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Characterisation of Upd2, a *Drosophila* JAK/STAT pathway ligand

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Abstract

The characterisation of ligands that activate the JAK/STAT pathway has the potential to throw light onto a comparatively poorly understood aspect of this important signal transduction cascade. Here, we describe our analysis of the only invertebrate JAK/STAT pathway ligands identified to date, the *Drosophila unpaired*-like family. We show that *upd2* is expressed in a pattern essentially identical to that of *upd* and demonstrate that the proteins encoded by this region activate JAK/STAT pathway signalling. Mutational analysis demonstrates a mutual semi-redundancy that can be visualised in multiple tissues known to require JAK/STAT signalling. In order to better characterise the in vivo function of these ligands, we developed a reporter based on a natural JAK/STAT pathway responsive enhancer and show that ectopic *upd2* expression can effectively activate the JAK/STAT pathway. While both Upd and Upd2 are secreted JAK/STAT pathway agonists, tissue culture assays show that the signal-sequences of Upd and Upd2 confer distinct properties, with Upd associated primarily with the extracellular matrix and Upd2 secreted into the media. The differing biophysical characteristics identified for Upd-like molecules have implications for their function in vivo and adds another aspect to our understanding of cytokine signalling in *Drosophila*.

Keywords: Unpaired; Domeless; STAT; Cytokine; Janus Kinase; *Drosophila melanogaster*; Ligand; Auto-regulation

Introduction

The JAK/STAT signal transduction cascade is named after its two major components, a receptor-associated Janus kinase (JAK) and the signal transduction and activator of transcription (STAT). In the canonical model of JAK/STAT signalling, extracellular ligands bind to a bi-partite transmembrane receptor complex that is itself non-covalently associated with the JAK tyrosine kinase via its intracellular C-terminal region. Upon binding of the ligand to the extracellular domain, a conformational change in the receptor is thought to lead to the activation of the JAK kinases which trans-phosphorylate tyrosine residues within both the receptors and one another. This phosphorylated receptor/JAK complex then acts as a docking site for cyotsolic STAT molecules which associate via their SH2 domain and are themselves phosphorylated on a C-terminally located tyrosine residue. STATs activated in this manner dimerise in a head to tail arrangement via their SH2 and phospho-tyrosine residues and translocate to the nucleus where they bind to DNA activating transcription of target genes (reviewed in Levy, 2003).

Both the major components and many of the functions of the JAK/STAT pathway appear to have been conserved throughout evolution with STAT-like molecules identified in the slime mould *Dictyostelium* (Araki et al., 1998) and the nematode *C. elegans* (Liu et al., 1999). The *Drosophila melanogaster* JAK/STAT pathway, the most intensively studied invertebrate example, contains a 'complete' canonical pathway. The *Drosophila* genome encodes a single STAT homologue, encoded by *stat92E* (Hou et al., 1996; Yan et al., 1996), as well as a JAK homologue *hopscotch (hop)* (Binari and Perrimon, 1994) and a receptor molecule called *domeless (dome)* (Brown

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et al., 2001). In vertebrates, JAK/STAT signalling is known to regulate multiple developmental processes including haematopoietic development, immune responses and cellular proliferation. The *Drosophila* pathway is required for segmentation, tracheal development, and also innate immune responses (Agaisse et al., 2003), cellular proliferation (Bach et al., 2003; Mukherjee et al., 2005), haematopoiesis (Hanratty and Dearolf, 1993; Harrison et al., 1995) and stem cell maintenance (Kiger et al., 2001).

One aspect of JAK/STAT signalling in *Drosophila* that has received less attention are the pathway ligands. In vertebrates, over 40 polypeptide ligands have been identified that function to activate JAK/STAT signal transduction. These include the α- and β-interferons, interleukins and a wide range of cytokines and growth factors (Boulay et al., 2003). This multitude of ligands stimulates a large number of diverse receptors which activate differing subsets of the four JAKs and seven STATs present in vertebrates. The situation in *Drosophila* is significantly more straightforward. By contrast to the rest of the pathway for which homologues can be identified, the fly genome does not appear to encode obvious interferon or interleukin-like proteins. However, the *unpaired* (*upd*) gene (Wieschaus et al., 1984), also known as *outstretched* (Muller, 1930), has been identified on the basis of its distinctive mutant cuticle phenotype that mirrors the segmentation defects produced by loss of other pathway components (Binari and Perrimon, 1994; Harrison et al., 1998; Hou et al., 1996). Genetic analysis of *upd* first indicated that the locus is likely to encode a molecule capable of acting at a distance (Gergen and Wieschaus, 1986) and subsequent molecular characterisation showed that it encodes a glycosylated secreted protein that associates strongly with the extracellular matrix in tissue culture assays and is capable of activating JAK/STAT signalling (Harrison et al., 1998). *upd* is expressed in regions known to require JAK/STAT pathway activity during development, has been visualised extracellularly in vivo (Zeidler et al., 1999) and is capable of stimulating target gene expression at a distance (Karsten et al., 2002; Tsai and Sun, 2004). Retrospective alignments of Type I cytokines have subsequently characterised Upd as being most closely related to the vertebrate leptins (Boulay et al., 2003).

In addition to the original *upd* locus, a recent study has characterised a role for the homologous protein Upd3 which also appears to function via stimulation of the JAK/STAT pathway and is central for the signalling of haemocytes to the fat body in response to septic injury (Agaisse et al., 2003). By contrast, no information regarding the biochemistry or developmental roles of the third *upd*-like gene, termed *upd2*, has been described and its potential functions are as yet unknown.

Here, we present a detailed analysis of the *upd* genomic interval present within the 17A region of the *Drosophila* X-chromosome. We define the extent of existing deletions and mutations in the region and show that the strength of the phenotypes caused by these mutations are related to the number of Upd homologues removed. Tissue culture-based assays show UpdGFP is secreted and associated with the adjacent extracellular matrix (ECM), while only minimal levels of Upd2GFP appear to associate with the ECM. This agrees with in silico analysis suggesting that Upd2 contains an N-terminal anchor-sequence present in membrane inserted, non-secreted proteins. However, the situation seems to be more complex, as both Upd and Upd2 activate JAK/STAT signalling in vivo. Moreover, in a tissue culture reporter assay system Upd2 is capable of strongly conditioning its overlying media suggesting that Upd2 is indeed secreted.

Our analysis therefore reveals an unsuspected level of complexity in the regulation of Upd and Upd2 that explains their mutual semi-redundancy and may affect their signalling potential in vivo.

Materials and methods

Molecular biology and cloning

upd2 expression constructs were generated by amplifying the open reading frame as predicted by flybase (http://fly.ebi.ac.uk:7081/), from genomic DNA using the primers TACGATGGCCAATCCACTAACGC and TCAAGACTCATTGGATCCGCCATC. The resulting 1.8 kb product was cloned into pCR-TOPO (Invitrogen) to give *pCR-upd2.* EGFP tagged Upd2 was generated by amplifying Upd2 from *pCR-upd2* using the SP6 and GGAAGATCTGACTCATTGGATCCGCCATC primers, trimming with *Eco*RI and *Bgl*II and subcloning into *pBS-EGFPA* (MPZ unpublished). Similarly, UpdGFP was generated by amplifying the coding region from the *upd* cDNA (Harrison et al., 1998) with the primers AGAATTCGATATCGGCGATGGCTCGTCCGCTG and CGGATCCGTGCGCTGCACGCGCTTC, trimming with *Eco*RI and *Bam*HI and cloning into *pBS-EGFPA*. The resulting plasmids were sequenced before subcloning into *pUAST* (Brand and Perrimon, 1993).

Signal swap constructs were generated by two step mega-primer amplification of both Upd and Upd2 using the primers GCCACCTGGTCGCGCAAGTGTCGCCCCTCGGCGAGGTGGGGCAAC (for Upd1SS2) and CCGCCGCTGCTGGTGGTGCTGCGCGCCTTGGTGAATGGCATCACG (for Upd2SS1) in conjunction with the existing forward and reverse primers (see above). Details available on request (MPZ).

Plasmids for expression in tissue culture were subcloned into *pAc5.1* (Invitrogen).

The *6x2DrafLuc* plasmid (Müller et al., 2005) is based on a multimerisation of the *2xDrafSTAT(wt)* plasmid (Kwon et al., 2000).

For deficiency break point mapping experiments hemizygous mutant male embryos were identified by use of a GFP expressing balancer chromosome and used for single embryo PCR reactions using primer pairs designed to amplify each predicted gene in the region.

dome-MESO was generated from an *Eco*RI genomic fragment flanking the $dome^{321}$ P-element insertion which was subdivided into a 0.6-kb *Kpn*I/*Not*I fragment containing the untranslated and upstream sequences and a *Not*I/*Bam*HI 2.8 kb fragment containing part of the first exon and most of the first intron. These fragments were subcloned into the *pCaspeR-lacZhs43* plasmid and transformed into flies. The 0.6-kb construct did not drive any consistent embryonic expression while the 2.8-kb construct gave the patterns of expression described in the results section as the *dome-MESO* reporter. Two insertions where kept that gave strong levels of *lacZ* expression in homozygous (for the X and third chromosome insertions) or hemizygous conditions (for the X insertion). In all lines, expression is observed in the pharynx, hindgut and precursors of the longitudinal visceral mesoderm. The expression in the latter is JAK/STAT independent.

upd-like genes present in other *Drosophilids* were identified using *D. melanogaster* Upd as a protein probe in tblastn searches of the available databases. Predictions of signal/anchor sequences were undertaken at http://www.cbs.dtu.dk/services/SignalP/ and prediction of potential N-linked glycosylation sites at http://www.cbs.dtu.dk/services/NetNGlyc/. Protein alignments and phylogenetic trees were constructed using the DNA Star software package.