Identification of Motifs in the Intracellular Domain of the Engulfment Receptor Jedi-1 required for Phagocytosis

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KEYWORDS. Apoptosis, engulfment, Jedi-1, ITAM, NPXY

BRIEF. This study focuses on a novel engulfment receptor Jedi-1, ITAMs, and the NPXY motif and their role in the engulfment process.

ABSTRACT. During development of the nervous system, approximately 50% of the nerve cells die as part of a normal pruning process. These dead cells must be cleared to prevent an inflammatory response and possible autoimmunity. A new engulfment receptor, Jedi-1, was recently discovered on glial cells that is necessary for the clearance of dead neurons. The purpose of this research was to determine how Jedi-1 signals engulfment. Jedi-1 is similar to the *Drosophila* engulfment receptor, Draper, which signals through a tyrosine kinase (a type of enzyme that transfers phosphate groups) that binds to an Immunoreceptor Tyrosine-Based Activation Motif (ITAM) on the receptor. In addition, Draper interacts with an adapter protein, engulfment adaptor PTB domain containing 1 (GULP), through an NPXY sequence. Jedi-1 contains both ITAMs and an NPXY motif; therefore, we hypothesized that these sequences are required for Jedi-1 mediated engulfment. To test our hypothesis, we developed an engulfment assay using HeLa cells transfected with Jedi-1 or Jedi-1 mutants and counted the engulfment of fluorescent microspheres. Expression of Jedi-1 led to a 6-fold increase in engulfment over control cells while mutants lacking either domain were significantly impaired in their engulfment ability. These results revealed that both the ITAM and NPXY motifs are necessary for Jedi-1 mediated engulfment.

INTRODUCTION.

In developing animals, a large percentage of neurons undergo apoptosis in order to remove unnecessary cells during development [1]. If these dead cells are not removed properly and quickly, they enter a second death stage called necrosis. In this stage, all the cell contents spill out of a cell and affect the healthy cells around them, possibly resulting in an inflammatory response or the onset of autoimmune disease. Therefore, it is very important to investigate and uncover the molecular mechanisms of this engulfment system.

For over 50 years, underlying mechanisms of programmed cell death have been studied and many genes were discovered by using the worm *Caenorhabditis elegans*. Studies in *C. elegans* led to the identification of a receptor essential for engulfment, CED-1 (cell death mutant-1), as well as the downstream gene CED-6 [2]. CED-1 was shown to bind to CED-6, a phospho-tyrosine binding domain-containing adaptor protein, through the NPXY motif on CED-1 [3]. Several mammalian receptors have been proposed to be similar to CED-1, but most have very different predicted structures; one example is LRP-1 (lipoprotein receptor-related protein-1) [3]. Although the extracellular domain of LRP-1 is different from CED-1, LRP still appears to be essential in the engulfment process in macrophages [4]. MEGF-10 was also reported to be a possible mammalian homolog of CED-1 and shows an increase in engulfment when overexpressed [5].

Recently Draper, a CED-1 homolog in *Drosophila*, was identified and shown to be required for glial cells in the fly nervous system to engulf dead neurons [6]. Draper was shown to signal by binding to a soluble tyrosine kinase, Shark. Shark binds to a sequence on Draper referred to as an ITAM based on its similarity to sequences found in receptors in the mammalian immune system [7]. In addition, similar to CED-1, Draper interacts with the *Drosophila* homolog of CED-6/GULP, which is necessary for engulfment process [8].

This research focuses on the recently discovered mammalian engulfment receptor, Jedi-1, which is expressed by glial precursor cells in the peripheral nervous system and mediates engulfment of dead neurons. Jedi is similar to Draper and CED-1, and the intracellular domain of Jedi-1 contains both ITAMs and an NPXY motif (Figure S1) [9]. The purpose of this research was to determine how Jedi-1 signals engulfment, specifically to identify sequences in the intracellular domain of the receptor required for it to mediate its effects, and to investigate the importance of these regions in Jedi-1 mediated engulfment. In this research, HeLa cells, an immortalized cell culture line, were used for the study of cellular processes; HeLa cells do not typically express Jedi-1. It was hypothesized that Jedi-1 would increase engulfment when expressed in HeLa cells; since ITAM and NPXY motifs are active sites, it was also hypothesized that Jedi-1 with mutated ITAM or NPXY motifs would show decreased engulfment in an engulfment assay.

MATERIALS AND METHODS.

In order to investigate the role of Jedi-1 in the engulfment of dead neurons and to clarify specific motifs which help the receptor to activate downstream signaling, a bead engulfment assay in HeLa cells was used to simulate engulfment of apoptotic cells. Mouse Jedi cDNA was subcloned into a modified pEGFP-N3 vector, a vector which can be used to create fusion proteins with a C-terminal GFP tag. HeLa cells were placed in four seperate plates to be transfected with different DNA plasmids. Each plate of HeLa cells was transfected with DNA plasmids encoding either wild-type Jedi-1-GFP, Green fluorescent protein (GFP) (negative control which should not enhance engulfment but it marks transfected cells), LRP (another engulfment receptor used as a positive control), or Jedi-1-GFP with ITAM or NPXY motif mutations. The cell transfection protocol was based on the Lipofectamine 2000 protocol according to the manufacturer's instructions (Invitrogen). The specific mutants that we used in the assay are described in Figure 1.

Carboxylated fluorescent beads were used in this research because they are negatively charged, and apoptotic cell surfaces are negatively charged due to exposed phosphatidylserine (a membrane lipid which becomes exposed as a normal part of cell death). Fluorescent beads were added to the media of the transfected HeLa cells in chamber slides and incubated with the cells for 2 hours. After rinsing away unbound beads, the cells were fixed using formalin. Then, immunostaining was performed using either a GFP primary antibody (to recognize GFP or GFP-tagged Jedi proteins) or a LRP antibody. A Cy-2 conjugated secondary antibody was used to visualize cells expressing the transfected proteins. Nuclei were stained using TO-PRO 3 staining after treatment with RNAse A. After immunostaining the cells, we determined the percentage of transfected cells which had engulfed beads by confocal microscopy. We used confocal microscopy because it allowed us to obtain high resolution, three dimensional images using the "z-stack" tool. This tool allowed us to ensure if beads were actually engulfed by cells or were just sitting on the surface of cells.

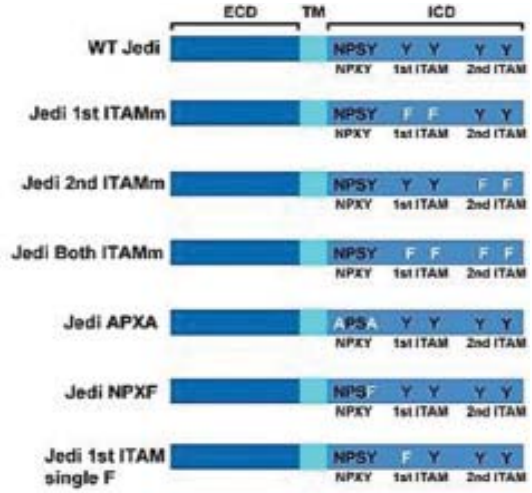

Figure 1. This image is a schematic of the mutants of Jedi motifs. They are mutants of ITAM domains and the NPXY domain. All mutants have a C-terminal GFP tag.

RESULTS.

In order to examine the importance of the engulfment receptor Jedi-1, the first experiment was created with one experimental and two control samples: Jedi-1, LRP, and GFP. LRP is known to enhance engulfment and therefore was a positive control [10]. GFP was a negative control because it does not show any enhanced engulfment. In Figure 2, two images show representative images of cells that have or have not engulfed beads. In Figure 2A, there is a fluorescent bead near the HeLa cell transfected with GFP (negative control), but the cell did not engulf the bead. However, in Figure 2B, many beads are engulfed in the HeLa cell transfected with GFP tagged Jedi-1.

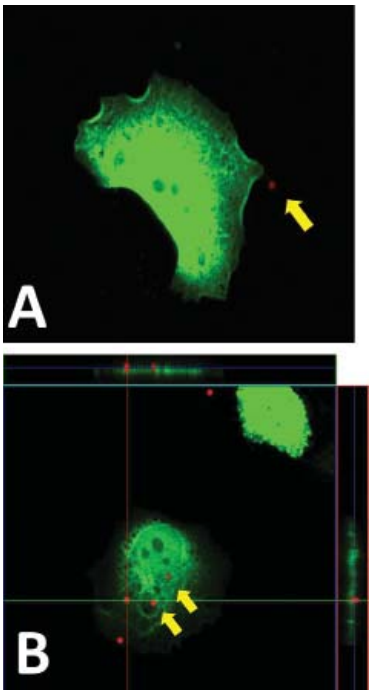

Figure 2. GFP-positive HeLa cell, showing no engulfment (**A**). Jedi-1-GFP positive HeLa cell engulfed several beads (**B**).

After analyzing the images of the first experiment samples, the engulfment graph (Figure 3A) showed that compared to the control samples, Jedi-1 transfected cells enhanced engulfment to 49.42%. GFP had 7.7% engulfment and LRP showed 40.91% engulfment. The results of a t-test indicate for the increase in engulfment by cells transfected with Jedi-1-GFP is statistically significant compared to the GFP control (Figure 3A).

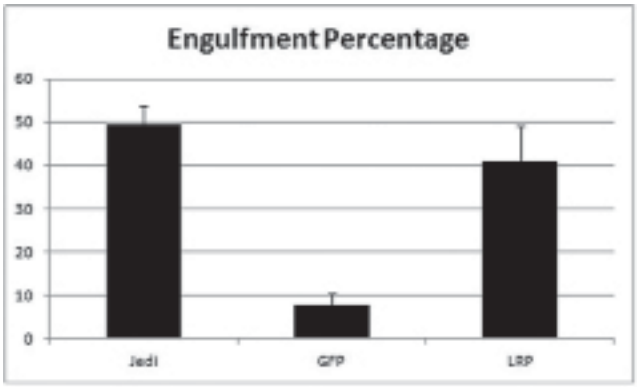

Figure 3A

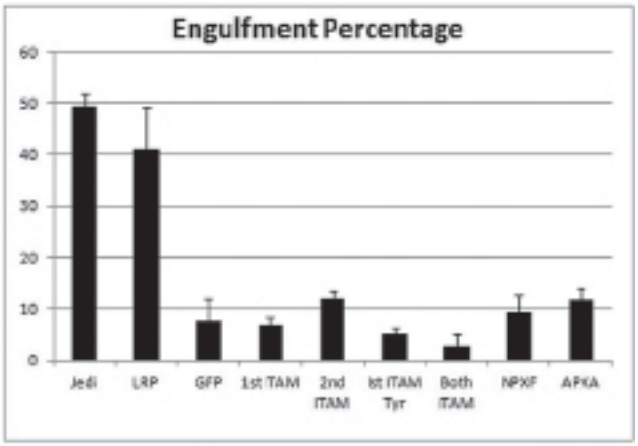

Figure 3B

Figure 3. 49.42% of Jedi-1 transfected cells engulfed beads, whereas 7.71% of GFP transfected and 40.91% of LRP transfected cells engulfed beads. The error bars on the graph represent the standard error of the mean of all experiments (calculated using Excel). * p-value for Jedi compared to GFP = 8.47E-06, Jedi compared to LRP = 0.341, and GFP compared to LRP = 0.0016 (**A**). This graph shows that Jedi-1 transfected cells engulfed 49.42%, whereas 1st ITAM showed 6.9% engulfment; 2nd ITAM 11.9%; 1st ITAM-tyrosine 5.1%; Both ITAM 2.7%; NPXF 9.4%; and APXA 11.6%. The p-values were obtained by using the Student's t-test in Excel: t-test between GFP-Jedi (8.5E-06); Jedi- Jedi 1st ITAM (0.00028); Jedi-Jedi 2nd ITAM (0.00061); Jedi-Jedi Both ITAM (0.00015); Jedi-Jedi 1st ITAM-tryosine (5.3E-05); Jedi-NPXF (0.00053); and Jedi-APXA (0.00065). N=3-5 (**B**).

The second experiment was focused on two motifs in Jedi-1: Immunoreceptor tyrosine-based activation motifs (ITAMs) and the NPXY motif. In this experiment, ITAMs were mutated in four different ways (1st ITAM, 2nd ITAM, 1st ITAM-tyrosine, and Both ITAM); the NPXY motif was mutated in two different ways (NPXF and APXA). Figure 3B shows the results obtained with these mutants in the HeLa cell bead assay. Similar to the results from the first experiment, Jedi-1 transfected cells showed the most engulfment. The engulfment of ITAM mutants dropped significantly from 50% to less than 10%. Also, NPXY mutants showed significantly lower engulfment.

DISCUSSION.

According to Figure 3, Jedi-1 transfected HeLa cells had enhanced engulfment, and cells transfected with the ITAMs and the NPXY mutants had less engulfment capability. This indicates that Jedi-1 promotes engulfment in our in vitro engulfment assay. Furthermore, the ITAMs and the NPXY motif are essential in the engulfment process mediated by Jedi-1.

This research was designed to study engulfment signaling mediated by Jedi-1 and to discover the molecular mechanisms that are involved in the engulfment process. Our *in vitro* engulfment assay allowed us to identify important signal-

ing motifs in Jedi. This assay will be useful in future studies involving potential signaling partners of Jedi-1.

Also, the ITAMs and the NPXY motif were both found to be essential in the engulfment process since mutating these motifs greatly decreased engulfment. These results suggest that they are working as mediators in a signaling process to promote engulfment by possibly recruiting signaling kinases or adaptor proteins. For example, it is known that ITAMs and the NPXY motif are present in Draper [7]. The ITAM motif of Draper is phosphorylated by a Srk family kinase which leads to binding of a Syk family kinase. Binding of a Syk family kinase to Draper seems to be essential for engulfment signaling in *Drosophila*. It has been found in the laboratory of Dr. Carter that a similar process occurs in mammals during Jedi-1 signaling. Furthermore, the NPXY motif might play a significant role in binding GULP, the homolog of CED-6.

CONCLUSION & FUTURE WORK.

The results demonstrate that Jedi-1 is able to mediate engulfment of micro beads when expressed in HeLa cells, suggesting that it functions as a phagocytic receptor. Also, the data show that the ITAMs and the NPXY motifs in Jedi-1 are important and necessary in the engulfment process. Studying the signaling pathway of engulfment by Jedi-1 will help elucidate the mechanisms by which apoptotic neurons are cleared in the developing nervous system and may shed light on how autoimmune disease could develop, since disruption of this phagocytosis may result in autoimmunity.

Studying Jedi-1 opens many doors to possibilities that other receptors or proteins are also involved and significant in engulfment signaling pathways. Also, there are countless factors to look at when it comes to analyzing a signaling pathway. For example, a recent paper suggests that the NPXY motif may be important for maturation of receptors like LRP-1; for future studies, we will determine whether this motif is important for Jedi maturation and processing [11]. Also, we will determine whether the NPXY motif is important for binding to GULP. GULP is an adaptor protein, and little is known about its role. Our future studies will also determine whether GULP is required for engulfment by knocking out GULP in our system. We will also find out if GULP is expressed in satellite glial cells during engulfment.

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SUPPORTING INFORMATION.

Figure S1. Predicted protein structure of Draper and Jedi-1

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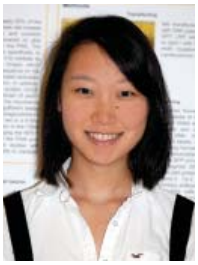

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