An Investigation of DNA-binding Properties of the Non-Receptor Tyrosine Kinase Rlk

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Abstract

Rlk, along with Tec family members Btk and Itk, are predominantly cytoplasmic, but have also been found localized in the nucleus. Rlk has been shown to have a specific DNA-binding relationship with the IFN-? promoter region –56/-36 region. In this study we investigate a shorter isoform of Rlk, one missing the cysteine-string motif of the N-terminus, and its capacity for DNA-binding.

Introduction

Cytoplasmic protein-tyrosine kinases are important components of cellular signaling pathways. This group can be subdivided into receptor and non-receptor kinases. Our research group is interested in the Tec kinases, the second largest nonreceptor protein-tyrosine kinase family. The Tec family is comprised of five related enzymes: Tec, Itk/Emt/Tsk, Btk, Bmx/Etk, and Rlk/Txk (1, 2, 3, 4). B cells mainly express Btk and T cells express Itk and Rlk, while Tec and Bmx are more widely expressed (4). Rlk is involved in signaling pathways of lymphocyte activation (5). Upon reception of signaling hormones, Src kinases activate Rlk, which can then phosyphorylate auxiliary signaling molecules (1). The movement of cellular signals through the Tec kinases is dependent on the family's specific domains. Common protein domains include the Pleckstrin homology (PH), Tec Homology (TH), a proline-rich region, Src Homology 3 (SH3), Src Homology 2 (SH2), and a kinase domain (1, 2, 3, 4). The PH domain is involved in lipid binding and, therefore, membrane association (2). Rlk differs from the rest of the Tec family in that it contains a cysteine-string motif in place of the PH domain. This suggests that Rlk performs a different function from the other Tec family members (6).

Rlk has recently been found to exist in two isoforms. One form contains the afore-mentioned cysteine-string motif, while the second form does not. It appears that the two forms of Rlk arise from alternate translation start sites, one at the beginning of the Rlk sequence and the other occurring after the cysteine-string motif. The presence of this particular region governs cellular protein localization. The longer, cysteine-string motif-containing Rlk is mainly cytoplasmic whereas the shorter Rlk form has been found in the nucleus. The cysteine-string motif of Rlk is the target for post-transcriptional palmitoylation, thereby behaving like a PH domain and effectively anchoring the enzyme to the cellular membrane. Rlk contains a nuclear localization signal that occurs after the cysteine-string motif and may aid in nuclear localization (5). Rlk has been shown to bind IFN-? promoter region and upregulate transcription (7). This specific amino acid binding sequence wil be used in conjunction with various protein domains to identify the specific region of Rlk involved in DNA binding. This information may lead to a better understanding of Rlk's involvement with T cell development and signal pathways. Our lab group has observed that the N-terminus of Rlk has many attributes associated with DNA-protein binding domains. When expressed and purified, the N-terminus of Rlk is

unstructured, suggesting that it requires another component, possibly DNA, to fold properly. In addition, it has been extremely difficult to purify the N-terminal protein region in that the protein appears to have DNA contamination after extensive purification techniques (unpublished results). The protein readily binds zinc (unpublished results), a common attribute of DNA-binding regions comparable to zinc fingers (8). Rlk exists as a dimer, with SH3-proline rich region binding between 2 proteins (unpublished results). This is a trait shared with Btk (9) and other DNA-binding regions, such as leucine zippers (8). We propose that the N-terminus of Rlk is involved in DNA-binding and protein nuclear localization. Biotin-labeled gel shift assays of varied Rlk N-terminus protein domains run with the known IFN-? promoter sequence will be used to test our hypothesis. Once the specific N-terminus domain has been identified, examination with nuclear magnetic resonance (NMR) techniques may be used to ascertain the structure of the DNA-binding domain.

Materials and Methods

Protein Expression and Purification

The shorter isoform of Rlk was generated by the Polymerase Chain Reaction (PCR) and subcloned into the *Bam*H1 site of pGEX-2T to attain a glutathione-*S*-transferase (GST) fusion protein. The 5' primer encodes Rlk beginning at amino acid 55 and has the sequence CGCGGATCCATGGGCAAAACTCAATCC. The 3' primer encodes through amino acid 143 and has the sequence CGCGAATTCTCAGGCGAGTC TGTTTTCTGTCACATAGTTGCTTGG. The RMPSH3 fusion protein was grown in *Escherichia coli* strain BL21 (DE3) cells at 37?C in minimal media containing 50? g/ml ampicillin. Once the optical density (600 nm) of the cells reached 0.5, protein expression was induced using 0.5 mM isopropyl ? -D-thiogalactopyranoside (IPTG) and the temperature was reduced to 15?C. Twenty-four hours post-induction the cells were collected by centrifugation and resuspended in 50 mM potassium phosphate buffer (pH 7.4), 75 mM NaCl and 2 mM DTT. Lysozyme was added and the cells were stored at ?80?C for 12 hours. The cells were lysed as a result of thawing at room temperature with the addition of a protease inhibitor (1 mM PMSF) and DNAse (50?l of 10mg/ml stock). The cell lysate was clarified by centrifugation, and the resulting supernatant was purified using a glutathione-agarose column (Sigma). The fusion protein was eluted using 5 mM glutathione, 50 mM potassium phosphate buffer, 75 mM NaCl (pH 7.4), 2 mM DTT and 0.02% (v/v) NaN_3. Protein was concentrated and loaded onto a gel filtration column (Sephacryl S-100 HR; Amersham Pharmacia Biotech). Fractions containing the fusion protein were combined and the NaCl concentration was increased to 150 mM. Thrombin was used at room temperature for 45 minutes to completely cleave the GST tag. After cleavage, the protein was passed over a glutathione column to remove GST from the solutions. The protein was then concentrated and loaded onto the gel-filtration column. Fractions were collected, analyzed for purity using SDS-polyacrylamide (16%) gel electrophoresis, and combined. Protein was stored at 4?C until used in a gel-shift assay.

DNA Probe

The DNA probe sequence was derived from IFN-? promoter already shown to bind to Rlk. The entire –56 to –36 region of IFN-? promoter was found to be essential for DNA-protein interaction (7). The DNA sense and anti-sense strands were synthesized by

the Iowa State University DNA Sequencing and Synthesis Facility with the sequences ACGTAATCCTCAGGAGACTTC (sense) and GAAGTCTCCTGAGGATTACGT (anti-sense). Each strand was biotinylated using a Biotin 3' End DNA Labeling Kit (Pierce Biotechnologies). In brief, the enzyme terminal deoxynucleotidyl transferase (Tdt) is used to incorporate 1-3 biotinylated ribonucleotides onto the 3' end of each DNA strand. Biotinylated DNA was then purified using a QIAquick PCR purification kit (QIAGEN) to remove impurities from the biotinylation reactions. The sense and anti-sense strands were annealed by incubating equal concentrations of both strands and heating to 85?C, followed by slow cooling to room temperature.

DNA-Protein-Binding Assay

A gel shift assay was performed using a LightShift Chemiluminescent EMSA kit (Pierce Biotechnologies). This assay is based on the fact that DNA-protein complexes migrate slower than unbound DNA in a native polyacrylamide gel, resulting in a shift in migration of the labeled DNA band. Biotin-labeled DNA was incubated with 2?l RMPSH3 for 20 minutes on ice. The DNA-protein complexes were separated from free DNA probes on a 6% native polyacrylamide DNA gel. The gel was electrically transferred to a zeta membrane using western blot techniques and detected using chemiluminescence.

Results

Direct binding reactions between the shorter isoform of Rlk and the IFN-? promoter region –56/-36 have shown no shift in gel shift assays performed. This implies that this region of Rlk does not bind the IFN-? promoter region and upregulate transcription. **(figures will be included, hopefully we will come up with more results in the next couple of weeks)**

Conclusions

We have not been able to show that the shorter form of Rlk binds the IFN-? promoter region. DNA-binding may require phosphorylation on a significant tyrosine residue (Y_{91}) located in the SH3 domain. Further experiments are required to investigate this possibility. Some possibilities include: Phosphorylation of Y_{91} in vitro with Src kinase, followed by gel shift assay with the IFN-? promoter region and mutation of Y_{91} to a glutamate or aspartate residue to simulate a local environment similar to phosphorylated Rlk. If shown to bind DNA, further studies using NMR techniques can be used to determine the structure of the Rlk DNA-binding pocket. This structure may lead us to a novel DNA-binding structure, suggest another role for Rlk in T-cell development and may suggest a DNA-interaction for a closely related Tec-family kinase, Itk.

References:

1) Smith CIE, Islam TC, Mattsson Pt, Mohamed AJ, Nore BE, Vihinen MA. The Tec Family of Cytoplasmic Tyrosine Kinases: mammalian Btk, Bmx, Itk, Tec, Txk and homologs in other species. BioEssays 2001; 23: 436-446.

2) Yang WC, Collette Y, Nunès JA, Olive D. Tec Kinases: A Family with Multiple Roles in Immunity. Immunity 200; 12: 373-382.

3) Lewis CM, Broussard C, Czar MJ, Schwartzberg PL. Tec Kinases: modulators of lymphocyte signaling and development. Current Opinions in Immunology 2001; 13: 317-325.

4) Schaeffer EM, Schwarzberg PL. Tec Family Kinases in Lymphocyte Signaling and Function. Current Opinions in Immunology 2000; 12: 282-288.

5) Debnath J, Chamorro M, Czar MJ, Schaeffer EM, Lenardo MJ, Varmus HE, Schwartzberg PL. *rlkTXK* Encodes Two Forms of a Novel Csteine String Tyrosine Kinase Activated by Src Family Kinases. Molecular and Cellular Biology 1999; 19: 1498-1507.

6) Kashiwakura J, Suzuki N, Nagafuchi H, Takeno M, Takeba Y, Shimoyama Y, Sakane T. Txk, a Nonreceptor Tyrosine Kinase of the Tec Family, Is Expressed in T Helper Type 1 Cells and Regulates Interferon ? Production in Human T Lymphocytes. Journal of Experimental Medicine 1999; 190: 1147-1154.

7) Takeba Y, Nagafuchi H, Takeno M, Kashiwakura J, Suzuki N. Txk, a Member of Nonreceptor Tyrosine Kinase of Tec Family, Acts as a Th1 Cell-Specific Transcription Factor and Regulates IFN-? Gene Translation. Journal of Immunology 2002; 168: 2365-2370.

8) Metzler DE. Biochemistry: The Chemical Reactions of Living Cells. San Diego: Harcourt/Academic, 2001.

9) Okoh MP, Vihinen M. Interaction between Btk TH and SH3 Domain. Bioppolymers 2002; 63: 325-334.