Characterization of Cortactin Expression in Tissue Types of the Eye

Magdalena Christoforou
Department of Biological Sciences, Fordham University
Bronx, NY

Abstract

The mammalian eye has evolved a renewal mechanism that maintains vision for life. One of the main actors in this process is the retinal pigment epithelium (RPE), which engulfs and degrades spent photoreceptor outer segment tips (POS) daily. This clearance process is called RPE phagocytosis. Failure of RPE phagocytosis can lead to retinal degeneration. The cellular mechanisms underlying RPE phagocytosis are still not well understood. The purpose of this study was to characterize cortactin expression in RPE cells since this protein is a molecular scaffold for F-actin assembly and organization, both of which take place during phagocytosis. Moreover, the aim of this project was to determine if cortactin expression is impacted by the absence of the avb5 integrin, a key receptor in the RPE membrane that mediates the binding of POS. RNA from wild type and β5 integrin knockout eyecups, and retinas were subject to RT PCR analysis. Two cortactin transcript variants were identified in the eyecups and retinas of wild type and β5 integrin knockout mice, one that contains exon 11 and one that does not contain exon 11. Moreover, the level of cortactin transcript appeared to be higher in β5 integrin knockout eyecups in comparison to wild type eyecups.

Introduction

The mammalian eye has evolved a daily renewal process that is vital for sustaining vision. One of the key actors involved in this mechanism is the retinal pigment epithelium (RPE), a continuous monolayer of epithelial cells located at the back of the eye interdigitating with light sensitive photoreceptor neurons (Boulton and Pierrette Dayhaw, 2001). The RPE performs many

functions vital for retinal preservation, including the prompt engulfment and degradation of spent photoreceptor outer segment tips, a process called RPE phagocytosis (Boulton and Pierrette Dayhaw, 2001). Photoreceptors are exposed to high levels of light on a daily basis, leading to the accumulation of photo-damaged lipids and proteins (Beatty et. al., 2000). To remain functional, the outer segments must undergo a constant renewal process, in which new materials are inserted at proximal ends of outer segments, while distal photoreceptor outer segment fragments (POS) are phagocytosed by underlying RPE cells in a diurnal rhythm (Young, 1967). Failure of RPE phagocytosis can lead to retinal degeneration, characterized by the accumulation of POS debris and eventual photoreceptor cell death (Edwards and Szamier, 1977; Chaitain and Hol, 1983).

The initial step in RPE phagocytosis involves the binding of POS to the apical membrane of the RPE. The initial phase of POS binding to RPE cells is mediated by αvβ5 integrin, a receptor embedded in the apical membrane of RPE cells. The recognition of POS binding is then communicated to the intracellular space through a signaling cascade via FAK, which is activated by the αvβ5 integrin (Finnemann et. al., 1997). FAK then phosphorylates and activates MerTK, a receptor tyrosine kinase located in the RPE apical membrane (Chaitain and Hol, 1983). MerTK, then activates its own downstream signaling that results in POS ingestion (Chaitain and Hol, 1983). The engulfment of shed POS occurs through the formation of a phagocytic cup, which is dependent on the reorganization of the RPE F-actin cytoskeleton (Law et. al., 2007).

The objective of this study was to characterize cortactin expression in RPE cells. The CTTN gene is located on the long arm of chromosome 11 and consists of 18 exons, which code for the cortactin protein, a monomeric protein that is involved in the polymerization and rearrangement of the actin cytoskeleton in cells (Kirkbride et al., 2011). Cortactin consists of an N-terminal acidic domain (NTA), followed by six and a half cortactin repeats, an alpha helix, a

proline rich region, and a SH3 domain at its C-terminus. The N-terminus of cortactin is critical for regulating branched F-actin assembly by interacting with the F-actin nucleating Arp2/3 protein complex at the NTA and binding to F-actin at its cortactin repeats (Kirkbride et al., 2011). The C-terminal SH3 domain can bind a nucleating promoting factor, N-WASP, which recruits G-actin monomers to promote F-actin assembly (Lua and Low, 2004). The aim of this study was to characterize cortactin expression in RPE cells since this protein is an important scaffold for F-actin organization. (Lua and Low, 2004). The aim of this study was also to characterize cortactin expression in the neural retina to determine whether cortactin's expression pattern is unique to the RPE. In addition, cortactin has been reported to interact with the protein FAK, a key component of the RPE phagocytic pathway that is activated by the POS recognition receptor avb5 integrin (Tomar et. al., 2012) Thus, the goal of this study was also to determine if cortactin expression is impacted by the absence of the avb5 integrin.

Methods

Animals

3 month-old Wild-type, β5 integrin knockout, and JNK2 knockout mice were sacrificed by CO2 asphyxiation at the same hour of the day. Eyeballs were carefully enucleated and rinsed in HBSS without Ca^{2+} and Mg^{2+}. The lens and vitreous humor were dissected out from each eye. Retinas were then removed from each eye, leaving a remaining eyecup containing the RPE/choroid layers. For one sample, three retinas or three eyecups were pooled together in RLT lysis buffer plus β-mercaptoethanol (Quiagen kit) and frozen at -80° C until further RNA extraction.

RNA Extraction

All lysate samples were purified from genomic DNA by a Qiashredder column from Qiagen. RNA was then extracted following the Qiagen RNeasy plus mini kit, following the manufacturer protocol. The Purity and concentration of each sample were then analyzed by spectrophotometry, and a 5ng/ul solution stock were made and stored at -20° C.

RT-PCR

RT-PCR was performed using the Qiagen One-Step RT-PCR kit. Ten nanograms of RNA were amplified in a 10ul reaction. RT PCR for cortactin in eyecups and neural retinas was performed with 0.5uM of the following primers:

For amplifying the region from Exon 2- Exon 6

exon2F: CATTGATCATCGCAGATGC

exon6R: CATCTGGACACCGAACTTGC

For amplifying the region from Exon 3-Exon 11

exon3F: GCTGATGACTGGGAGACTG

exon11R: CTTGTCCTGTCTGTCTGTCTGC

For amplifying the region from Exon 10-Exon 18

exon10F: GACAGACAGGACAAGTGTGC

exon18R: AGCTCCACATAGTTGGTG

RT PCR for the housekeeping gene GAPDH was performed with 0.5uM of the following primers:

GAPDHF: ACCAGGGCTGCCATTTGC, GAPDHR: TGGAAGATGGTGATGGGCTTCC

Products were then analyzed in a 1% agarose gel with Ethidium bromide. Products were then gel purified using the QIAquick gel extraction kit and set out for Sanger sequencing by Genewiz.

Results

In order to determine if cortactin expression is impacted by the absence of the avb5 integrin, an RT PCR for cortactin was performed in wild-type and b5 integrin knockout mice eyecups and neural retinas using primer pairs that spanned exons 2-6, 3-11, and 10-18. The results depicted in Figure 1 indicate that the level of cortactin transcript appears to be higher in β5 integrin knockout eyecups in comparison to wild type eyecups in the regions amplified using primer pairs for exons 3-11 and for exons 10-18. This same increase in cortactin expression was not observed in the neural retinas amplified using primer pairs for the same regions. The level of cortactin expression appeared to be the same in both the eyecups and the neural retinas of the region amplified using primers spanning exons 2-6. Moreover, the results in Figure 1 indicated the presence of two bands in the region amplified using primer pairs spanning exons 10-18, suggesting that two alternative cortactin transcripts were being produced in this region.

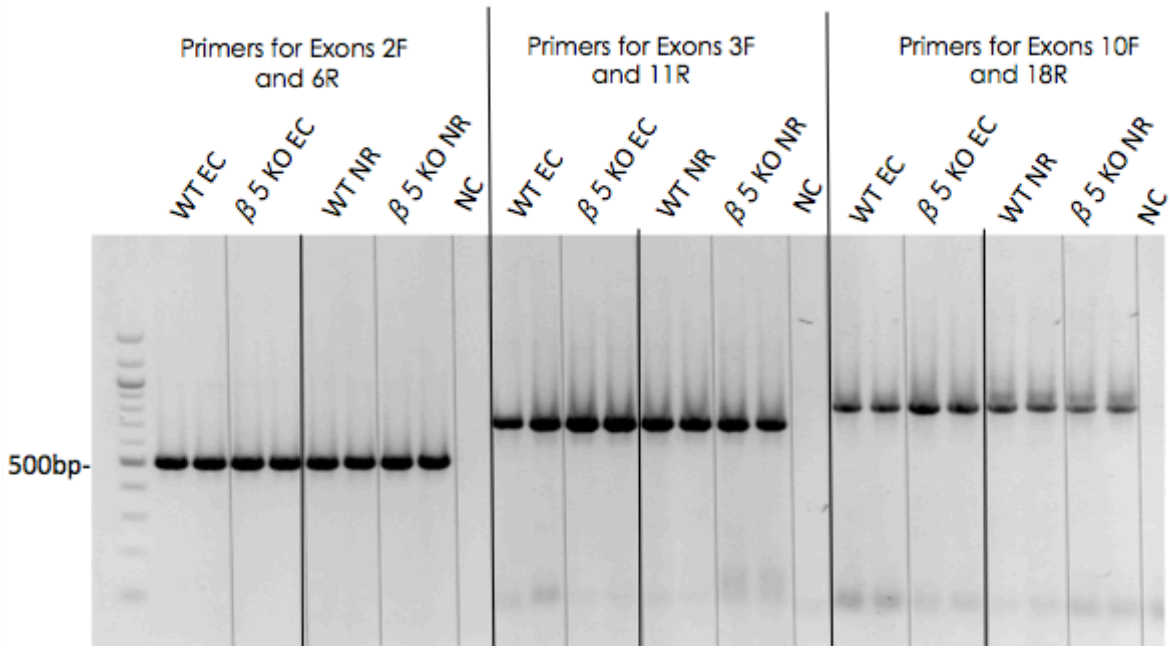

Figure 1: RT PCR for cortactin in Wild Type (WT) and β5 integrin knockout eyecups (EC) and neural retinas (NR). Amplified using primers spanning exons 2-6, 3-11, and 10-18. Two transcripts are being produced using primers for exons 10-18. Cortactin expression appears to be higher in β5 integrin knockout eyecups versus wild-type eyecups.

In order to determine whether two alternative cortactin transcripts were being produced, another RT PCR for cortactin was performed using primer pairs spanning exons 10-18. This analysis utilized eyecups and neural retinas from wild-type and β5 integrin knockout mice. This analysis also utilized eyecups and neural retinas from JNK2 knockout mice, which were originally used to test primer pairs, but were employed in this experiment to characterize the expression of the two cortactin transcripts observed in the regions amplified using primer pairs for exons 10-18 as well as controls to determine whether the increase in cortactin expression observed in β5 integrin knockout eyecups was unique to that knockout or simply a knockout artifact. As is depicted in Figure 2, two bands were once again produced in both eyecups and neural retinas of wild-type, β5 integrin knockout, and JNK2 knockout mice. To characterize these two bands, products were gel purified and sent out for sequencing analysis. Sequencing analysis revealed that the two bands were in fact two cortactin alternative transcripts that differed by 111 base pairs, corresponding to exon 11 of cortactin. Thus, while the 911 base pair cortactin transcript contains exon 11, the 800 base pair cortactin transcript does not contain exon 11. In addition, the results in Figure 2 once again indicated higher cortactin expression in β5 integrin knockout eyecups in comparison to wild type eyecups, or JNK2 knockout eyecups. This same increase in cortactin expression was not observed in the neural retinas. To confirm the increase in cortactin expression seen in β5 integrin knockout eyecups in comparison to wild type eyecups, an RT-PCR for the housekeeping GAPDH gene was performed. Eyecups exhibited similar GAPDH expression, confirming the increase in cortactin expression.

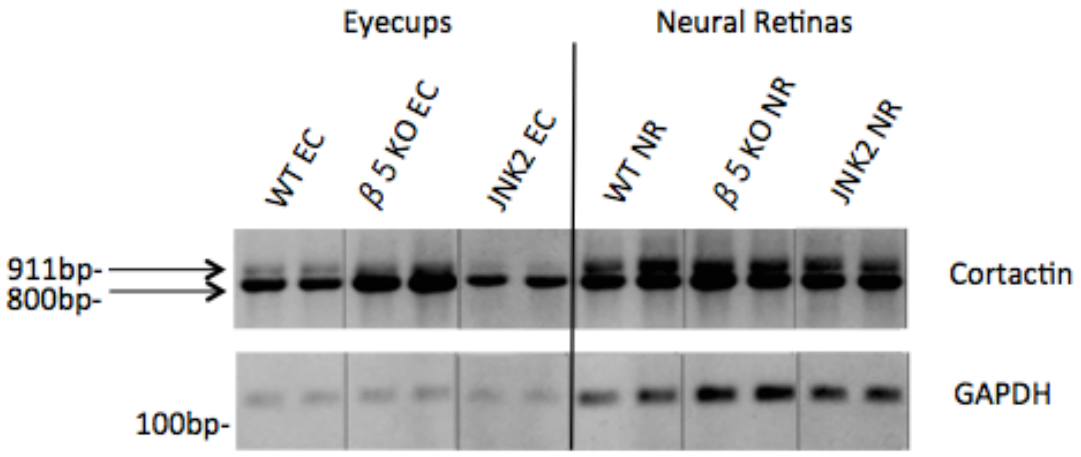

Figure 2: RT PCR for cortactin and GAPDH in Wild Type, β5 integrin knockout, and JNK2 knockout eyecups (EC) and neural retinas (NR). Amplified using primers spanning exons 10-18. Two transcripts are being produced, one that contains exon 11 (911bp) and one that does not contain exon 11 (800bp). Cortactin expression appears higher in β5 integrin knockout eyecups versus wild-type JNK2 knockout eyecups.

Discussion

The purpose of this study was to characterize cortactin expression in RPE cells as well as to determine if cortactin expression is impacted by the absence of the avb5 integrin. The results of this study depicted both in Figure 1 and Figure 2 indicate that the expression of cortactin appears to be higher in β5 integrin knockout eyecups in comparison to wild type eyecups in the regions amplified using primer pairs for exons 3-11 and for exons 10-18. An increase in cortactin expression in β5 integrin knockout eyecups was not observed in the region amplified using primer pairs for exons 2-6. One explanation for such an observation could be that a cortactin transcript may be produced beginning at exon 3 and may be transcribed more frequently than the transcript produced beginning at exon 2. Moreover, the increase in cortactin expression observed in eyecups was not observed in the neural retinas, suggesting that this increase in cortactin expression in β5

integrin knockout mice was specific to tissues of the eyecup where the RPE cells reside. The results of this study thus suggest that cortactin expression may be impacted by the absence of the avb5 integrin in the eyecup where RPE cells reside and may be up-regulated in b5 integrin knockout eyecups, possibly to compensate for the loss of this receptor. Future studies on the protein level could provide more insight into this observation.

Moreover, this study identified the production of two cortactin transcript variants in eyecups and neural retinas of all mice examined, one that is missing exon 11 (111bp) and one that contains exon 11. Exon 11 encodes a cortactin repeat of 37 amino acids in length that is responsible for binding F-actin (Van Rossum et al., 2003) (Figure 3). A previous study found that the transcript variant lacking exon 11 is found co-expressed with the transcript that contains exon 11 in all tissues and cell lines examined (Van Rossum et al., 2003). This study also found that cells over-expressing the form of cortactin that does not contain exon 11 were significantly less motile than cells over-expressing the form of cortactin containing exon 11, due to their reduced F-actin cross-linking capacity (Van Rossum et. al., 2003). The expression of this variant in cells has been suggested to provide a mechanism in which cortactin can modulate F-actin dynamics and cell migration. Future studies to examine the protein expression of these alternative transcripts in both the retina and RPE as well as their physiological roles in these tissue types could provide more insight into these mechanisms.

Figure 3: Schematic of cortactin proteins produced from two alternative transcripts. The top figure represents the cortactin protein produced from the transcript that contains exon 11. The bottom figure represents the cortactin protein produced from the transcript that does not contain exon 11, which corresponds to the sixth cortactin repeat which is 37 amino acids in length.

References

Beatty S, Koh H, Phil M, Henson D, Boulton M. The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol* **45**: 115–134, 2000.

Boulton, M., Dayhaw-Barker, P. The role of the retinal pigment epithelium: topographical variation and ageing changes. *Eye* **15.3**: 384-389 2001.

Chaitin, M. H., Hall M. O. Defective ingestion of rod outer segments by cultured dystrophic rat pigment epithelial cells. *Investigative Ophthalmology Vis. Sci* 24.7: 812-820, 1983.

Finnemann, S. C., et al. Phagocytosis of rod outer segments by retinal pigment epithelial cells requires αvβ5 integrin for binding but not for internalization. *Proceedings of the National Academy of Sciences* **94.24**: 12932-12937, 1997.

Kirkbride, K. C., Sung, B. H., Sinha, S., Weaver, A. M. (2011). Cortactin: a multifunctional regulator of cellular invasiveness. *Cell adhesion & migration* **5.2**: 187-198, 2011.

Law, A. L., Ling, Q., Hajjar, K. A., Futter, C. E., Greenwood, J., Adamson, P., ... & Hayes, M. J. (2009). Annexin A2 regulates phagocytosis of photoreceptor outer segments in the mouse retina. *Molecular biology of the cell* **20.17**: 3896-3904, 2009.

Lua, B. L., Low B. C. Cortactin phosphorylation as a switch for actin cytoskeletal network and cell dynamics control. *FEBS letters* **579.3**: 577-585, 2005.

Tomar, A., Lawson, C., Ghassemian, M., Schlaepfer, D. D. Cortactin as a target for FAK in the regulation of focal adhesion dynamics. *PloS one* **7.8**: e44041, 2012.

Van Rossum, A. G., de Graaf, J. H., Schuuring-Scholtes, E., Kluin, P. M., Fan, Y. X., Zhan, X., ... & Schuuring, E. (2003). Alternative splicing of the actin binding domain of human cortactin affects cell migration. *Journal of Biological Chemistry*, *278*(46), 45672-45679.