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Differential activities of the *Drosophila* JAK/STAT pathway ligands Upd, Upd2 and Upd3

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ABSTRACT

JAK/STAT signalling in vertebrates is activated by multiple cytokines and growth factors. By contrast, the *Drosophila* genome encodes for only three related JAK/STAT ligands, Upd, Upd2 and Upd3. Identifying the differences between these three ligands will ultimately lead to a greater understanding of this disease-related signalling pathway and its roles in development. Here, we describe the analysis of the least well characterised of the Upd-like ligands, Upd3. We show that in tissue culture-based assays Upd3–GFP is secreted from cells and appears to interact with the extracellular matrix (ECM) in a similar manner to Upd, while still non-autonomously activating JAK/STAT signalling. Quantification of each of the Upd-like ligands in conditioned media has allowed us to determine the activity of equal amounts of each ligand on JAK/STAT *ex vivo* and reveals that Upd is the most potent ligand in this system. Finally, investigations into the effects of ectopic expression of Upd3 *in vivo* have confirmed its ability to activate pathway signalling at long-distance.

1. Introduction

The JAnus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) cascade mediates intracellular signalling in response to multiple cytokines and growth factors. In the canonical model of JAK/STAT signalling, binding of extracellular ligand to the receptor induces a conformational change leading to the activation of receptor-associated JAK kinases. The activated JAKs trans-phosphorylate tyrosine residues on the receptor and themselves, creating docking sites for the SH2 domain containing STATs. Cytoplasmic STATs are then recruited to the membrane and activated by tyrosine phosphorylation allowing dimerisation to occur via interactions between their SH2 domains and phospho-tyrosines. The dimerised STATs then translocate to the nucleus to regulate transcription [reviewed in 1].

In vertebrates, misregulation of JAK/STAT signalling has been associated with several diseases including haematopoietic disorders [2], leukaemias [3] and cancers [4] due to its roles in cellular proliferation, haematopoiesis and the immune response. These roles of JAK/STAT signalling have also been conserved throughout evolution with alterations in JAK/STAT activity in invertebrates such as *Drosophila*, also affecting cell proliferation [5], haematopoieic cell regulation and differentiation [6,7]. In addition, the *Drosophila* JAK/STAT cascade also plays roles in embryonic development, and stem cell maintenance [reviewed in 8,9].

An advantage of studying JAK/STAT in *Drosophila* is the presence of a complete and yet low complexity version of the canonical JAK/STAT pathway, encoding a single receptor called *domeless* (*dome*) [10,11], a JAK kinase, *hopscotch* (*hop*) [12] and a STAT transcription factor, referred to as *stat92E* [13,14]. JAK/STAT signalling in vertebrates can be activated by a large range of ligands including, interferons, interleukins, cytokines and growth factors whereas the *Drosophila* genome encodes only three ligands, Unpaired/Outstretched (henceforth termed Upd) [15], Upd2 [16] and Upd3 [17]. Sequence alignments suggest that the *Drosophila* JAK/STAT ligands show some similarity to the vertebrate leptins [18], and the predicted secondary structure for Upd is similar to that of other cytokines with stretches of ∝-helices [15] suggesting a similar function.

The *upd* locus was initially suggested as a potential JAK/STAT pathway component as loss-of-function mutants produce embryonic segmentation defects similar to both *hop* and *stat92E* mutants [15]. Biochemical characterisation confirmed Upd as an activator of the JAK/STAT pathway with Hop tyrosine phosphorylation observed only in the presence of ectopic Upd expression [15]. Subsequent *in silico* searches identified two Upd-like homologues referred to as Upd2 and Upd3 [16,17] and both *ex vivo* and *in vivo* JAK/STAT assays have also shown that Upd2 is capable of activating JAK/STAT signalling [16]. Although not yet studied in detail, Upd3 has also been shown to play a role in JAK/STAT signalling. Upon septic injury, Upd3 is up-regulated and is responsible for the JAK/STAT activation that results in the expression of the stress response actor TotA [17].

Signalling in response to Upd and Upd2 occurs non-autonomously as tissue culture-based assays have demonstrated that both ligands can be secreted into media to activate JAK/STAT signalling in cells containing a JAK/STAT luciferase reporter [15,16]. In addition, long-

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range pathway activation has been observed, in the *Drosophila* eye in response to ectopic Upd expression [19,20] and in the embryo with Upd2 expression in the ectoderm activating the *in vivo* JAK/STAT activity reporter, *dome-MESO*, in the mesoderm [16]. By comparison, little is known about the mechanisms by which Upd3 can activate the JAK/STAT pathway. Furthermore, determining how each of the ligands results in the downstream effects of JAK/STAT signalling will be important in understanding the pathway roles in development and how misregulation of JAK/STAT can result in disease.

Here, we investigate the mechanism by which Upd3 can activate JAK/STAT signalling. Using tissue culture-based assays we show that Upd3 is a secreted molecule that can activate the JAK/STAT pathway non-autonomously. In addition, we have generated media conditioned with specific Upd-like molecules and developed assays to quantify the amount and activity of each ligand. We show that there are differences in the strength and temporal dynamics of each of the Upd-like ligands to activate an *in vitro* JAK/STAT reporter with Upd being the most potent and Upd2 producing the longest response. Finally we move our analysis *in vivo* and show that ectopic expression of Upd3 in different *Drosophila* tissues results in similar effects to the expression of Upd, with studies in the ovary demonstrating that Upd3 can activate the JAK/STAT pathway non-autonomously *in vivo*.

2. Results

2.1. Upd3, a secreted protein that activates JAK/STAT in cell culture-based assays

It has been shown that Upd and Upd2 are secreted proteins [15,16] and both cytokines can non-autonomously activate signalling at a distance [16,20]. By comparison, relatively little is known about Upd3, the nature of its secretion and ability to activate the JAK/STAT pathway.

The *upd3* gene is situated between *upd* and *upd2* at polytene band 17A on the X chromosome and consists of 4 exons (Fig. 1A) encoding the smallest of the Upd-like ligands with a length of 401 amino acids and includes several predicted N-linked glycosylation sites (Fig. 1B). Post-translational N-linked glycosylation has been previously predicted for Upd [15]. Homologues of *Drosophila melanogaster* (*Dmel*) Upd3 are encoded by all sequenced *Drosophilid* genomes (Fig. 1C and D) with the *Dmel* Upd3 remaining more closely related to all other Upd3 homologues than to the other *Dmel* Upd-like ligands (Fig. 1D) suggesting that the Upd group of ligands radiated into three distinct molecules before the evolutionary divergence of the *Drosophilidae*. However, Upd-like proteins diverge quite considerably between these closely related flies (Fig. 1D) and no *upd*-like genes could be identified in other insects suggesting that Upd-like ligands represent a rapidly evolving group of proteins.

Given that a secreted ligand should include an N-terminal signal sequence we examined *Dmel* Upd3 for such a motif. However, SignalP 3.0 [21] predicts an N-terminal anchor sequence of 63 amino acids (C-terminal boundary indicated by the light grey arrow Fig. 1C), suggesting that Upd3 may not be secreted—a striking contrast to the signal sequence correctly predicted for *Dmel* Upd. However, analysis of other Upd3-like molecules found that *Drosophila yakuba* Upd3 contains a high confidence signal sequence located at amino acid 29 and is found at the N-terminus of a region conserved in most Upd3s (black arrow in Fig. 1C). As such, this predicted site may well represent the physiological cleavage site *in vivo*.

Given the previously described differences between the cell localisation and secretion of Upd and Upd2 [16], we first investigated whether Upd3 is in fact secreted. We therefore generated a C-terminal GFP fusion construct, *pAc–upd3–GFP* (see Experimental procedures) and transiently transfected this into *Drosophila* Kc_{167} cells. Upd3–GFP can be visualised both within the cytoplasm and also as a 'halo' around the cell (Fig. 2A), an effect previously described for Upd–GFP (Fig. 2A, [16]). Extracellular Upd3–GFP can only be visualised at the most basal confocal sections and, given the similarity to Upd–GFP which has previously been shown to be ECM-associated [15,16], may indicate an interaction between Upd3 and the extracellular matrix (ECM).

We next used transcriptional reporters of JAK/STAT activity in Kc_{167} cells to further characterise Upd3. The *6x2xDrafLuc* reporter contains twelve STAT92E binding sites upstream of the firefly luciferase gene to report STAT92E activity via changes in firefly luciferase activity [22]. Transfection of plasmids constitutively expressing Upd, Upd2 or Upd3 GFP fusion proteins significantly increased *6x2xDrafLuc* activity when compared to cells transfected with empty vector (Fig. 2B) demonstrating that Upd, Upd2 and Upd3 can all activate STAT92E in this tissue culture model.

Given the ambiguity regarding the signal sequence of *Dmel* Upd3 we next designed a paracrine assay to determine if Upd3 expression in one population of cells is capable of signalling to a second population of cells. Using Upd and Upd2 paracrine assays as a baseline, Upd3 demonstrated a similar ability to signal non-autonomously and was able to induce a 15-fold increase in STAT92E activity compared to control cells transfected with an empty vector (Fig. 2C).

Finally assays were set up in which media previously conditioned by cells expressing the Upd-like ligands is added to cells transfected with the *6x2xDrafLuc* reporter (Fig. 2D). It has previously been shown that the addition of heparin to media being conditioned with Upd can increase the amount of soluble ligand released into the media, presumably by competing with the ECM for Upd binding (Fig. 2D, [16]). By contrast, heparin has no significant effect on media conditioned by Upd2 which, when considered together with the cell localisation results, suggests that Upd2 does not interact with the ECM. However, while Upd3 appears to condition media, the addition of heparin almost completely ablates activity (Fig. 2D)—a result opposite to Upd. Given the normal signalling elicited by Upd and Upd2 in the presence of heparin, this effect is unlikely to be caused by a general effect of heparin on the Dome receptor. Rather, heparin could inhibit Upd3 production, directly affect Upd3 or interfere with the Upd3/Dome interaction. In order to differentiate between these possibilities we treated Upd3 conditioned media with heparin and added this to cells transfected with the *6x2xDrafLuc* reporter (Fig. 2E). Compared to mock, Upd3 conditioned media gives around a 17-fold increase in reporter activity while media with added heparin is essentially inactive (Fig. 2E). This suggests that the effect occurs after secretion and that heparin impairs Upd3 either directly or via its interaction with Dome. A similar sensitivity to heparin has been previously reported for human cytokines [23] and given this interaction heparin was not used in subsequent experiments.

We have demonstrated that Upd3 is a secreted cytokine that is able to activate STAT92E *ex vivo*. In order to determine if Upd3 signals through the same canonical pathway as Upd we undertook autocrine assays as above following knockdown with dsRNA targeting known JAK/STAT pathway components [22,24]. RNAi knockdown of the core pathway components *hop*, *stat92E* and *dome* resulted in a significant decrease in JAK/STAT pathway activity in response to all three ligands (Fig. 2F). In addition, knockdown of the negative regulators, *socs36E* and *ptp61F* result in a significant increase in JAK/STAT reporter activity (Fig. 2F). These results confirm that all three ligands, including Upd3, activate signalling via a single activating receptor, JAK and STAT molecule. Furthermore, known regulators such as SOCS36E and Ptp61F mediate the down-regulation of Upd3-activated pathway signalling as has previously been shown for Upd and Upd2 [22,24].

2.2. The Upd-like ligands exhibit different abilities to activate JAK/STAT signalling

Although the JAK/STAT assays (Fig. 2B–E) demonstrated an activity for each of the Upd-like ligands, they do not allow the absolute ability of each of the ligands to stimulate pathway activity to be determined. We