2), human Janus Kinase 1 (hJAK1), murine JAK1 (mJAK1), rat JAK1 (rJAK3), chicken JAK1 (cJAK1), carp JAK1, zebrafish JAK1, hJak2, mJAK2, rJAK2, hJAK3, mJAK3, rJAK3, cJAK3, drosophila JAK (hopscotch), viral Src (vSrc), human p56 tyrosine kinase (hp56), murine tyrosine phosphatase Syp (mSyp), human GTPase-activating protein ras p21 (GAP_N_), and human signal transducer and activator of transcription 1 (hSTAT1) were obtained from GenBank. All conserved amino acids are shown as white on a black background and all similar amino acids are shaded gray. The invariant arginine in βB5 (asterisk) that forms the bottom of the phosphotyrosine binding pocket and the βD5 residue (# sign) that determines group classification of the SH2 domains are shown. Alignments were performed using CLUSTAL W.

MATERIALS AND METHODS

Prediction of patterns and profiles. The sequences of all JAK family members were compared to PROSITE (19, 20) and Pfam (21, 22) in order to identify any known patterns or profiles. The returned SH2 domains were aligned using the Clustal W 1.8 algorithm (23).

Prediction of secondary structure. The SH2 domains identified by PROSITE and Pfam for human JAK1, 2, and 3 (amino acids 427–532, 401–482, and 375–454, respectively) were submitted to three independent secondary structure programs (24–26). NNPredict assigns a secondary structure type for each residue and uses the tertiary class (none, all-α, all-β, or α/β) of the protein in making the prediction (27, 28). PredictProtein uses neural networks to assign helix, strand, or loop to each residue based upon a multiple sequence alignment (29–31). PSIPred uses a PSI-BLAST generated profile for secondary structure prediction (32).

Prediction of tertiary structure. The tertiary structure of human JAK 1, 2, and 3 (amino acids 427–532, 401–482, and 375–454, respectively) were evaluated using three independent methods, 3D-PSSM, UCLA-DOE Fold Recognition Server, and SWISS-MODEL (33–35). 3D-PSSM uses one- and three-dimensional sequence profiles combined with secondary structure (PSI-Pred) and solvation potential information to perform protein fold recognition (36, 37). The results are sorted by E-value, where E-values below 0.05 are highly confident. The UCLA-DOE Fold Recognition Server (38) computes a Z-score using a distribution of raw scores of all folds. The Z score represents the number of standard deviations above the mean alignment for other sequences of similar length and a Z-score of 5.0 $\pm$ 1.0 is considered the confidence threshold. SWISS-MODEL is a protein modeling server, which runs on the software ProModII (39–41). Reference structures are used to perform homology modeling.

Construction of three dimensional homology model. The homology model was constructed (35, 42) using the template structures of vSrc (1sha) and mouse Syp (1aya) (coordinates obtained from the PDB). These two proteins were superimposed and the resulting sequence alignment generated by Swiss-Pdb Viewer was manually edited so that it was identical to the results obtained from the Clustal W analysis (see Fig. 1). Then the amino acid sequences of either human JAK2 or 3 (aa 401–500 and 375–474, respectively) were threaded onto the templates using the Magic Fit option. Again, any discrepancies in the overall alignment, as compared with Fig. 2, were adjusted. The resulting model was submitted to Swiss Model optimize (project) mode where models are generated with ProModII and energy minimization is performed using Gromos96 (39).

Plasmid construction. A partial cDNA encoding wild-type chicken JAK3 (cJAK3) was previously cloned from a T cell library and full length sequence was obtained using 5′ RACE. Full length cJAK3 (aa 1–1107) and JH4-3 domains (aa 285–513; the potential SH2 domain) were PCR amplified and sub-cloned in frame into pGEX-4T (Amersham Pharmacia Biotech) for expression of glutathione (GSH) sulfotransferase (GST)-fusion proteins.

Cell culture and cell lysate preparation. Concanavalin A (Con A) activated T cell-enriched splenocytes were isolated from 4-week-old

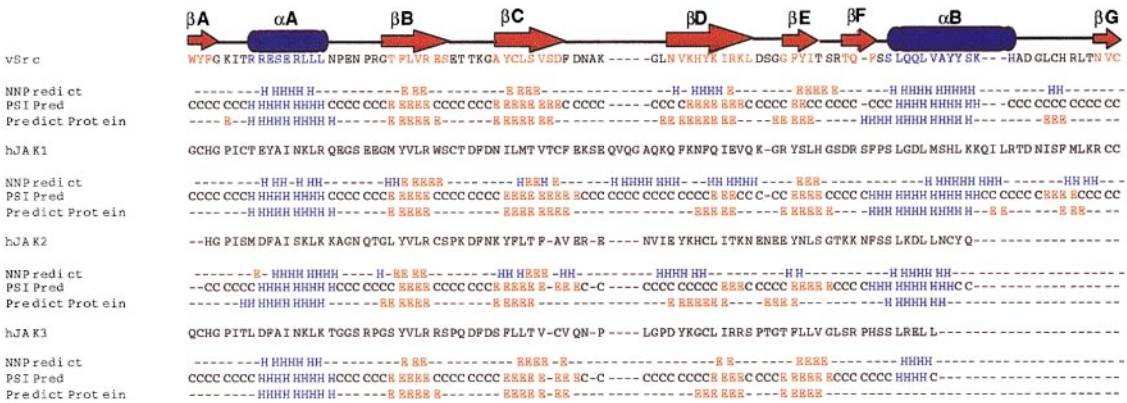

FIG. 2. Comparison of secondary structure predictions of vSrc and hJAKs using three methods. All H's represent an α helix element, all E's represent a β strand element, all -'s represent a turn element (NNPredict), all C's represent a coil (PSIPred), and all -'s represent a loop (PredictProtein). The nomenclature depicted by the β sheets (red arrows) and the α helices (blue barrels) are based upon the consensus nomenclature for the secondary structure elements of SH2-containing proteins and was generated based upon the prediction. The exact amino acids corresponding to the secondary structure elements, as described in the crystal structure of v-Src, are color coordinated.

chickens as previously described (43). After 24 h, Con A was neutralized by the addition of α-methylmannopyrannoside (0.05 M), and recombinant chicken IL-2 (44) was added (10^{-9} M) for 15 min. Cells were collected, washed once in ice-cold phosphate buffered saline (PBS), solubilized in lysis buffer (150 mM NaCl, 50 mM Tris [pH 8], 2.5 mM $MgCl_2$, 10 mM NaF, 5 mM NaPyrophosphate, 1% NP-40, 1.0 mM Na_3VO_4, 0.8 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) on ice for 20 min and centrifuged at 14,000*g* for 10 min at 4°C. The supernatant was used for the *in vitro* binding assay. The buffers used in the lysate preparation and the binding assay were adapted from Pollack *et al.* (45).

In vitro binding assay. GST fusion proteins were purified from bacteria by affinity chromatography with glutathione-agarose beads in Buffer A (150 mM NaCl, 50 mM Tris-Cl [pH 7.4], 10.3 mM NaF, 5 mM NaPyrophosphate, 0.8 mM PMSF, 1.05 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The immobilized fusion proteins were washed twice in Buffer B (Buffer A containing 500 mM NaCl) and three times in Buffer C (25 mM Tris-Cl [pH 7.4], 50 mM KCl, 10 mM $MgCl_2$, 2% glycerol, 0.1% Triton X-100, 10.3 mM NaF, 5 mM NaPyrophosphate, 100 μg/ml BSA, 0.8 mM PMSF, 1.05 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). T cell lysate (pre-cleared twice with GSH-linked beads (Pierce) followed by GSH-linked beads with GST bound) was added and incubated with the immobilized GST fusion proteins at 4°C for 30 min followed by washing twice with Buffer C. The proteins bound to the immobilized GST-fusion proteins were solubilized by boiling for 5 min in SDS–PAGE sample buffer (250 mM Tris-HCl [pH 6.8], 10% SDS, 10% β-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue), separated by SDS–PAGE, transferred to nitrocellulose, and immunoblotted with α-pY (1:2500, Zymed).

RESULTS

Profiles and Pattern Predictions of the Primary Structure of JAKs by PROSITE and Pfam

PROSITE identified a putative SH2 motif in the majority of JAK proteins and an SH2 domain was recognized in the three JAKs not detected by the PROSITE analysis (mouse JAK3, rat JAK3, and human Tyk2) using Pfam-B (PB002025). The profile used by PROSITE to detect SH2 domains (PDOC50001) includes JAK1 and JAK2 as recent additions, but they are not traditionally included in this protein domain profile. Pfam-B contains SH2 domains that are not currently detected by the Pfam model, and identified an SH2 domain in all JAKs except the *Drosophila* homolog, hopscotch.

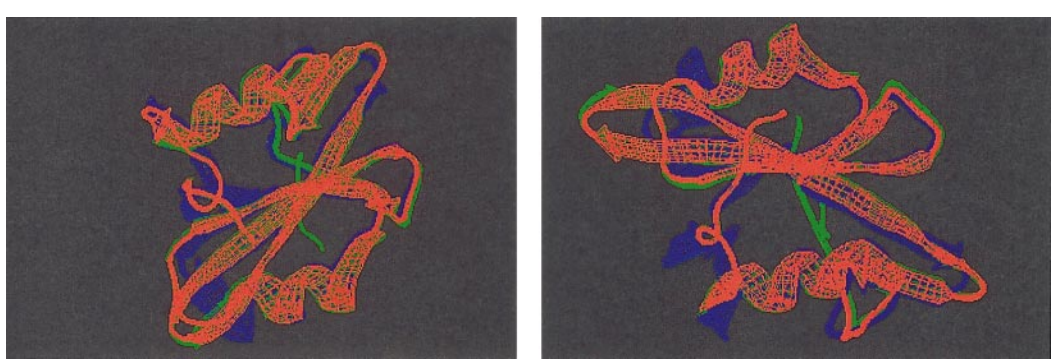

FIG. 3. Tertiary structure prediction of the SH2 domain of (A) hJAK2 (aa 401–500) and (B) hJAK3 (aa 375–474). Ribbon diagram generated by Swiss-PdbViewer. hJAK2 and hJAK3 are indicated in red, vSrc (PDB 1sha) in green, and mSyp (PDB 1aya) in blue. Both vSrc and mSyp coordinates were obtained from the Protein DataBank.

TABLE 1

Reference Structures Identified Using 3D-PSSM and UCLA-DOE Fold Recognition Server

JAK	SH2-containing proteins identified	ID#[a]	3D-PSSM E value[b]	UCLA-DOE Z score[c]
hJAK1	p56 tyrosine kinase	1lkk	2.70e-03	—
	tyrosine kinase Syp	1aya	2.75e-03	—
	Grb2	1tze	2.83e-03	—
	phosphatidyl inositol 3-kinase	1pic	3.01e-03	—
hJAK2	p56 tyrosine kinase	1lkk	2.72e-03	—
	p56 tyrosine kinase	1lck	—	3.68
	tyrosine kinase Syp	1aya	2.79e-03	3.60
	phosphatidyl inositol 3-kinase	1pic	3.62e-03	5.20
	Grb2	1tze	2.80e-03	—
	Abl tyrosine kinase	2abl	2.92e-03	—
	v-Src tyrosine kinase	1sha	—	5.30
hJAK3	p56 tyrosine kinase	1lkk	2.73e-03	—
	p56 tyrosine kinase	1lck	—	4.08
	tyrosine kinase Syp	1aya	2.73e-03	—
	phosphatidyl inositol 3-kinase	1pic	5.41e-03	4.20
	Grb2	1tze	2.77e-03	—
	Abl tyrosine kinase	2abl	2.90e-03	—
	v-Src tyrosine kinase	1sha	—	4.36

[a] ID# indicates the protein data bank ID code.
[b] An E value below 0.05 is a highly confident score from the 3D-PSSM server.
[c] A Z score of 5.0 ± 1.0 is within the confidence threshold from the UCLA-DOE server.

A multiple sequence alignment of the putative SH2 domains of the JAKs and known SH2 containing proteins was performed using Clustal W (Fig. 1). This alignment shows that JAKs contain the invariant arginine (βB5) located in the core sequence of all SH2 domains (indicated by the asterisk, *). The terminal guanidinium nitrogens of this arginine bind to the two phosphate oxygens of the phosphotyrosine moiety of the cognate binding protein. The importance of this residue is indicated by the fact that it is the only invariant residue in all SH2-containing proteins, and is required for binding activity (46, 47). The residue (βD5) which is critical in determining the subclassification of SH2 domains is indicated by the number sign (#) in Fig. 1. The corresponding residue in JAK2s and JAK3s is a cysteine, thus assigning them to subgroup III of SH2 domains. This predicts that JAKs 2 and 3 would interact with a protein containing the motif pY-hydrophobic-X-hydrophobic, with strong selection for a methionine at the +3 position. JAK1s, TYK2, and hopscotch have a phenylalanine in this position, which is characteristic of subgroup I and predicts recognition of a pY-hydrophilic-hydrophilic-hydrophobic phosphopeptide (48). This difference among JAK family members provides insight into the specificity of their interactions with other proteins.

Pattern analysis combined with sequence alignments suggests that JAKs do contain an SH2 domain. However, a more thorough assignment of structure can be performed using secondary structure predictions.

Secondary Structure Prediction of Human JAK1, 2, and 3 by NNPredict, PSIPred, and PredictProtein

A secondary structure diagram indicating the predicted location of the α helices and β sheets in the putative SH2 domain of human JAK1, 2, and 3 is displayed in Fig. 2, and compared to the secondary structure of vSrc SH2 domain. The secondary structure predictions for vSrc appear to be reasonably correct when compared to the experimentally determined crystal structure (49), although these programs failed to predict the three small β strands, βA, βF, and βG (3–4 amino acids each). These same three β strands were not identified in JAKs either. By using the multiple sequence alignment as a guide, the majority of the secondary structural elements of JAKs mirrored those of vSrc. Based upon these results, it is reasonable to propose that these proteins would fold in a similar manner. We therefore investigated the predicted tertiary fold of JAKs.

Tertiary Structure Prediction of Human JAK1, 2, and 3 by 3D-PSSM and UCLA-DOE Fold Server

Table 1 shows results returned by 3D-PSSM and UCLA-DOE Fold Recognition Servers, when used to analyze the tertiary structure of human JAK 1, 2, and 3. All of the proteins returned by 3D-PSSM have E values below 0.05 and therefore are potential reference structures for the SH2 domain in JAKs. However, the proteins returned by the UCLA-DOE fold server are

not all above the confidence threshold. In fact, for human JAK1, no proteins were identified by this program. For human JAK2, two proteins (v-src and phosphatidyl inositol 3-kinase) were above the confidence threshold. For human JAK3, three proteins (v-src, phosphatidyl inositol 3-kinase, and p56 tyrosine kinase) were above the threshold (Table 1). Based on these results, the SH2 domains of two proteins were chosen as templates for constructing the three-dimensional homology model: v-src tyrosine kinase transforming protein (PDB 1sha) and murine tyrosine phosphatase syp N-terminal SH2 domain (PDB 1aya).

Construction of Three-Dimensional Homology Models with Swiss Model Server and Swiss Pdb-Viewer

In constructing the three-dimensional model, we found that inclusion of 20 additional amino acids C-terminal to the SH2 domains of human JAK2 and 3 (amino acids 401–500 and 375–474, respectively), improved the SH2-type fold. In fact, classical SH2 domains are approximately 100 amino acids in length (50) but PROSITE and Pfam results contained only ~80 amino acids. Therefore, the second α helix and the last β strand would be missing if additional C-terminal amino acids were not included.

The coordinates of the reference structures (src, 1sha and syp, 1aya) were obtained from the PDB (39), the two reference structures were superimposed, and the sequence alignment generated by this software was edited to match that generated by the Clustal W analysis (Fig. 1). Following manual editing in accordance with the Clustal W results, the amino acid sequence of human JAK2 or human JAK3 was threaded onto these two reference structures. The resulting model (Fig. 3) (obtained with Swiss Model optimize (project) mode to perform energy minimization) shows the overall fold of an SH2 domains, with antiparallel β sheets flanked by α helices. This represents the first three-dimensional view of an SH2 domain in JAK proteins; confirmation awaits determination of the crystal structure.

In order to determine if this model of an SH2-type fold in JAKs is simply a result of a "forced fit," another phosphotyrosine interaction domain (PID or PTB for phosphotyrosine binding domain) was used as a test (data not shown). PID/PTB domains are slightly larger than SH2 domains (approximately 160 amino acids) and the topography is a globular domain with an antiparallel beta sheet. Shc transforming protein and Sck kinase PID domains were superimposed and human JAK2 and 3 (amino acids 401–560 and 375–534, respectively) were threaded onto the model. The returned model contains no real defined beta sheets or helices. Instead, there were large unlikely bond lengths and angles indicating no real fold was possible.

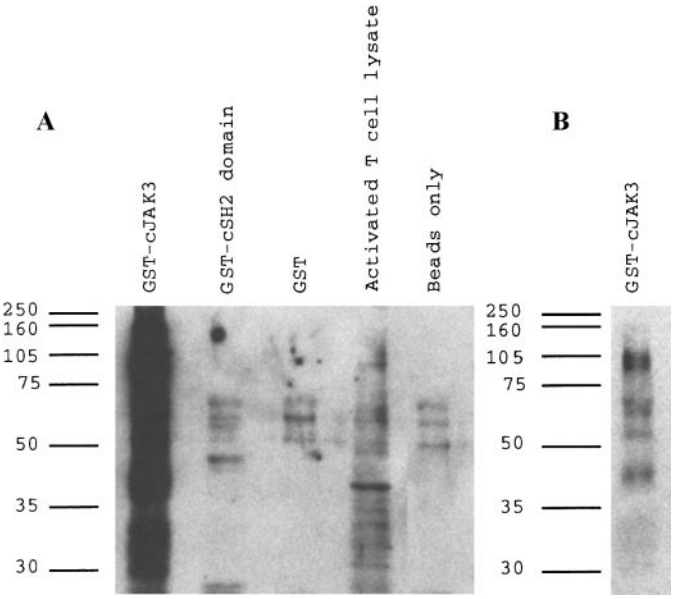

FIG. 4. Tyrosine phosphorylated proteins bind to full length and the putative SH2 domain of cJAK3. (A) The indicated GST fusion proteins, GST (empty vector), and GSH-linked beads only were incubated with activated T cell lysate. Following washing, bound proteins were solubilized by boiling in loading buffer. Proteins were separated by SDS–PAGE, transferred to nitrocellulose, and immunoblotted with αpY. (B) A shorter exposure of the GST-cJAK3 lane. Molecular mass markers (kDa) are indicated on the left.

Functional Analysis of SH2 Domain in Chicken JAK3

The presence of an SH2 domain in JAK proteins predicts the presence of interacting proteins in cells. We have used chicken JAK3 (cJAK3) in an *in vitro* binding assay, to evaluate proteins interacting with the putative SH2 domain. Full-length cJAK3 and the SH2 domain (amino acids 285–513) were expressed as GST fusion proteins, immobilized on agarose, and incubated with lysates from activated splenic T cells, as a source of JAK3 interacting proteins. Proteins bound to the beads were eluted, electrophoresed, transferred to nitrocellulose, and blotted with α-phosphotyrosine antibody. As shown in Fig. 4, several tyrosine-phosphorylated proteins were found to interact with the SH2 domain, and with full-length JAK3. GST-agarose or agarose beads alone show non-specific binding of three phosphorylated proteins of approximately 86, 76, and 67 kDa. These proteins also bind to the GST-SH2 fusion construct. However, five additional bands (MW 72, 58, 34, 27, and 26 kDa) are seen in addition to the three non specific bands in GST-SH2. Full length cJAK3 shows a stronger and more complex pattern of binding. Because of the intensity, it is not possible to determine if the same proteins that interacted with the SH2 domain are present in the cJAK3 lane. Although this assay does not allow for identification of these bound proteins, these results suggest that

the putative cJAK3 SH2 domain is functional in protein–protein interactions.

DISCUSSION

In this study, we have shown, with the use of web-accessible structure determination programs, that the primary, secondary and tertiary structure of JAK proteins predict the presence of an SH2 domain. In addition, a functional role for this putative SH2 domain is suggested by our preliminary experiments demonstrating the binding of tyrosine phosphorylated proteins to a GST fusion protein expressing this domain. The concept that JAKs contain an SH2 domain has been ignored in the literature for several years and most publications on JAKs actually make a statement that these proteins lack an SH2 domain (3–7). However, in the initial identification of this class of PTKs, the presence of an SH2 domain was suggested (1, 13). In addition, primary sequence analyses using more contemporary pattern search tools support the original observation (9, 10) and PROSITE has recently included JAKs, albeit with a question mark, in the SH2 domain profile (PDOC50001). Based on these studies, the existence of a functional SH2 domain in JAKs should be experimentally evaluated.

We found at least five tyrosine-phosphorylated proteins from activated splenic T cells bind to the cJAK3 SH2 domain. Identification of these proteins, either by affinity purification and protein sequencing, or by techniques such as the yeast two-hybrid system, would shed more light on the role of this putative SH2 domain in JAK signaling.

Numerous tyrosine-phosphorylated proteins bind to the full-length cJAK3 construct. This is consistent with reports of several different proteins interacting with JAK proteins. Recently, a tyrosine phosphorylated JAK3 substrate STAM (signal transducing adaptor molecule) was identified from cells stimulated with IL2 (51), and is thought to form a complex with a novel STAM-associated molecule (AMSH, associated molecule with the SH3 domain of STAM) (52). Another protein known to interact with JAK3 is Pyk2, a nonreceptor PTK (53). Pyk2 is activated following IL2 stimulation in a JAK dependent manner. Other proteins that have been identified as JAK interacting proteins include SH2-Bβ and SHP-2 phosphatase (54, 55). In addition, a family of antagonists that interact with JAKs, the JAB/CIS/SOCS family, have been identified (56–58). The N-terminal region of JAK3 binds to the γc chain of the IL2 subfamily of cytokine receptors (59) and it is thought that receptor binding by JAKs is mediated by two domains, with the JH7-6 region conferring general binding to the receptor subunits and the JH5-3 region conferring specificity. Since the putative SH2 domain spans JH4-3, SH2 interacting proteins could play a role in this specificity. Similarly, the JH7-6 domains of JAK2 bind to the granulocyte-macrophage colony-stimulating factor receptor β chain (60), although the JH7-3 domains of JAK2 appear to be required for binding to the growth hormone receptor (61). For Tyk2 and the interferon α receptor (IFNAR), the major interaction surface lies within JH7-6, but additional JH regions (JH5-3) contribute specificity to the assembly of the Tyk2/IFNAR complex, and to maintenance of the protein levels of IFNAR (62–64). More specific interactions with the SH2 domain have not been rigorously examined.

It should be noted that the function of this domain was evaluated by mutating the invariant arginine at position βB5 in murine JAK2 (65). This mutation had no effect on STAT1 activation when monitored by electrophoretic mobility shift assay in response to interferon-γ. While this argues against a functional SH2 domain in JAKs, it is possible that this domain does not have a critical function in that particular signaling pathway, and under the experimental paradigm of that study.

It is now becoming clearer that the JAK homology domains originally assigned by sequence identity between family members, actually consist of known protein–protein interaction modules. The structure of JAKs has recently been depicted not by the original seven homology domains but rather by these protein–protein interaction modules: from N- to C-terminus, the JAKs are comprised of a band 4.1/ERM domain, an SH2 domain, a Kinase-like domain, and a Kinase domain (11, 12, 66).

In summary, we conclude that the region corresponding to domains JH4-3 represents a structural and functional SH2 domain and this predicts that there are other proteins that specifically interact with JAKs and participate in the intracellular signaling pathway. This has implications for a more complete understanding of signaling by important cytokines and growth factors and for identification of chemical mediators that modulate the different pathways using JAKs. Current studies in our laboratory are aimed at the identification of proteins that interact with this putative SH2 domain in chicken JAK3.

ACKNOWLEDGMENTS

We would like to thank Roy S. Sundick for providing the recombinant chicken IL-2. We are grateful to L. A. Cogburn and B. J. Bahnson for careful reading of the manuscript. This work was supported by USDA Grant 98-35206-6595. D.K. is supported by USPHS Grant T32 GM08550.

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