ANALYSIS OF QK-1 BINDING TO mRNA TARGETS IMPLICATED IN SCHIZOPHRENIA PATHOLOGY

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ABSTRACT

Quaking (QK-1) is a recently identified candidate for the gene that gives rise to enhanced schizophrenia susceptibility. An apparent reduction of QK-1 levels was observed in post-mortem brain tissue samples taken from schizophrenic patients in two independent studies. Down regulation of QK-1 correlates with reduced levels of SFRS5, hnRNP A1, NCAM, hnRNP D, and SRRM 1RNA. QK-1 is an RNA binding protein that may regulate the localization and stability of these mRNA transcripts. To test if QK-1 can bind to these candidate targets, quantitative binding experiments were performed using titration fluorescence polarization, competition fluorescence polarization, and gel mobility shift assays. The data show that QK-1 binds with the highest affinity to hnRNP A1, NCAM and SRRM 1 transcripts, consistent with direct role for this protein in the regulation of these mRNAs. Interestingly, like QK-1, NCAM is also suspected to play a role in schizophrenia. The data show that QK-1 binds tightly to NCAM RNA, establishing a new link between these two schizophrenia candidates.
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BACKGROUND

Schizophrenia

Schizophrenia (SCZ) is a complex and elusive brain disorder. Approximately one percent of the population develops this disease in their lifetime, with nearly two million Americans affected (REF). The disease presents itself equally in men and women, but the onset in men occurs earlier, generally in the late teens or early twenties, while affected females become symptomatic in their late twenties into their thirties. SCZ can present in individuals as early as five years of age, but cases of adolescent SCZ are rare.

SCZ does have a heritable component to its expression (information obtained from schizophrenia.com). The risk associated to a person from a family with no history of SCZ is only about one percent, but that percentage increases to roughly ten percent when a child is born to a schizophrenic parent. The highest percentage of heritability lies in identical twins. Persons who have an identical twin with the disorder elevate their chance of inheriting the disease to nearly forty or fifty percent.

Symptoms of the disease are extremely debilitating and include hearing voices and extreme paranoia. Symptoms including hallucinations can affect all five senses, manifesting themselves in a distorted perception of reality that actively affects social interaction and daily life. Other symptoms involve distorted thinking and emotional expressiveness. Persons affected with SCZ may demonstrate an inability to control their own thoughts, with fleeting thoughts and reactions. They may also appear to be devoid of emotion and express a dulled and sullen outward expressiveness.
These symptoms leave affected individuals fearful of contact with other human beings and cause them to be withdrawn. Early onset symptoms are not extremely severe, and are not noticeably apparent to onlookers. A drastic change in mood may precede or follow a psychotic episode. The most obvious symptom of schizophrenia is the debilitating and long-lasting psychosis that leaves the affected individual unable to interact in normal societal situations. While persons with SCZ are withdrawn and paranoid, they are not typically outwardly violent. Crimes of violence associated with mental illness do not normally coincide with individuals that have this disease. Despite this fact, suicidal tendencies need to be addressed immediately. A consequence of the internal voices and delusions, these thoughts can be manifested and present as a viable option to many affected persons.

Current treatments for SCZ are somewhat effective, but only about 20% of those affected receive a full recovery. Treatment with antipsychotic drugs has been available since the 1950’s, but this approach is only able to alleviate some of the symptoms. These drugs are able to stifle hallucinogenic episodes, and often can help control the outbreak of new psychotic episodes, but do not aid in the treatment of all symptoms. A new, more effective treatment plan needs to be implemented to ensure that patients with SCZ can enjoy an active and productive adult life; a treatment with more direct effects on the main causes of SCZ.

Identification of Quaking as a Schizophrenia Candidate Gene

Two independent detailed microarray analyses and haplotype mapping suggest Quaking protein may underlie SCZ susceptibility. Both the Jazin Lab (Uppsala
University) and project collaborator Alison McInnes (Mount Sinai School of Medicine) have independently demonstrated that QK-1 is down regulated in post-mortem SCZ brain samples (Aberg et al., 2006; Lauriat et al., 2006). The down regulation of the known QK-1 regulatory targets MAG, PLP, and MBP is also observed. Further proof that QK-1 correlates with SCZ was obtained by haplotype mapping of a large SCZ pedigree from northern Sweden, which shows that a link between the haplotype and the QK-1 locus on chromosome 6 (Aberg et al., 2006).

The Quaking Protein

In mice, QK-1 is an important regulator of the structural components of myelin (MBP, MAG, PLP). QK-1 controls the splicing pattern of these components and is responsible for transporting MBP mRNA to the myelin compartment where it can be properly translated (Larocque et al., 2002; Li et al., 2000; Wu et al., 2002). Myelin is composed of phospholipid bilayer rings formed by oligodendrocytes that are tightly packed in concentric circles around the neuronal axons in the brain (Figure 1). The myelin rings aid in increasing the speed of electrical messages across the axon. Oligodendrocytes and myelin examined from patients with SCZ demonstrate serious defects (Lauriat et al., 2006) in which the phospholipid bilayer rings that form the myelin sheath fail to pack into tight circles. A viable QK-1 mutant in mice (qkv) demonstrates similar myelin pathophysiology, but has a severe ataxia phenotype not observed in SCZ patients (Sidman et al., 1964).
The human Quaking gene is located on chromosome 6q26-q27 (Li et al., 2002). The protein encoded by this gene is an mRNA-binding protein from the STAR/GSG family. Proteins in the STAR (Signal Transduction Activators of RNA) family have a distinct RNA-binding feature that allows them to only bind specific targets. Deletions, polymorphisms and point mutations can have a great impact on the binding capability and specificity of these proteins. Normal Quaking protein contains a RNA-binding KH domain, and functions as a homodimer (Figure 2). The STAR domain (underlined in the diagram) contains three functional regions, the QUA 1 and QUA 2 regions that flank the distinct KH domain. The QUA 1 region encompasses amino acids 11-82 of the protein, the KH domain, amino acids 83-180, and the QUA 2 region 181-205.
The mouse homolog is alternatively spliced into three major isoforms (Kondo et al., 1999). The three isoforms are Qk1-5, Qk1-6, and Qk1-7. Qk1-6 and Qk1-7 are cytoplasmic. The Qk1-5 isoform is the shortest of the three major isoforms, and is the major form that is responsible for the mislocalization of myelin basic protein mRNA to the nucleus (Larocque et al., 2002). These isoforms contain C-terminal variances of 8-30 nucleotides due to alternative splicing which is responsible for targeting QK1 proteins to different cellular compartments (Hardy et al., 1996). QK1-5 changes in the C-terminal end demonstrate a unique nuclear localization signal, and target and retain QK1 in the nucleus (Larocque et al., 2002). QK1-6 and QK1-7 are suspected to act as shuttle proteins that bind to the 3' UTR of mRNA’s they are compartmentalizing and function as both homo- and heterodimers (Chen et al., 1997; Larocque et al., 2002).

Mice with the Quaking-viable mutation (qkv) demonstrate a distinct twitching phenotype. Quaking-viable mice develop normally until about ten days after birth, when the pronounced twitch in the hind limbs begins. This phenotype can be attributed to the lack of maturation in the myelin sheath or the arrest of myelination (Sidman et al., 1964). Homozygous recessive mutants are recessive lethal during early stages of development (Justice and Bode, 1988). The death of homozygous recessive mice, ENU (N-ethyl-N-
nitrosourea) mutated mice, occurs before the neural progenitor cells commit to the neuronal or glial cell fate. This demonstrates that QK-1 proteins have an essential role in proliferation, migration, and/or differentiation of multipotential neural progenitor cells. The same phenotype in the human Quaking homolog is suspected to induce mislocalization of MBP to the myelin compartment and, in part, induce SCZ in humans. The point mutation that prevents the dimerization of QK-1 is a glutamic acid to glycine switch at amino acid position 48, the QUA1 region (Chen et al., 1997). The failure to dimerize prevents localization of mRNA and causes death in qkv. Another single point mutation has been isolated in a large Swedish pedigree of known SCZ susceptibility but the effect of the single nucleotide polymorphisms identified in the human quaking locus in the Swedish family on QK-1 function is not known.

The method by which Quaking mutants encounter problems in myelin localization presents a multi-step process (Ryder and Williamson, 2004). This process demonstrates that QK-1 protein has many potential targets, and provides a dramatic regulatory function in neuronal axon myelination. The first step is the reduction of myelin basic protein (MBP) mRNA. Next, the remaining mRNA undergoes a variation in localization, leaving the myelin compartment with reduced levels of mRNA, and its subsequent transcripts and elevated mRNA levels in the nucleus (Larocque et al., 2002). Simultaneously, the splicing pattern of MBP, proteolipid protein (PLP), and myelin-associated glycoprotein (MAG) are affected (Wu et al., 2002). Ryder and Williamson (2004) propose that mis-localization of MBP mRNA depends upon the interaction of QK1 with the 3’-untranslated (UTR) region of MBP mRNA via a small regulatory motif that they term the Quaking Star Binding Element, or QSBE.
Quaking Protein and mRNA Targets

Reduced Quaking levels in SCZ correlates with the down regulation of several additional transcripts normally present in oligodendrocytes that may represent the basis for SCZ pathology (Aberg et al., 2006; Lauriat et al., 2006). The targets include SFRS5, hnRNP A1, hnRNP D, SRRM 1, and NCAM. The first four are genes involved in the global regulation of RNA splicing, and the last is required for the initiation of myelination and has previously been implicated in SCZ (Vawter, 2000). These candidate targets were identified by mining the microarray data set of post-mortem brain tissue samples of SCZ patients performed by project collaborator Alison McInnes at Mount Sinai School of Medicine. If these genes are direct regulatory targets of QK-1, then the 3’ UTR of each candidate gene should contain the QSBE sequence 5’-A(A/C)UAA-3’, and QK-1 should bind to these RNA with high affinity. In this report, I present the detailed analysis of the QK-1 interaction to binding sites present in each of these candidate targets.
METHODOLOGY

QK-1 Protein Growth and Purification

A plasmid encoding the functional binding domain of the QK-1 gene, amino acids 1-205, fused to maltose binding protein was transformed into the *E. coli* strain JM-109. A two liter culture of the transformed *E. coli* cells was inoculated and measured periodically by taking an OD$_{600}$ reading. At 0.6 OD, expression was induced by the addition of 1 mM IPTG. The cells were kept on a shaker at 37°C for four additional hours and then harvested by centrifugation. The cell pellets were stored at -80°C overnight.

The cell pellet was lysed using sonication in a lysis buffer by dissolving two pellets of Complete EDTA-free protease inhibitor into 100 mL of chilled Buffer A (50 mM Tris, pH 8.8, 200 mM NaCl, 2 mM DTT), and the lysate was clarified by centrifugation at 8500 x g for 20 minutes. The soluble lysate with the target protein was then passed through an amylose column equilibrated with lysis buffer. After loading and washing the column, bound protein was eluted with lysis buffer supplemented with 10 mM maltose. A polyacrylamide gel was run with the fraction samples to monitor the presence of the protein. Fractions containing the protein were dialyzed overnight in Dialysis Buffer #1 (50 mM MOPS pH 6.0, 20 mM NaCl, 2 mM DTT).

The protein was then further purified using an ion exchange S column using a buffer gradient of buffers B (50 mM MOPS pH 6.0, 20 mM NaCl, 2 mM DTT) and C (50 mM MOPS pH 6.0, 2 M NaCl, 2 mM DTT). The protein was eluted with a gradient of sodium chloride from 20 – 2000 mM. Fractions contained the protein dialyzed into
Dialysis Buffer #2 (50 mM Tris, pH 8.8, 20 mM NaCl, 2 mM DTT) overnight. Lastly, the QK-1 protein was run over another ion exchange Q column to purify the final contaminants away from the QK-1 protein. This was done with a sodium chloride gradient of 20-2000 nM. The gradient was run using Buffer D (50 mM Tris, pH 8.8, 20 mM NaCl, 2 mM DTT) and Buffer E (50 mM Tris, pH 8.8, 2 M NaCl, 2 mM DTT). The pure QK-1 fractions from this ion exchange chromatography were dialyzed into Dialysis Buffer #3 (50 mM Tris, pH 8.0, 20 mM NaCl, 2 mM DTT) overnight. Protein was aliquoted into 500 μl amounts and stored at 4°C until use. A polyacrylamide gel was run of the included fractions from the S and Q columns, as well as the wash and flow-through from each column to assess the purity of the protein.

The concentration of the resulting Quaking protein was measured with a UV Spectrophotometer at 280 nanometers using a calculated extinction coefficient of 76810. The yield was 31.25 mg, at a concentration of 45.1 micromolar, which is acceptable for QK-1 binding experiments. The results are comparable to previous QK-1 purifications by Professor Ryder.

**Titration Fluorescence Polarization Assay**

The titration fluorescence polarization (tFP) assay was used to measure the QK-1/QSBE complex affinity to ensure that the protein was binding with equivalent affinity to previously determined values (Ryder and Williamson, 2004). The tFP was carried out using a serial dilution of QK-1, beginning at 20 μM and continuing at a 2/3 dilution over a twenty-four well dilution, with the final well being a no protein control. 10 μL of this dilution series was added to wells of a 96 well plate that contained 90 μL of a master mix
to give a final concentration of 1000 nM fl-QSBE RNA, 10 mM Tris, pH 8.0, 0.1 mM EDTA, pH 8.0, 25 mM NaCl, 0.01 mg/ml tRNA, and 0.01% IGEPAL CA630, and a range of protein concentrations from 2 uM to .122 nM. The master mix was heated for two minutes in a 60°C water bath, and let cool for ten minutes prior to addition to the plate.

Each plate was read in a Perkin-Elmer Multilabel counter (Figure 4). The fluorescence polarization process begins by shining white light through a spectral filter and then a polarizing filter. This orients the light in a specific direction and wavelength. The polarized light is then reflected off of a mirror, and down into the sample well. The light that shines down in the well is oriented so that it can only read molecules in a specific angle. The mRNA without the protein is able to move much more quickly than the protein/mRNA complex, and therefore more efficiently depolarizes the light. Once the light has been absorbed by the fluorescein molecule, it is emitted at a different wavelength. The emitted light passes through two more polarizing filters, a parallel polarizing filter followed by a perpendicular polarizing filter, in order to assess the degree of depolarization. A second spectral filter is used so that the detector is able to read the new wavelength. The units used to measure the data output are polarization units (mP). The mP is determined by the intensity of readings assessed through the parallel and perpendicular filters. The formula is the reading of the parallel filter minus the reading of the perpendicular filter, over the reading of the parallel filter plus the reading of the perpendicular filter.
The data measured by the plate reader was exported to the program IGOR. Using a macro in IGOR set up with the Hill Equation (Hill, 1910); I was able to define the binding affinities from the mP values collected. The Hill equation is:

\[ f = b + \left[ \frac{m - b}{1 + 10^n (\log K_d - \log[P])} \right] \]

The equation determines the equilibrium dissociation constant for each experiment. In the equation \( f \) is the fraction bound, which is the determined mP value, \([P]\) is the concentration of the fusion protein, \(K_d\) is the equilibrium dissociation constant, \(n\) is the Hill coefficient, \(m\) is the maximum signal, and \(b\) is the base signal. The determination of this equation was fitted to a graph of the data and the binding affinity was determined through the equilibrium dissociation constant.
Fluorescence Polarization Competition Assay

In competition experiments, the QK-1 protein was introduced to a Master Mix containing a labeled QSBE RNA. The Master Mix was prepared using a 10x Buffer consisting of 100 mM Tris pH 8.0, 250 mM NaCl, and water to bring the final volume to 10 ml. The master mix also contained 1 mg/ml tRNA, 1% IGEPAL CA630, the labeled QSBE (2 M) and the QK-1 protein (20 uM). The QSBE RNA is tagged with a 5'-fluorescein molecule that could be used to measure QK-1 binding to the target. This master mix is different from the titration polarization experiments with the QK-1 protein being introduced directly in the mix. The ability of a sequence to compete for binding was assessed by adding a serial dilution of competitor sequence with the highest concentration at 30 micromolar. The master mix was loaded into the 96 well plate at 70 microliters, and the target mRNA dilution series was added at 30 microliters per well, with the final volume in each well at 100 microliters. Each trial was run in triplicate and the assay was measured using the program Igor Pro Carbon.

The fluorescent polarization competition experiments run were always competed against fl-QSBE RNA. The binding capacity was measured with the Lin and Rigs Equation (Lin and Riggs, 1972). The Lin and Rigs Equation is:

\[
mP = \frac{(mP_{max} - mP_{base})}{2R} \{K_d + \left(\frac{K_d}{K_c}\right)C + P + R - \sqrt{[K_d + \left(\frac{K_d}{K_c}\right)C + P + R]^2 - 4RP}\} + mP_{base}
\]

The Lin and Rigs equation was used to determine the equilibrium dissociation constant for each competitor RNA using a quadratic solution. In the equation, \(K_c\) is the competitor
RNA, $mP_{\text{max}}$ is the polarization at saturation, $mP_{\text{base}}$ is the polarization in the absence of protein, and $R$ is the concentration of fluorescein-labeled RNA (Ryder, year).

Once the baseline Kd of QK-1 for fl-QSBE was established by titration FP, we began testing mRNA targets suspected to bind with QK-1. These targets were NCAM (4 versions), hnRNP A1 (5 versions), SFRS5 (5 versions), SRRM1 (3 versions) and NCAM variants engineered by Professor Ryder labeled SPR 097, SPR 098, SPR 099, SPR 100, SPR 101, SPR 102.

NaCl and pH dependence of QK-1 Binding to NCAM RNA

The fluorescence polarization titration assay was altered slightly and used to demonstrate the [NaCl] dependence on the QK-1/mRNA complex. The same procedure was followed from the fluorescence polarization titration assay with the change in [NaCl] in the 2x Buffer. In these experiments the concentration ranged from 0 mM NaCl, to 200 mM NaCl. The QK-1 concentration remained at 2 μM in a 2/3 serial dilution series, and none of the other components of the master mix were altered. The experiments were carried out in triplicate in 96 well plates and tested in the Perkin-Elmer Multilabel Counter. The results were imported into IGOR and the data were analyzed using the Hill Equation.

The pH dependence experiments also followed the same protocol as the fluorescence polarization titration assay. The change in the 2x Buffer for these experiments involved the pH of the buffer. At pH values of 6.0, 6.4, and 6.8, MOPS Buffer was used in place of the Tris Buffer. Tris was used at pH 7.2, 7.6, 8.0, 8.4, and 8.8. The QK-1 concentration remained at 2 micromolar in a 2/3 serial dilution series. The experiments were carried out in triplicate in a 96 well plate and read using the Perkin-
Elmer Multilabel Counter. The results were imported into IGOR and the data were analyzed using the Hill Equation.

**Gel Mobility Shift Assay**

The gel mobility shift assay was chosen for a target NCAM mRNA for further investigation beyond the titration fluorescence polarization. In this assay the QK-1/fl-mRNA complex was performed by loading the equilibrated reactions from a QK-1 dilution series starting at 2 μM and carried over a 2/3 serial dilution series. The NCAM sequence was labeled with a 5’-fluorescein molecule and purchased from IDT. The gel shift was performed on a 2% agarose gel with 1X TAE and a final volume of between 300 and 400 milliliters. The gel was run at 120 V for thirty to forty-five minutes. Running the gel at over 120 V caused the complex to dissociate in the gel and provide inaccurate binding data.

No ethidium bromide was added to the gel, as it would cause complex dissociation and background fluorescence. Three experiments were carried out on each gel with three 20 well combs. The combs were spaced equally throughout the gel. Each well was loaded with 40 microliters of the equilibrated sample complex. The loading dye was added directly to the 96 well plate with the equilibrated samples at a volume of 12.5 microliters.

A variety of loading dyes were used in an attempt to reduce the auto-fluorescent shadow produced many of the dyes when the gel was read in the FUJI FLA-500 imager. Early dyes, including xylene cyanol and bromophenol blue exhibited co-migration with the QK-1/mRNA complex and made quantitative analysis of the shift difficult. Gels were
attempted with no loading dye and results were inconclusive due to experimenter error. The time needed to load the 60 well gel caused the complex to dissociate into the gel while the remainder of the gel was being loaded. Several additional dyes were tried, and the final loading dye that demonstrated no auto-fluorescence or blue laser absorption was bromocresol green.

Each gel shift experiment used three separate mRNA sequences, each run over twenty wells. SPR 097, SPR 098 and QSBE were all run on the fluorescent gel shift. QSBE was used as a control, while the SPR 097 and SPR098 are NCAM RNAs that demonstrated high affinity binding using the fluorescence polarization assay. The SPR 097 and 098 oligos used in these gels shifts is 30 nucleotides, longer than the mRNAs used in the FP experiments, but still a sequence present in the 3’ UTR of the mRNA.

The gel mobility shift assay was used to test the equilibrium dissociation constant and binding affinity demonstrated through the competition fluorescence polarization assay. To do this the equilibrium dissociation of this assay was measured by plotting the intensity of the free RNA versus the QK-1 concentration. The bound mRNA versus free mRNA could not be used to accurately measure the binding affinity due to the presence of the light absorbing loading dye in the gel and multiple shifts of the bound RNA at high RNA concentration.

*Miniaturization for Compound Screening*

Miniaturization of the FP assay was performed using 50 microliter reactions in a 384 well plate. The miniaturization procedure used the same master mix as the normal FP assay but in reduced volumes. One half of the plate was set up with QK-1 in a
concentration of 15 nanomolar. The other half of the plate was set up with no protein in each well. The Z’ factor equation was used to demonstrate the effectiveness of the assay (Zhang et al., 1999). The Z’ factor equation is:

\[ Z\text{factor} = 1 - \frac{3 \times (\sigma_p + \sigma_n)}{|\mu_p - \mu_n|} \]

This equation measures three times the standard deviation of the protein bound wells plus the values of those wells without protein over the absolute value of the assay window. The assay window is present as the space between the bound and free protein represented in the Gaussian peaks (Fig. 11 in the Results section).
RESULTS

Purification Results

The purification process of the QK-1 protein gave an overall yield of 31.25 mg at 45.1 μM concentration. This purification experiment demonstrated the properties of the QK-1 protein. The purification process involved purifying the maltose binding protein fused to the QK-1 protein in an amylase affinity column (Fig. 5). The binding to this affinity column demonstrated that the QK-1 protein had been grown correctly and was presenting with the maltose binding protein tag present. The S-column and Q-column steps used ion exchange to bind and elute the protein to the column (Fig. 5). One column utilized high salt concentration to bind the protein, and the other used low salt to separate out contaminants. The presence of the protein after these column steps demonstrates that it is folded correctly and able to withstand the varied salt concentrations and remain active. If the protein had misfolded it would not have eluted off the columns during the correct steps and would have likely been solubilized in the ion exchange steps.
Titration Fluorescence Polarization Assay Results

The titration fluorescence polarization assay demonstrated the high affinity binding of the QK-1 protein to the known regulator sequence, QSBE (Fig. 6). The experiments demonstrate the equilibrium constant of this reaction averaged over three measurements is $5 \pm 2$ namomolar (Table 1). The Hill coefficient, which should be at a numerical value of 1.0 assuming bimolecular association of the QK-1 dimer with QSBE RNA, is $1.1 \pm 0.2$ (Table 1). The value reported in this assay is similar to the published values of QK-1 binding to the QSBE sequence (Ryder and Williamson, 2004). This demonstrates that the preparation is active and can be used to test other mRNA targets with the QSBE sequence in their 3’ UTR. From this experiment we learned that QK-1 binds with high affinity to the QSBE sequence. Demonstration of binding to this
sequence illustrates QK-1 ability to bind to mRNA targets and potentially function in regulating their localization in the neuronal axon.

Table 1: Parameters determined by a fit to the Hill equation

<table>
<thead>
<tr>
<th>QSBE Titration</th>
<th>Kd</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>5.1E-09</td>
<td>0.7</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>5.2E-09</td>
<td>1.3</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>7.5E-09</td>
<td>1.3</td>
</tr>
<tr>
<td>Average</td>
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</tr>
<tr>
<td>St. Dev.</td>
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</table>

*Competition Fluorescence Polarization Assay Results*

The competition fluorescence polarization assays determined that the QK-1 protein binds to RNA fragments from the 3’-UTR’s of NCAM, hnRNP D, hnRNP A1, and SRRM1 (Figure 7, Table 2). The mRNA fragments that demonstrate the highest binding affinity are the NCAM v.2, NCAM v.3, and hnRNP A1 v.1 fragments with average Kd of 2.4 E-08, 1.2 E-08, and 5.5 E-08 respectively. These fragments bind almost as well as the QSBE sequence. The mRNA fragments that demonstrated the highest binding affinity for the QK-1 protein do so at binding capacities very close to the
known regulatory QSBE sequence. The average binding to the QSBE mRNA is 5.9 nM, and four mRNA sequences from the potential targets tested here demonstrate binding at only 10 fold higher concentration. NCAM v.3 is only two fold weaker, while NCAM v.2, a shorter variant of NCAM v.3, is only 4-fold weaker. This demonstrates that QK-1 binds this target with sufficient affinity, and the magnitude of the affinity correlates with the length of the competitor sequence. The ability of the QK-1 protein to bind the QSBE sequence in the competitor mRNA shows that the specificity for QK-1 is not selective to only the STAR binding sequence in the 3’ UTR of the Myelin Basic Protein, but also interacts with high affinity to mRNA’s with the same sequence in their 3’ UTR (Table 2). The effectiveness of QK-1 to bind consensus sequence in the longer NCAM, SPR 097, (Fig 8) variant sequence shows that QK-1 has the potential to bind to other targets with affinity as high as the QSBE mRNA. The ability of the NCAM mRNA to compete with the QSBE mRNA exhibits specificity for QK-1 that may affect localization. This binding demonstrates the potential for QK-1 to have a regulatory effect on these target mRNAs.

![Fig. 7](image) Competition fluorescence polarization data. hnRNP A1 v.1 sequence titrated into the complex of QK-1 and fl-QSBE RNA. The error bars are the standard deviation of five reads of the plate. The red line is a fit of the data to the Hill equation, the blue dashed line is a fit of the data to the Lin and Riggs equation.
Table 2. Parameters determined by a fit to the Lin and Riggs equation

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>$K_d$ (M) (Average)</th>
<th>Standard Deviation</th>
</tr>
</thead>
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<td>SFRS5 v.1</td>
<td>UGUAAUAUAAACUU</td>
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<td>SFRS5 v.4</td>
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<td>1.15E-08</td>
<td>5.286E-09</td>
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<td>NCAM v.4</td>
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<td>3.14E-06</td>
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<tr>
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<td>3.806E-07</td>
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<tr>
<td>SRRM1 v.3</td>
<td>TTTAAACTAAAGC</td>
<td>6.36E-07</td>
<td>1.044E-07</td>
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</table>

Fig. 8 Direct titration of QK-1 into SPR097, a longer fragment of the NCAM 3'-UTR. Error bars represent the standard deviation of five reads of the plate. The red line is a fit of the data to the Hill equation.
NaCl and pH Dependence of QK-1 binding to NCAM RNA

QK-1/mRNA complex interactions with varying pH demonstrate that side chain interaction between the range from pH 7.2-8.8 does not contribute to binding. At pH values below 7.0 the fluorescein fluorescence is quenched, prohibiting analysis of these data. At pH above 8.8 the RNA in the complex begins to degrade.

NaCl concentration experiments (Figure 9) demonstrate that high ionic strength can decrease the binding affinity, likely by disrupting salt bridge interactions between the protein and the RNA. These experiments demonstrate that between 50 and 150 nM NaCl concentration there is very little correlation between affinity and NaCl. Below 50 mM NaCl there is much greater variability of the equilibrium dissociation constant of the complex. These low concentrations demonstrate a definitive impact on the QK-1/mRNA complex, but the exact cause of this problem is not fully understood.
Gel Mobility Shift Assay Results

At low concentration, the RNA migrates quickly through the gel. At higher concentration, some of the RNA appears to migrate more slowly. At very high concentration, a shift to a second species is observed. The gel mobility shift assay demonstrated that the binding affinities determined in the competition fluorescence binding experiments were accurate. The binding constant determined by the gel shift assay was 12 nanomolar. This binding constant is very close to the 20 nanomolar constant determined by the polarization assay.
Miniaturization for Compound Screening

The Miniaturization experiments were run in a 384 well plate at 50 microliters per well. The success of this assay demonstrates that the QK-1/mRNA complex is stable at low volumes and can be used in high throughput compound screening. This is important because it allows many more compounds to be successfully screened at once and verifies the stability of the QK-1/mRNA binding complex.

This quality of the assay was assessed using the Z’ factor equation (Fig. 11). This equation uses the mP values of the assay window presented by the well with protein and adding that to the wells without protein. This number is multiplied by three and divided by the absolute value of the standard deviation of the wells with protein and subtracting the standard deviation of the wells without protein. In an ideal assay the result of this
equation would be 1. The Z factor of our experiment was 0.9. The assay is generally considered successful if the Z factor value is between 0.5 and 1 (Zhang et al., 1999).

\[
Z_{\text{factor}} = 1 - \frac{3 \times (\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}
\]

\[
Z \text{ factor} = 0.9
\]

**Fig. 11** Miniaturization of the FP was performed using 50 uL reactions in a 384 well plate. **A.** One half of the plate was set up with QK-1 concentration of 15 nM, just above the Kd (B), and the second half was set up without protein. **C.** The assay window and the standard deviations were determined by fitting a histogram of the data to gaussian distributions. The fitness of the assay for inhibitor screening was determined by measuring the Z factor. Z factors greater than 0.5 are suitable for high throughput screening.
DISCUSSION

These results indicate that the consensus binding sequence present in QK-1, 5’-A(A/C)UAA-3’ doesn’t show preferential treatment to either the A or C in the second position, with each consensus sequence having at least one target that bound with high affinity. The analysis of QK-1 binding to mRNA targets in the SCZ pathway proved through quantitative binding experiments that QK-1 has high affinity binding to NCAM, hnRNP A1 and SRRM1 transcripts. The use of the complementary competition fluorescence polarization assay and the gel mobility shift demonstrated the accuracy of the binding results. All of the assays performed to demonstrate binding of the protein proved successful and showed that QK-1 targets have definitive binding sites in their 3’ UTR. These experiments, however do not directly demonstrate that QK-1 is a regulator of any of these targets. It is known that QK-1 is a regulator of MBP, and the QSBE sequence exhibited the tightest binding affinity; but it is unclear from these experiments whether the QK-1 protein functions to regulate the localization and expression of these targets. Though the in vitro binding data presented here are consistent with the model that QK-1 regulates SFRS5, hnRNP A1, and NCAM derived from the microarray data, more experiments will be necessary to demonstrate regulation directly.

Future experiments should demonstrate that NCAM, SFRS5, or hnRNP A1 co-immunoprecipitate from mouse brains with anti-QK-1 antibody. Also requiring further investigation to demonstrate the QK-1 has a regulatory function is to demonstrate that
NCAM, SFRS5, and hnRNP A1 mRNA levels are modulated in qkv mice. Another experiment that demonstrates that QK-1 directly interacts and regulates these targets is to show that QK-1 regulates a reporter construct with SFRS5, NCAM, or hnRNP A1 UTR sequences in cell culture. This direct correlation between QK-1 and these mRNA targets, including NCAM would demonstrate a definitive regulatory component in QK-1 schizophrenia pathology.

The determination of the inhibitors of QK-1 through high throughput compound screening is also an important step to understanding the regulation of QK-1. Because most QK-1 mutants are embryonic lethal, and because the qkv is not a knock out mutation, a chemical inhibitor could be used to test the role of QK-1 inactivation after its critical role in development. Once potential QK-1 inhibitors have been identified, the QK-1/mRNA quantitative binding experiments can be applied to test the binding affinity of the inhibitor to QK-1 in the presence of its target RNA.

The role of QK-1 in the SCZ pathology is still unclear. The QK-1 protein binds to many mRNA targets implicated in the myelination of the central nervous system and to various NCAM variants. The NCAM mRNAs have been implicated to the SCZ pathology in previous studies and the correlation of QK-1 to NCAM provides a direct link to QK-1 as a regulator of many mRNA targets involved in SCZ. This connection of QK-1 to NCAM, along with the proven high affinity binding of the QSBE sequence in the 3’ UTR of other mRNA targets demonstrates that QK-1 may have a definitive regulatory role in the myelination of the neuronal axons and in the SCZ pathology.
BIBLIOGRAPHY


