

Investigating the role of a *Cryptosporidium parvum* apyrase in infection

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Abstract

This project attempted to characterize the function of a *Cryptosporidium parvum* apyrase termed, Similar to Riken (SRK), and its human ortholog, CANT-1,. First, we looked at SRK's effect on gene expression in host cells with a microarray analysis, which revealed significant changes in both adenosine and apoptosis pathways. We also attempted to elucidate the biological function of CANT1 using RNAi, indirect immunofluorescence, and flow cytometry assays.

Introduction

The ubiquitous protozoan parasite *Cryptosporidium parvum* causes severe diarrhea in millions of people every year by infecting and destroying epithelial cells of the small intestine. Transmitted through water supplies, infected animals, and oral-fecal contact, the disease is especially harmful to immunocompromised individuals. Recently, a parasite apyrase named SRK has been shown to be critically important for successful invasion likely by disrupting intracellular signaling pathways associated with the prevention of invasion. Immunoprecipitation and mass mass spectrometry data indicate that SRK binds to its human ortholog, CANT1, *in vitro*. Unfortunately, little is known about the biological function of CANT1. Unlike other apyrases, it lacks ADP hydrolysis activity and is soluble (1). However, like other apyrases, its activity is dependent upon homodimerization (2).

The goal of our research project was two fold:

1. To characterization the gene expression of both SRK in *Cryptosporidium* and its effects on host cells by microarray and RT-PCR.
2. To examine the biological function of CANT-1 by investigating its role during infection and its localization in host cells.

Methods

RNA extraction for RT-PCR and Microarray experiments

RNA was extracted using TRIzol Reagents (Invitrogen) according to the manufacturer's instructions.

Microarray analysis of SRK's effect on the transcriptional profile of human epithelial cells

We scanned and hybridized 4 Human U1333 v2 arrays (Affymetrix) with total RNA extract from 10^6 HCT-8 cells (human epithelial cell line) treated with either 0.1 μ M of recombinant SRK for 3 hours (2 microarrays) or no treatment (2 microarrays). The resulting CEL files were normalized using the RMA method (3) and the differentially expressed genes were identified by pairwise t-tests.

RT-PCR of SRK

RT-PCR was performed on RNA extracted 3 hours, 6 hours, 20 hours, 24 hours, and 48 hours 5×10^5 HCT-8 after infection with 3×10^6 *Cryptosporidium parvum* sporozoites using the Superscript III One Step RT-PCR System kit (Invitrogen) and the following primers:

SRK forward (5' \rightarrow 3'): AGGAAAGGAGGGTTTGCCT
SRK reverse (5' \rightarrow 3'): CCATCCCTCTCCCATATTCA

Control gene: GAPDH
GAPDH forward (5' \rightarrow 3'): GTTTCGGACGTATTGGTCGT
GAPDH reverse (5' \rightarrow 3'): CCATCCCTCTCCCATATTCA

For every sample, a no reverse transcriptase control reaction was performed with the Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) kit.

Indirect immunofluorescence of CANT1 in epithelial cells

5×10^5 HCT-8 cells were fixed with 4 % paraformaldehyde in PBS for 1 hour, washed three times with PBS, and blocked with NH_4Cl for 15 minutes. After 3 washes with PBS, cells were then blocked overnight with PGN, followed by three PBS washes. If the cells were permeabilized, they were treated with 0.01% Triton X-100 solution for 5 minutes. Cells were then washed again, and incubated with serum immunized with recombinant SRK protein diluted 1:80 in PBS. This serum contains antibodies against SRK. Control treatments were incubated with non-immunized serum ('normal serum'). Then, after 3 washes 0.005 % Tween 20 (Fischer Scientific) in PBS, cells were treated with anti-mouse IgG FITC conjugated antibody (Sigma) diluted 1:100 in PBS. Cell were washed once again, treated with DAPI and Texas Red- X Phalloidin (Molecular Probes) solutions, with 1:50 and 1:100 PBS dilutions, respectively. Cells were then visualized on the IX81 confocal microscopy (Olympus).

Flow Cytometry

Cells were prepared in the same manner as the immunofluorescence, expect that the 5×10^6 cells were used, and the cells were not stained with DAPI or Texas Red. Cells were analyzed on the FC 500 cell analyzer (Cytomics).

RNAi of CANT1 In epithelial cells

10⁶ HCT-8 cells were transfected with CANT1, GAPDH, or non-target siRNAs using Oligofectamine Reagents (Invitrogen) according to the manufacturer's instructions. CANT1 protein levels were assessed using standard western blotting procedures and serum obtained from mice injected with recombinant SRK protein.

Results

Microarray Analysis of SRK's effects on host cells

To elucidate possible effects of SRK on host cells, we analyzed the transcriptional profile of host cells after treatment with recombinant SRK protein with Human U1333 v2 arrays. Statistical analysis revealed that 102 genes were differentially expressed, including genes involved in apoptosis and AMP signaling. Several altered genes in an apoptotic network are shown in Figure 1.

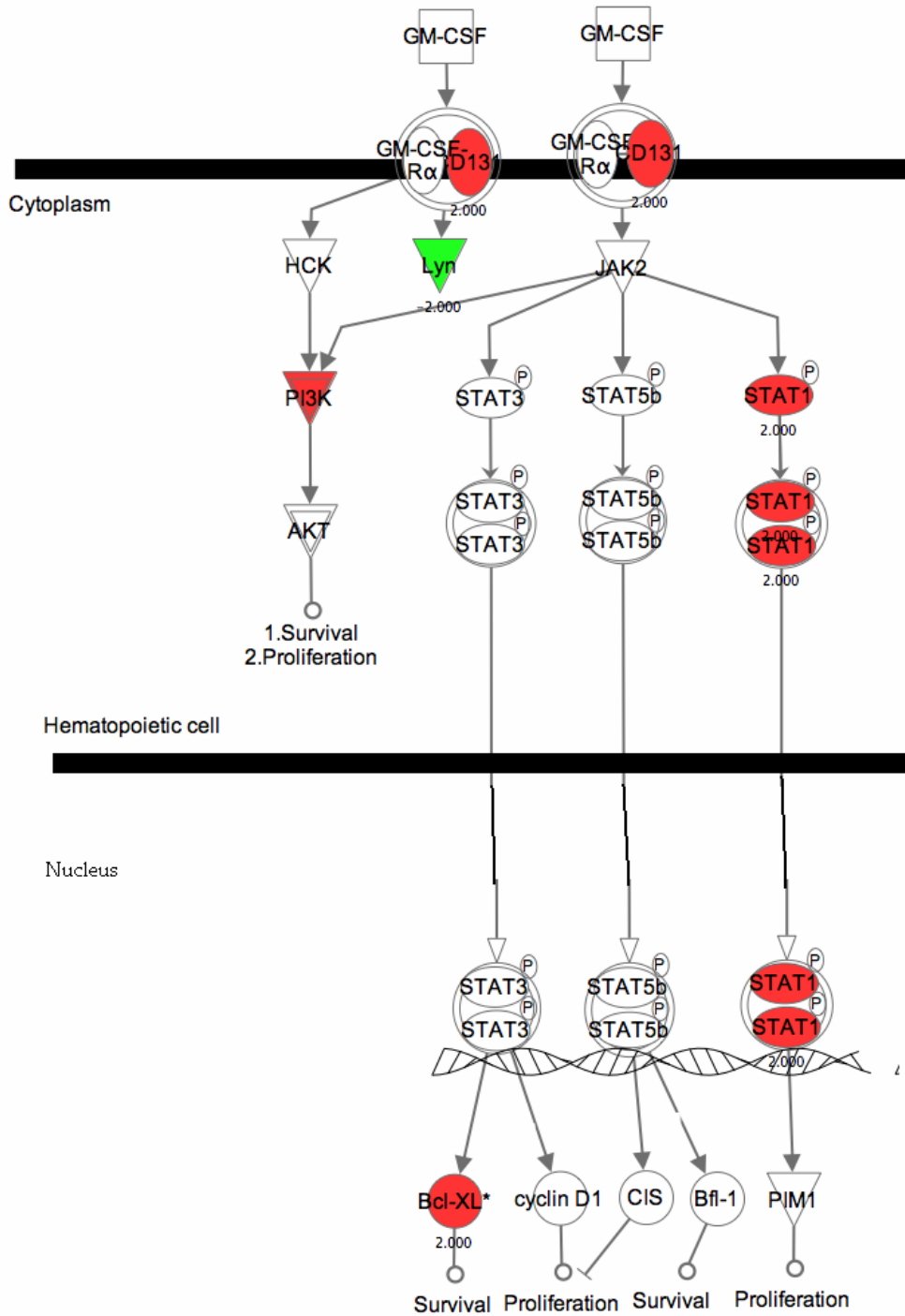


Figure 1. Pathway analysis of differential expressed genes after treatment with SRK. After statistical analysis, selected genes were analyzed using Ingenuity pathways. Genes that are upregulated are in red, and in green, those that are down regulated.

RT-PCR of SRK gene

To assess the expression level of the SRK gene during infection, we performed RT-PCR of *Cryptosporidium* sporozites before infection, and 3 hours, 6 hours, 20 hours, 24 hours, and 48 hours after infection. Preliminary results showed no amplification either of SRK or the positive control GAPDH gene. After increasing the total RNA concentration from 10 ng to 100 ng, amplification for the GAPDH gene could be seen at 20 hours, 24 hours, and 48 hours (Figure 2a), but not below these time points, and no SRK amplification was seen (Figures 2b). To test the specificity of the SRK primer, PCR was performed on a previously prepared SRK plasmid and *Cryptosporidium* DNA, and the plasmid did amplify, but the DNA did not (Figure 2b and 2c). The *Cryptosporidium* DNA did amplify the GAPDH gene, however.

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

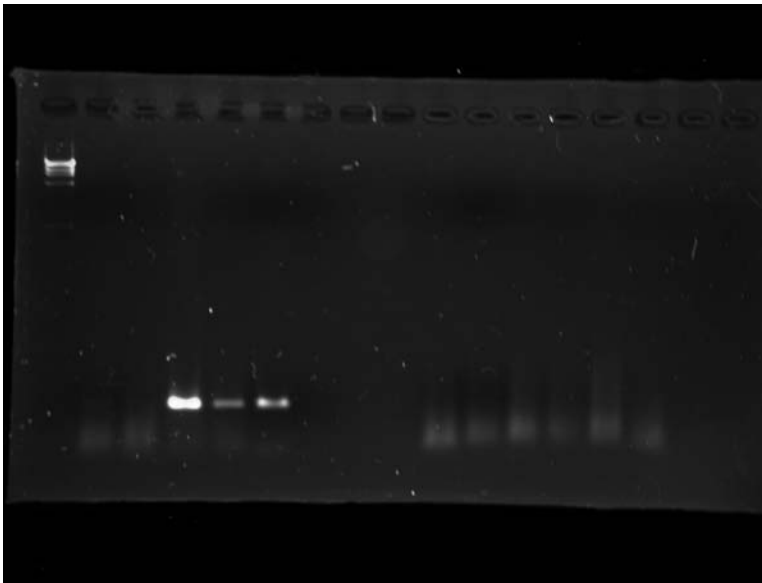


Figure 2a. RT-PCR of GAPDH gene in *Cryptosporidium* during infection. 1% agarose gel stained with ethidium bromide. Lane 1 contains a digested λ plasmid, lanes 2-6 contain RNA extracted 3 hours, 6 hours, 20 hours, 24 hours, 48 hours, and 0 hours after infection, respectively. Clear bands at the predicted amplicon size are seen in 20 hours, 24 hours, and 48 hour infected samples. Lanes 10-15 contain RNA in the sample orientation as lanes 2-6, but lacked reverse transcriptase in the PCR reaction.

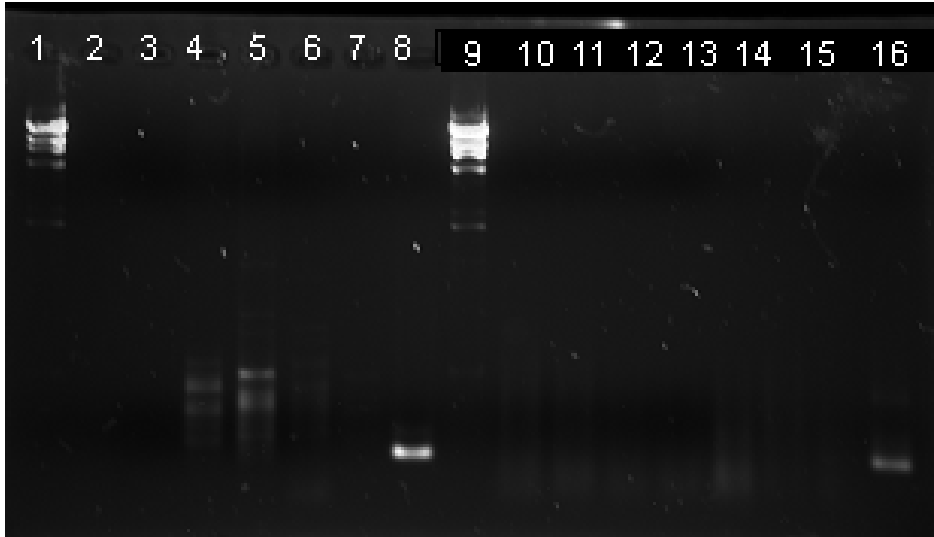


Figure 2b. RT-PCR of SRK gene in *Cryptosporidium* during infection. 1% agarose gel stained with ethidium bromide. Lanes 1 and 9 contains a digested λ plasmid, lanes 2-6 contain RNA extracted 3 hours, 6 hours, 20 hours, 24 hours, 48 hours, and 0 hours after infection, respectively. Lane 8 was loaded with SRK DNA plasmid, and has a clear band at the predicted amplicon size. Lanes 10-16 contain samples in the orientation as lanes 2-7, but lacked reverse transcriptase in the PCR reaction. Lane 16 was loaded with SRK DNA plasmid, and has a clear band at the predicted amplicon size.

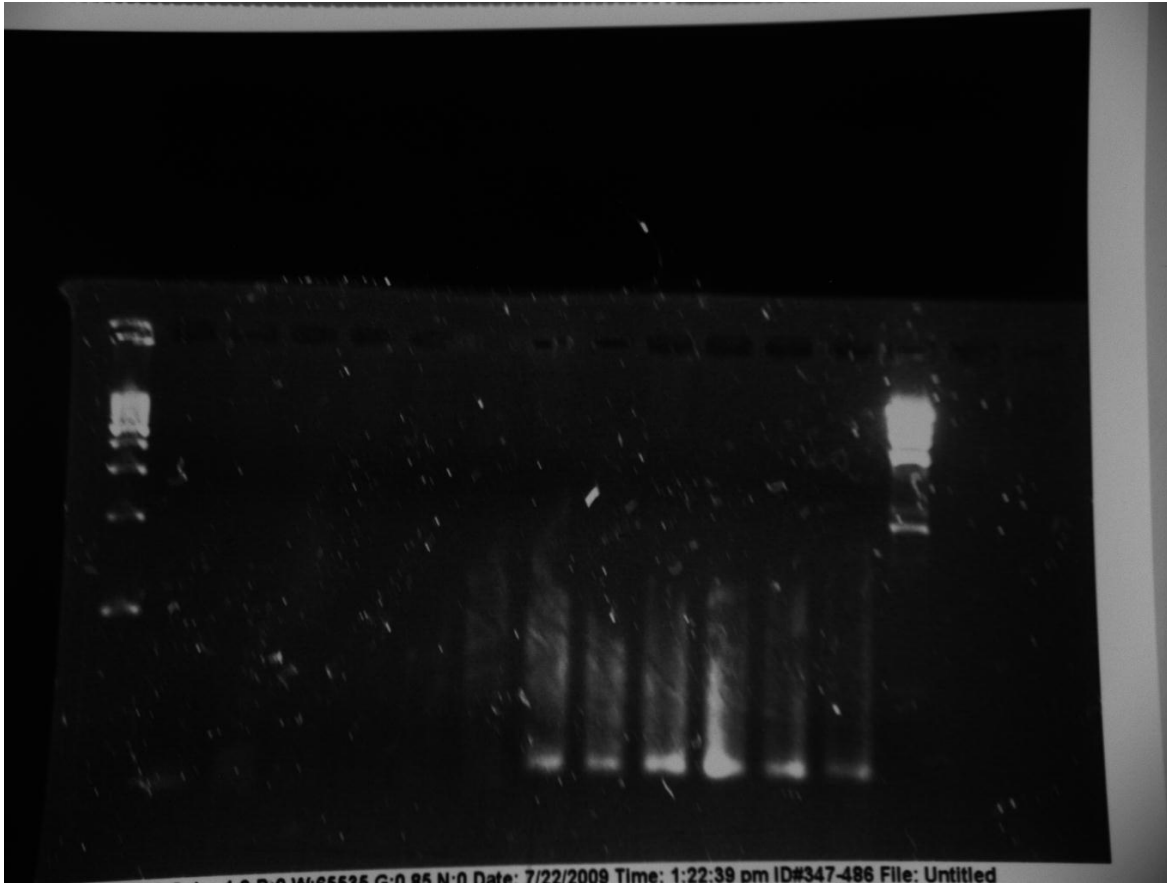


Figure 2c. PCR of SRK and GAPDH in *Cryptosporidium*. 1% agarose gel stained with ethidium bromide is shown. The first and last lanes contain a digested λ plasmid. The 6 lanes on the left contain PCR product using *Cryptosporidium* DNA as a template and SRK primers. A gradient of annealing temperature between 57° and 65 ° degrees (increasing from left to right lanes) was used in the PCR reaction. The 6 lanes on the right contain PCR product using *Cryptosporidium* DNA as a template and GAPDH primers. A gradient of annealing temperature between 57° and 65 ° degrees (increasing from left to right lanes) was used in the PCR reaction. For all 6 lanes, a clear band was seen.

Localization of CANT1 in epithelial cells

To locate CANT1 in epithelial cells, an indirect immunofluorescence assay was employed. Because of the high degree of sequence similarity between SRK and CANT1, polyclonal antibodies against SRK also bind to CANT1 (western blot data not shown). CANT1 appears to be located at outside of the nucleus of permeabilized HCT-8 cells (Figure 3a), but due to non-specific binding of the secondary antibody in the normal serum control (Figure 3b) this result can not be attributed solely to antibodies binding to CANT1.

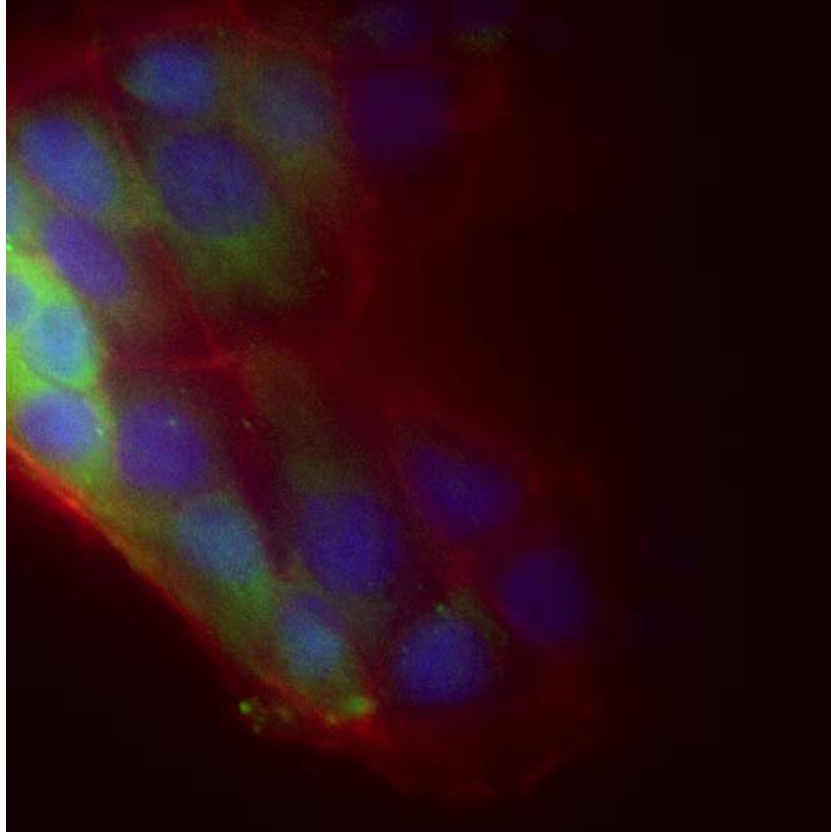


Figure 3a. Indirect immunofluorescence of CANT1. Micrograph showing permeabilized HCT-8 cells treated with anti-SRK and anti-mouse IgG antibodies (FITC label, green). The cytoskeleton (, Texas Red-phalloidin red) and nucleus (DAPI, blue) were also stained. Note the green staining around the nucleus.

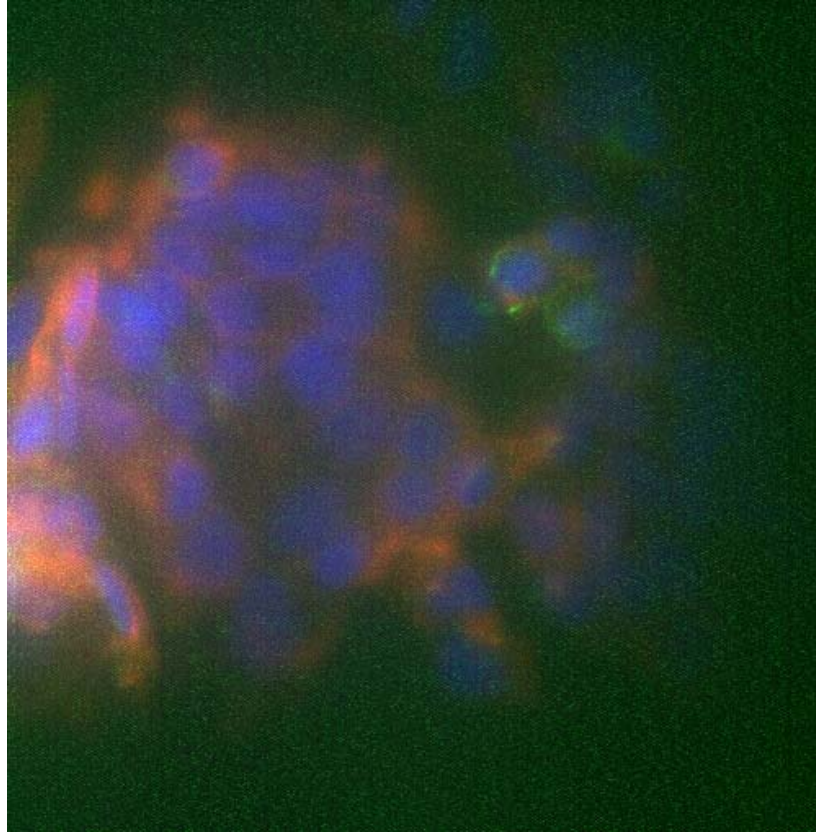


Figure 3b. Indirect immunofluorescence contro. Micrograph showing HCT-8 cells treated normal serum and anti-mouse IgG antibody (FITC label, green). Cytoskeleton (, Texas Red-phalloidin red) and nucleus (DAPI, blue) were also stained. Not the green staining surrounding cells.

Flow cytometry was also performed on permeablized and non-permeablized with the same antibody treatment used above. If CANT1 was located at the surface of the cell, we hypothesized that non-permeablized cells would have high fluorescence, and permeablized cells would have low fluorescence. If CANT1 was located on the inside of the cell, we would expect the opposite results. Cell populations treated with mouse normal serum had high levels of fluorescence in non-permeablized (Figure 4a) and permeablized cells (Figure 4b and 4c), masking any fluorescence caused by SRK antibody binding to CANT1.

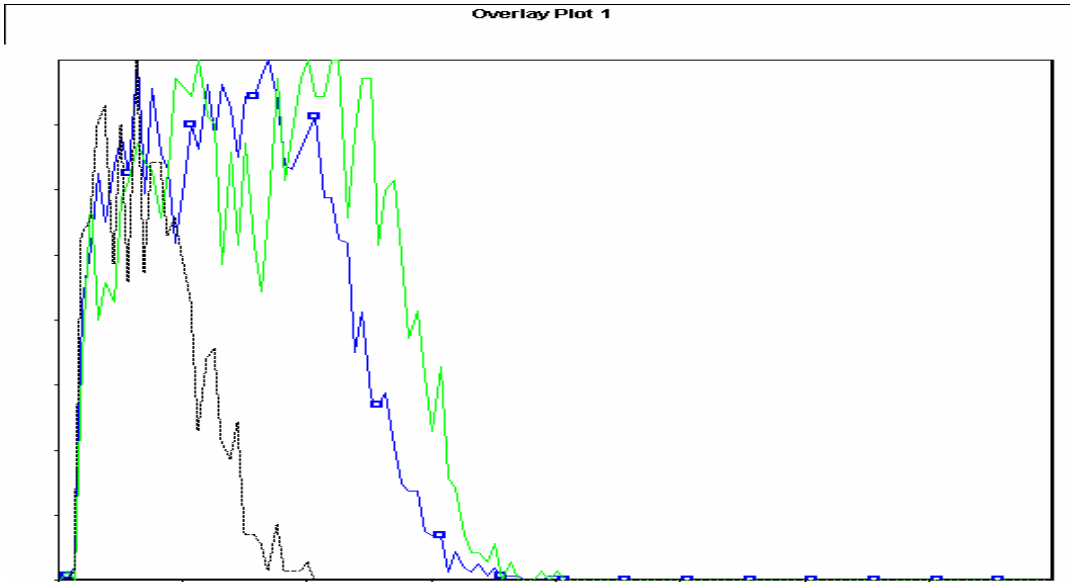


Figure 4a. Flow cytometry analysis of non-permeabilized HCT-8 cells. Shown here is a histogram plot, with X-axis indicates FITC emitted fluorescence intensity of individual cells (log scale) and y-axis indicates cell number. Black line represents autofluorescence cells (no antibody treatments), blue line represents cells treated with normal serum and anti-mouse IgG antibody conjugated with a FITC molecule, and green line represents cells treated with anti-SRK antibody and anti-mouse IgG antibody conjugated with a FITC molecule. Note that in both cell populations treated with normal serum and the secondary antibody, there a large number of cells that fluoresce at high intensity.

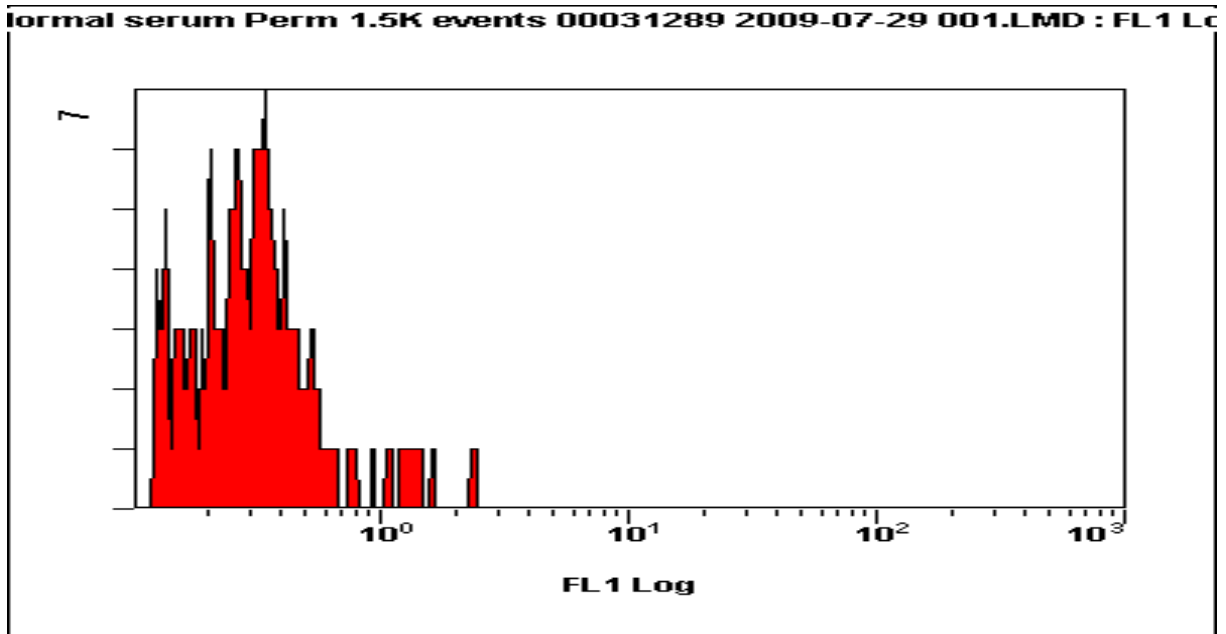


Figure 4b. Flow cyotmetry analysis of permeablized HCT-8 cells. Shown here is a histogram plot, with X-axis indicates FITC emitted fluorecence intensity of individual cells and y-axis indicates cell number. Cells were treated with normal serum and anti-mouse IgG antibody conjugated with a FITC molecule.

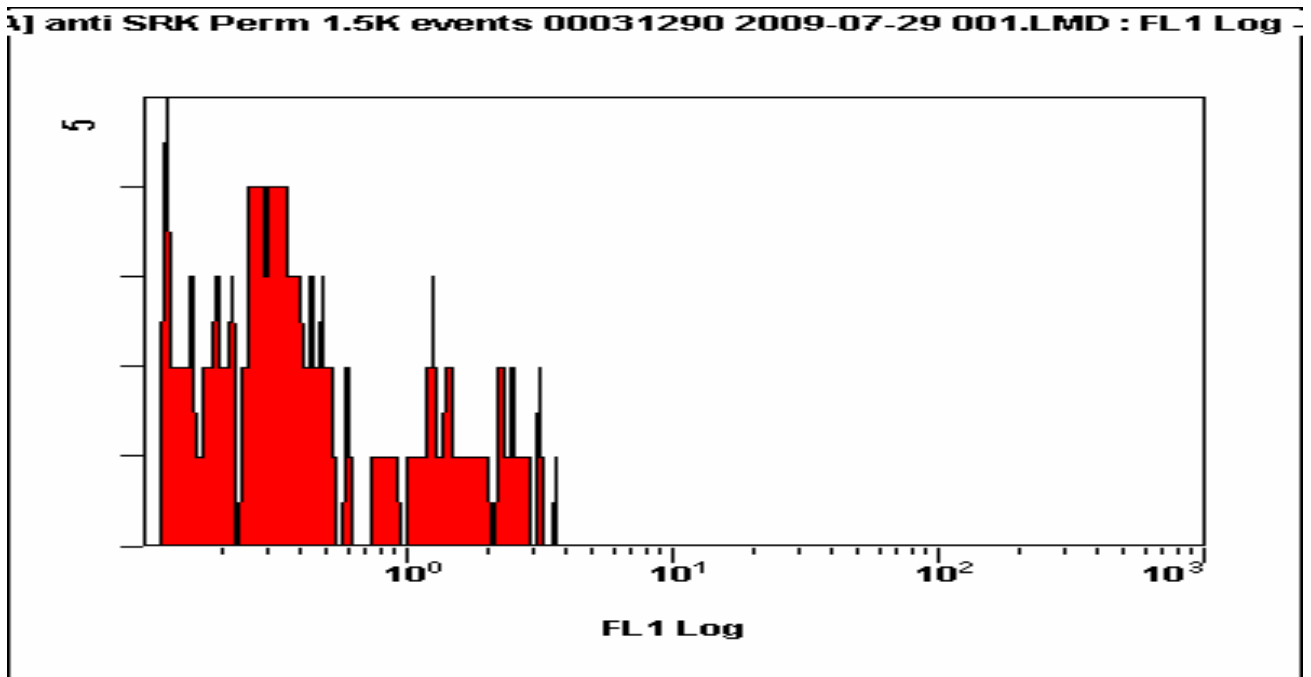


Figure 4c. Flow cyotmetry analysis of permeablized HCT-8 cells. Shown here is a histogram plot, with X-axis indicates FITC emitted fluorecence intensity of individual cells and y-axis indicates cell number. Cells were treated with anti-SRK antibody and anti-mouse IgG antibody conjugated with a FITC molecule.

RNAi of CANT1 in epithelial cells

In order to investigate a possible role of CANT1 during infection, we attempted to HCT-8 cells deficient for CANT1 protein with RNAi technology. However, we were able to reduce CANT1 protein levels (Figure 5).

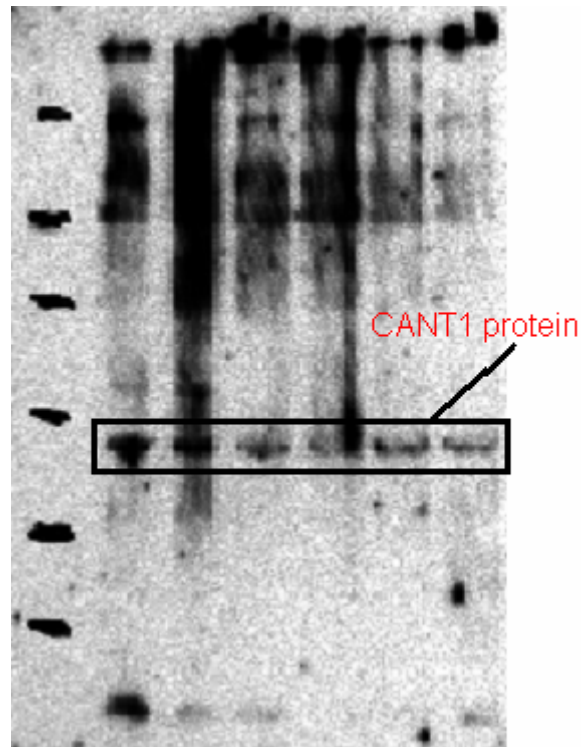


Figure 5. RNAi of CANT1. Shown here is a western blot of HCT-8 protein extract. From left to right, lane 1 contains a protein ladder, the next 4 lanes extract from cells treated siRNA directed against CANT1 and last the two lanes contain extract from cells treated siRNA directed GAPDH. Equal amounts of protein were loaded into each well, and the membrane was probed with antibodies against SRK. Note that there was no reduction in CANT1 protein in the knockout cell extracts.

Future directions

Because the primers failed to amplify the SRK in total genomic *Cryptosporidium* DNA, it is very likely that the primers are non-specific. A bioinformatic tool for the *Cryptosporidium* database similar to Primer-Blast for the human genome would be very useful.

Because of the fluorescence of normal serum controls in the indirect immunofluorescence and flow cytometry experiments, it is highly probable that the secondary antibody is

binding to another immunoglobulin in the mouse serum other than the SRK antibody. Three possible solutions to this problem are:

1. Use a different secondary antibody.
2. Treat HCT-8 cells with goat serum (species in which secondary antibody was obtained from) to block murine immunoglobins from binding to F_C receptors.
3. Develop a monoclonal antibody.

The protocol for CANT1 knock down by RNAi needs to be optimized. One might do this by experimenting with different concentrations of siRNA and oligofectamine reagents.

The microarray experiments suggest that SRK might influence apoptosis pathways. It has been already established that *Cryptosporidium* represses host cell apoptosis (4), but the mechanism in which this occurs remains to be elucidated. The microarray results need to be validated by RT-PCR, and it would be interesting to investigate possible changes in apoptosis levels in host cells infected with parasites coated in anti-SRK antibodies. If SRK prevents apoptosis, one would predict these cells to have a higher rate of apoptosis.

References

1. Smith TM, Hicks-Berger CA, Kim S, Kirley TL: **Cloning, expression, and characterization of a soluble calcium-activated nucleotidase, a human enzyme belonging to a new family of extracellular nucleotidases.** *Archives of Biochemistry and Biophysics* 2002, **406**:105-115.
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4. McCole DF, Eckmann L, Laurent F, Kagnoff MF: **Intestinal epithelial cell apoptosis following *Cryptosporidium parvum* infection.** *Infection and Immunity* 2000, **68**:1710-1713.