Transfection of Caco-2 Cells With siRNA Using the siLentFect™ Lipid Reagent

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Introduction

The tight junctions between intestinal epithelial cells are an important component of the permeability barrier separating the potentially harmful contents of the intestinal lumen from the internal milieu. Cultured monolayers of polarized epithelial cell lines, such as MDCK, T84, and Caco-2, are important model systems for the study of tight junction structure and function. The Caco-2 cell line in particular is a well-established model of the intestinal epithelium (Rousset 1986), and the study of tight junctions using this cell line has important implications for the understanding of normal intestinal physiology as well as diseases such as inflammatory bowel disease, celiac disease, and enteric infections.

Tight junction permeability can be modified in response to physiological and pathophysiological stimuli, and one important regulator of permeability is myosin light chain kinase (MLCK). Although the molecular mechanisms are incompletely defined, it is clear that increased MLCK activity can increase tight junction permeability in epithelia and endothelia. This mechanism appears to have direct relevance to physiological tight junction regulation as well as dysfunction in a variety of infectious and inflammatory diseases (Clayburgh et al. 2004b).

The recent development of siRNA technology to reduce expression of specific target genes has provided a new method of probing tight junction regulatory pathways in cell models. Unfortunately, the Caco-2 cell line is relatively resistant to transfection, making the use of siRNA technology technically difficult. In particular, Caco-2 cells must be transfected in suspension before plating, after which the cells typically require a week or more of culture to develop functional tight junctions. Given that most siRNAs have a half-life within cells of less than 4 days, traditional methods of Caco-2 transfection are not adequate for siRNA-mediated knockdown.

We used siLentFect lipid reagent to provide efficient siRNA transfection of Caco-2 cells. Knockdown using siLentFect-mediated transfection of siRNA and high-density plating of Caco-2 cells allowed the early development of tight junctions, permitting the study of tight junction physiology. With this method, we were able to interfere with expression of MLCK1, a splice variant of MLCK, and to study the effect on electrophysiology.

Methods

Tissue Culture

Cells from the BBe clone of the Caco-2 cell line (Peterson and Mooseker 1992) expressing the intestinal Na^+-glucose cotransporter SGLT1 (Turner et al. 1996) were plated on Transwell permeable supports (Corning Inc., Corning, NY, USA) as described previously (Turner et al. 1997).

siRNA Design and Transfection

A 207 bp sequence (nucleotides 1,428–1,634) unique to the MLCK1 splice variant was used to design the MLCK1 *SMART*pool siRNA (Dharmacon, Lafayette, CO, USA). For transfection, 10 µl of 50 µM MLCK1 *SMART*pool siRNA or 25 µl of a 20 µM nonspecific control siRNA mix was added to 500 µl Opti-MEM medium (Invitrogen Corp., Carlsbad, CA, USA) and allowed to incubate for 5 min at 25ºC. The siRNA solution was then added to 500 µl of Opti-MEM containing 30 µl of siLentFect lipid reagent and allowed to incubate for 30 min at 25ºC to create the transfection mix. At the same time, approximately 10^7 Caco-2 BBe cells were incubated with 1.5 ml trypsin for 20 min. After trypsinization, the cells were resuspended in 5 ml of DMEM with 4.5 g glucose/L (Mediatech, Herndon, VA, USA). The cells were recovered by gentle centrifugation at 500 x g for 5 min, washed once more in DMEM followed by Opti-MEM, separated into two equal aliquots, and resuspended in 800 µl of Opti-MEM. This suspension was added to 1 ml of previously prepared transfection mix. The cell suspension and siRNA mix was then plated at high density (375,000 cells/cm^2) on Transwell permeable supports and cultured for 4 days to allow tight junction assembly and polarization before use in electrophysiology experiments.

Analysis of Transfected Monolayers

To isolate RNA for RT-PCR, monolayers were scraped into TRIzol reagent (Invitrogen), and RNA was extracted with chloroform, precipitated with isopropanyl alcohol, and resuspended in DEPC-treated water. Quantitation of MLCK1 and MLCK2 mRNA levels was performed using primers TCTGAGAAGAACGGCATG and ACTTCAGGGGGTGGATTC. All reactions were cycled 36 times using an iCycler® thermal cycler, with an annealing temperature of 57°C. The PCR products were separated on a 1% agarose gel and visualized using ethidium bromide. The band intensity was measured using ImageQuant software (Amersham Biosciences, Piscataway, NJ, USA), and the MLCK1 content was calculated as (MLCK1 intensity)/(MLCK1 + MLCK2 intensity). See Clayburgh et al. (2004a) for more details.

Cell monolayers were lysed, and the lysate was separated by SDS-PAGE and immunoblotted using MLCK1-specific antisera. The band corresponding to MLCK1 was detected with horseradish peroxidase-conjugated secondary antisera (Cell Signaling Technology, Inc., Beverly, MA, USA), and the blot was visualized by enhanced chemiluminescence.

Electrophysiological measurements were made with agar bridges and Ag/AgCl calomel electrodes, as previously described (Turner et al. 1997). Briefly, monolayers were transferred from culture medium to Hank's balanced salt solution (HBSS) with 15 mM HEPES (pH 7.4) and 25 mM glucose to activate Na^+-glucose cotransport. Electrical potential differences were measured before and after application of a 50 µA current, and transepithelial resistance (TER) was determined using Ohm's law.

Results and Discussion

To determine the effectiveness of siRNA-mediated knockdown of MLCK1 in transfected Caco-2 cells, we performed semiquantitative RT-PCR of RNA from cells transfected with either nonspecific control siRNA or MLCK1-specific siRNA (Figure 1A). We observed a significant decrease in MLCK1 mRNA in cells transfected with MLCK1-specific siRNA. MLCK1 mRNA content of monolayers transfected with control siRNA was 52±3% of the total MLCK expressed. MLCK1 mRNA was reduced to 27±2% of total MLCK mRNA, a 47±4% reduction, in monolayers transfected with MLCK1-specific siRNA. MLCK2 mRNA content was unchanged by the MLCK1-specific siRNA.

We confirmed that siRNA reduced expression of MLCK1 by immunoblotting cell lysates with MLCK1-specific antisera (Figure 1B). Cells transfected with the specific siRNA, but not the control siRNA, showed a significant reduction in the amount of MLCK1 protein. This reduction in MLCK1 expression had a significant effect on the tight junction permeability of the Caco-2 monolayers: Monolayers of cells transfected with MLCK1 siRNA exhibited a significant increase in transepithelial resistance compared to those transfected with nonspecific siRNA (Figure 2). Since MLCK activity is known to decrease TER (Turner et al. 1997), this result suggests that MLCK1 makes an important contribution to tight junction regulation in Caco-2 cells.

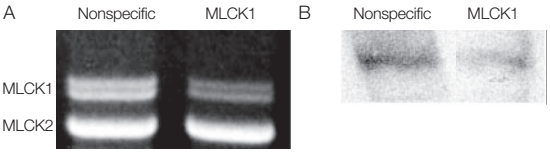

Fig. 1. Knockdown of MLCK1 in transfected Caco-2 cells.* A, RT-PCR of MLCK splice variants MLCK1 (upper band) and MLCK2 (lower band) from Caco-2 monolayers transfected with nonspecific or MLCK1-specific siRNA. B, immunoblots of lysates from monolayers transfected with nonspecific and MLCK1-specific siRNA with MLCK1-specific antisera. A significant drop in MLCK1 mRNA and protein expression is observed in monolayers transfected with MLCK1-specific siRNA.

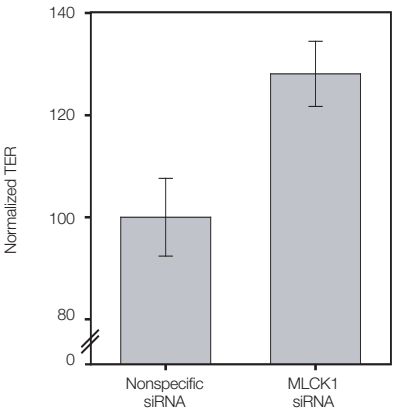

Fig. 2. Normalized TER in Caco-2 monolayers transfected with nonspecific and MLCK1-specific siRNA.* TER was normalized to monolayers transfected with nonspecific siRNA. TER was significantly higher in monolayers transfected with MLCK1-specific siRNA. Error bars represent standard error.

* Data from Clayburgh et al. (2004a).

Conclusions

In this study, we successfully knocked down the expression of a single splice variant of MLCK in Caco-2 cells using siLentFect lipid reagent to transfect siRNA. The use of siLentFect in conjunction with high-density plating allowed us to measure the effects of siRNA transfection on tight junction physiology. This method permits the use of siRNA technology in the study of Caco-2 cell physiology and barrier function and can contribute to generating further insight into molecular regulation of intestinal permeability.

References

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