1.) ABSTRACT

Gurken (Grk) is a protein translated at the dorsal anterior corner of the oocyte during the oogenesis of the developing follicle. It associates with the oocyte surface to facilitate proper dorsal/ventral patterning. Keeping grk mRNA translationally silent until needed requires several factors. One such factor is Spd, a translational repressor that binds to grk mRNA and inhibits translation until it reaches the dorsal anterior end of the developing oocyte. Thus, Spd plays a role in establishing proper polarity and viability of the oocyte. It is therefore important to determine where and when this interaction occurs in order to better understand the grk mRNA development. The specific localization and interaction of grk mRNA and Spd protein has been the focus of this study. A real-time analysis approach, TriFC Fluorescence Complementation (TriFC), Venus fluorescent protein was split into two domains and each was incorporated into one of two different constructs. The first construct contained one half of Venus and a viral protein that binds strongly to the comparable terminus of the mRNA. Each of a second set of constructs contains a gene encoding one of the isoforms of Spd (Spd or Spd). Each of these constructs has been used to create separate transgenic fly lines. After analyzing these lines, Venus fluorescence was captured when and where domains were in juxtaposition due to protein-mRNA interaction. Fluorescence appeared in the developing oocytes, ring canals, anterior cortical and later localized to the dorsal anterior corner. These observations suggest a pathway of movement of the spd-grk complex that starts in the nurse cells. However, auto-fluorescence contamination by egg yolk was observed, causing complications in reading the fluorescence.

2.) BACKGROUND

Gurken-The Eggn signal Protein

Gurken (Grk) protein is translated from grk mRNA and localized at the dorsal anterior corner during the later stages of Drosophila (fruit fly) development. Once translated, Grk protein activates epithelial growth receptors (EGFR) that signal for the formation of dorsal appendages on the eggshell of Drosophila. Both improper localization and concentrations of Grk protein result in phenotypic abnormalities in the dorsal appendages, resulting in improper localization and death. A major challenge in Grk function is that Grk protein lacks specific appendages due to auto stimulation of EGFR by Grk. (Figure 1). Due to the importance of Grk quantity and quality, researchers must control all of the signals involved in Grk translation as genetic engineering protocols for transgenesis (Fig. 1).

Advantages of Fluorescent Protein (FPLC) Reporting

A simple protein that functions in developing Drosophila that plays an important role in the recognition of axon polarity and dorsal development through the regulation of Grk. More specifically, there is a large regulatory function in the oocyte via its ability to bind to grk mRNA and preclude translation until the proper time and location. It is known that spd binds grk mRNA and prevents Grk protein in the dorsal anterior corner and controls leading one or more Trichescient protein (TRIC). Spd has been found to play a role in both somatic cell function, and oogenesis. In contrast, SPD is thought to function only in the ovary, in somatic cells, while SPD functions only in oocytes. Consequently, experiment by Newbey et al., 1999 has revealed that eggs lacking Spd do not have a significant change in protein or cell patterning in eggs because they do not interact with grk to direct localization. However, eggs lacking in Spd and Spd exhibit severe misregulation of grk, and thus dorsalization occurs (Figure 1). Differences also exist between Spd and Spd. Spd has the ability to restrict Grk protein to the dorsal anterior, yet does not significantly block grk mRNA. Therefore, it is believed that Spd has a role in the same region as Spd.

Each form of Spd plays a role in Drosophila, and many specific of these roles are not understood. The emphasis in this manuscript is to put in situ specific factors make contact with grk to further understand the Grk pathway in development.

TriFC Technology

TriFC Fluorescence Complementation (TriFC) technology was pioneered by Kieczman and Green at the University of Chicago, (Newbey and Brown, 2001). TriFC provides a method to visualize the real-time interaction between protein and mRNA in mammalian cell culture. In this study, TriFC technology was used to determine Spd protein and grk mRNA interactions in real-time and in vivo using Drosophila oocytes. Adaptation of this system to in vivo Drosophila has allowed for real-time observation of Grk mRNA interaction. This method makes the TriFC technology particularly advantageous. Drosophila oocytes develop in a track-like pattern within the ovary, and so stages can be easily assessed and may provide a window to the future. It will be interesting to see if there are advantages that feed more complex and disease management with mouse models.

Previously, Buenton et al. described the first construct necessary for TriFC. This construct (pQaFpcSpd-sqdB-VP16, 1.54, or TriFC) mimics the Drosophila grk mRNA and Grk protein (translation) and results in a 1:1 mRNA:protein ratio which is required for the creation and incorporation of the suicidal VP16-253 construct was effective (Figure 4). The TriFC technology was used to evaluate the expression of different mRNAs and proteins within the oocyte. Western blot analysis showed prevalent bands in all lanes when anti-Spd was used. However, the bands were not as sharp as those witnessed in the live analysis and the intensity was lower when using anti-Spd antibodies. Therefore, the failure to detect bands in the live analysis may be due to the creation of the suicidal VP15-253 construct was effective (Figure 4). This was also evident through the various crosses where expected phenotypes were not observed. In the future, Western blot analysis of MCF 1-55 will be used to confirm the presence and absence of grk mRNA and grk protein expression in the newly transgenic Drosophila line that will be made, which will also contain a hemagglutinin (HA) tag construct. However, this will not be used for Western blot analysis, but the hemagglutinin tag can also be used for immunohistochemical analysis of the transgenic-containing oocytes.

4.) RESULTS

Through the phenotypic marker analysis of grk mRNA with grk protein analysis it was clear that previous protocols including genetic engineering, transforming, and transgenesis of the various constructs was sound. Western blot analysis showed prevalent bands in all lanes when anti-Spd was used. The bands were not as sharp as those witnessed in the live analysis and the intensity was lower when using anti-Spd antibodies. Therefore, the failure to detect bands in the live analysis may be due to the creation of the suicidal VP15-253 construct was effective (Figure 4).

Microscope analysis supported further the effectiveness of TriFC technology on Drosophila oocytes. As visualized at various stages of development, fluorescence microscopy revealed significant areas of fluorescence throughout the oocyte. Fluorescence was seen only within cells containing all three TriFC components. This could be due to the core of the system: natural localisation such as at the anterior dorsal and ventral poles, and areas over the anterior dorsal region (not revealed) a clear fluorescent signal, clearly indicating a functioning TriFC system. These signals were easily compared and confirmed against controls, which lacked fluorescence (Figure 6). Results from fluorescence analysis were physiologically relevant and developmentally consistent with the current literature and understanding of the grk pathway during egg development. The most obvious interactions, for instance, occurred in the zygotic membrane ring in late stage 8 and 9 oocyte and in the dorsomembrane cap in early stage 8-9 oocyte. This localization is consistent with protein localization.

5.) FUTURE WORK

As the extension wavelength of Venus enable auto-fluorescence of the yolk in Drosophila oocytes, new transgenic constructs will be designed that utilize GFP in the yolk in order to reduce the interfering background fluorescent signal for immunohistochemical analysis of the oocyte using TriFC. The new constructs will also encourage increase transgenic expression levels, as our interests in gene expression levels. To avoid the problems experienced previously in finding an appropriate primary antibody for the detection of grk, we will use two more TriFC components and Grk with different antibodies in the fusion of the new transgenic Drosophila lines that will be made, which will also contain a hemagglutinin (HA) tag construct. However, this will not be used for Western blot analysis, but the hemagglutinin tag can also be used for immunohistochemical analysis of the transgenic-containing oocytes.

After characterizing transgenic crosses for each of the lines (the both the transgenic construct and the MCF1-55 construct), Spd will be used to amplify the sequences flanking the transgene insertion site for each transgenic line. The location of the transposase-mediated transgene insertion can then be elucidated. By determining which genomic locations convey the strongest expression for the grk promoter, the studies can then be used to position these potential sites and optimize fluorescence levels, for immunohistochemical analysis of oocytes.

REFERENCES: