Biochemical and Biophysical Research Communications 436 (2013) 66–72

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Upd3 – An ancestor of the four-helix bundle cytokines

Mirja Oldefest [a,1], Jana Nowinski [a,1], Chien-Wen Hung [b], Denis Neelsen [a], Ahmad Trad [a], Andreas Tholey [b], Joachim Grötzinger [a,*], Inken Lorenzen [a]

[a] Biochemisches Institut der Christian-Albrechts-Universität Kiel, Olshausenstr. 40, 24118 Kiel, Germany
[b] Institute for Experimental Medicine – Division of Systematic Proteome Research, Christian-Albrechts-Universität zu Kiel, Niemannsweg 11, 24105 Kiel, Germany

ARTICLE INFO

Article history:
Received 17 April 2013
Available online 22 May 2013

Keywords:
Cytokine
Drosophila melanogaster
Unpaired-like protein
Upd3
Four-helix bundle cytokine

ABSTRACT

The *unpaired*-like protein 3 (Upd3) is one of the three cytokines of *Drosophila melanogaster* supposed to activate the JAK/STAT signaling pathway (Janus tyrosine kinases/signal transducer and activator of transcription). This activation occurs via the type-I cytokine receptor *domeless*, an orthologue of gp130, the common signal transducer of all four-helix bundle interleukin-6 (IL-6) type cytokines. Both receptors are known to exist as preformed dimers in the plasma membrane and initiate the acute-phase response. These facts indicate an evolutionary relation between vertebrate IL-6 and the *Drosophila* protein Upd3. Here we presented data which strengthen this notion. Upd3 was recombinantly expressed, a renaturation and purification protocol was established which allows to obtain high amounts of biological active protein. This protein is, like human IL-6, a monomeric-α helical cytokine, implicating that Upd3 is an "ancestor" of the four-helix bundle cytokines.

1. Introduction

The JAK/STAT signaling pathway is evolutionary highly conserved; all players involved are already present in the fruit fly *Drosophila melanogaster*, although in a more simplified fashion compared to mammals. In human there are four different JAK and seven different STAT molecules. Additionally, in mammals there is a bunch of receptors and ligands present, such as cytokines and growth factors, inducing this pathway. This huge number of factors orchestrates a complex and redundant signaling network. In contrast, in *Drosophila* there is only one JAK (*hopscotch*), one STAT (Stat92E) and one type-I cytokine receptor (*domeless*) known [1,2]. Three putative ligands of *domeless* are described so far, *unpaired* (Upd) [3], *unpaired*-like protein 2 (Upd2) [4,5] and *unpaired*-like protein 3 (Upd3) [6].

The receptor *domeless* is the orthologue to the mammalian signal-transducer gp130. Like gp130, *domeless* consists extracellular of several fibronectin type-III like domains, two of them constitute the classical cytokine binding module [1,2,7]. For both molecules it was described that even without ligand they do exist as preformed dimers on the cell surface [8–10].

The ligands of gp130 belong to the interleukin-6 (IL-6) type cytokine family. Members of this family like IL-6, IL-11, ciliary neurotrophic factor (CNTF) and leukemia-inhibitory factor (LIF), although not exclusively, signal via the JAK/STAT pathway. Beside their roles in the immune system and inflammation these cytokines are involved in hematopoiesis, metabolism, regeneration, and development [11,12]. Similar functions are regulated by the JAK/STAT pathway in *Drosophila*. For example, IL-6 is able to induce the acute-phase response after onset of an infection in mammals. In analogy, Upd3 is expressed by *Drosophila* blood cells after septic injury leading to the expression of an acute-phase protein in the fat body [6]. Pathophysiological similarities are also observed in misregulated JAK/STAT pathway signaling. In mammalian this phenomena is well known to be associated with autoimmune diseases and cancer [13,14]. A dominant gain-of-function mutation in the *Drosophila* JAK (*hopscotch*) leads to a leukemic phenotype, caused by hyper-proliferative *Drosophila* blood cells [15,16].

Cytokines are classified by their structural features into four different folds. IL-6 type cytokines belong to the four-helix bundle cytokines, which share a characteristic up-up-down-down topology [17]. Other cytokines share the cysteine-knot fold, like nerve growth factor [18,19] or the β-trefoil fold, like the acid fibroblast growth factor [20,21]. The fourth fold is constituted by the tumor-necrosis factor (TNF) family [22]. In contrast to the first fold the later three are characterized mainly by β-sheets.

Until now nothing is known about the structural properties of the *Drosophila* proteins Upd, Upd2 and Upd3. By in silico methods

Abbreviations: CD, circular-dichroism; CNTF, ciliary neurotrophic factor; IL-6, interleukin-6; IAA, iodoacetamide; JAK, Janus tyrosin kinases; LIF, leukemia-inhibitory factor; STAT, signal transducer and activator of transcription; Upd, unpaired.

* Corresponding author. Fax: +49 431 880 2007.
E-mail addresses: moldefest@biochem.uni-kiel.de (M. Oldefest), jkieper@biochem.uni-kiel.de (J. Nowinski), cw.hung@iem.uni-kiel.de (C.-W. Hung), dneelsen@biochem.uni-kiel.de (D. Neelsen), atrad@biochem.uni-kiel.de (A. Trad), a.tholey@iem.uni-kiel.de (A. Tholey), jgroetzinger@biochem.uni-kiel.de (J. Grötzinger), ilorenzen@biochem.uni-kiel.de (I. Lorenzen).

[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.04.107

helical secondary structural elements were predicted for Upd [3]. In addition, the exon structure of Upd is similar to the exons encoding helices B/C and helix D in mammalian four-helix bundle cytokines [23,24], suggesting that Upd and Upd-like proteins might be the orthologous of mammalian four-helix bundle cytokines [3,23]. This notion is in line with the fact that all ligands of mammalian gp130 are members of the four-helix bundle cytokine family and that this feature might be evolutionary conserved.

Here we present that Upd3 can be recombinantly expressed in *Escherichia coli*. A renaturation/purification procedure allowed us to obtain native folded monomeric Upd3. This cytokine is biological active and allowed the *in vitro* characterization of an Upd-like protein. From these data we conclude that Upd3 is an IL-6 type like cytokine with an α-helical fold that is able to activate a type-I cytokine receptor which implicates that Upd3 is an "ancestor" of the four-helix bundle cytokines.

2. Materials and methods

2.1. Cloning of Upd3-expression constructs

For the expression of Upd3 in *E. coli* the sequence of the "mature" protein (T93-L401) was codon optimized and cloned into pET28a via NheI and NotI. Thereby the protein was N-terminal His-tagged.

2.2. Production of recombinant Upd3

Upd3 was expressed in *E. coli* strain BL21 (DE3) grown in LB medium. Most of the protein accumulates in inclusion bodies (IBs). The purified IBs were incubated with Benzonase (Novagen, Germany) at 4 °C and dissolved in 6 M GuHCL, 50 mM at Tris pH 7.2 and purified using Ni-NTA agarose beads (Qiagen, Germany). After elution using IB buffer containing 250 mM imidazole the protein was refolded by dialysis (20 mM Tris pH 8.0, 500 mM sodium chloride, 2 mM reduced and 0.2 mM oxidized glutathione) and further purified by size-exclusion chromatography (Superdex75 GE Healthcare, Germany).

2.3. STAT92E luciferase-reporter assay

Drosophila Kc_{167} cells were cultured in Schneiders medium (Genaxxon, Germany), 10% FBS (PAA, Germany) and 1% Pen-Strep (PAA, Germany) at 25 °C. For transfection 3×10^6 cells were seeded in medium containing 0.5% FCS and transfected with 30 ng 10xSTAT92E-luciferase and 300 ng Act-*Renilla* (Perrimon-Lab, Howard Hughes Medical Institute, Boston) using Effectene (Qiagen, Germany). One day after transfection cells were incubated with purified Upd3. One day later cells were lysed and the luciferase assay was performed using Dual Luciferase Reporter® Assay System (Promega, USA) according to manufactures' instructions and analysed in a luminometer (GloMax from Promega, USA).

2.4. Mass spectrometry

A lyophilized Upd3 (50 μg) was redissolved in 100 μl of digestion buffer (50 mM HEPES, pH 7.0). The protein was alkylated with IAA (2 μL of 375 mM IAA) for 30 min at room temperature in the dark. One μg Chymotrypsin was added, incubated at 37 °C and 10 μl aliquots were taken after 1, 2 and 23 h. Digestion was terminated by addition of 5% formic acid; the digests were stored at −20 °C until analysis.

Samples were analyzed by LC-MS/MS using an Ultimate 3000 nanoLC (Dionex, Idstein) with a trapping column (Acclaim PepMap 100; 100 Å,5 μm, 300 μm i.d. × 5 mm), followed by an analytical reverse phase C18 column (PepMap; 100 Å, 3 μm, 75 μm i.d. × 15 cm) coupled to a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Germany) via a nanospray ion source (New Objective, MA) equipped with an online nanospray emitter (Picotip, New Objective, MA). Mobile phase A was 0.05% formic acid (water), mobile phase B was 0.04% formic acid (80% acetonitrile). Gradients used were: 0–4 min at 5% B (sample loading and desalting); linear from 5% to 60% B (30 min); linear from 60% to 95% B (4 min); and isocratic elution at 95% B (6 min) at a flow rate of 300 nL/minutes. The mass spectrometer was operated in data-dependent mode to switch automatically between full-MS scan and MS/MS scan (either CID-MS^2 or HCD-MS^2 scan). The normalized collision energy (NCE) used was set at 35% in CID-MS^2, and at 40% in HCD-MS^2. Reaction time was 30 ms for both CID-MS^2 and HCD-MS^2 scan. After a full MS scan, the top five ions were selected for either CID-MS^2- or HCD-MS^2-scan in a separate run. Full-MS spectra were acquired at 60,000 resolution and CID-MS^2/HCD-MS^2 spectra were acquired at 7500 resolution.

2.5. CD-spectroscopy

Circular-Dichroism (CD) measurements were carried out with a Jasco-J-720-CD spectropolarimeter (Japan Spectroscopic Company). Upd3 was dissolved in 20 mM Tris, 500 mM sodium chloride, pH 8.0 with or without 0.4 mM TCEP. The spectral bandwidth was 2 nm. The spectropolarimeter was calibrated according to Chen and Yang [25]. The measurements were performed at 20 °C in a 0.01 cm quartz cuvette (for thermal stability: 0.1 cm). The CD spectrum was analysed with respect to the main-chain conformation with the CONTIN-program [26]. For the melting curve the temperature was increased 1 °C/min and the spectral changes at 222 nm were recorded. The signal intensity at 20 °C was set to 100% of native protein, the intensity of the denatured sample to 0%.

3. Results

3.1. Design of an Upd3 expression construct

To analyze the structural and functional properties of Upd3 we aimed to express the protein recombinantly in *E. coli*. Since Upd3 is supposed to be a secreted cytokine the first task was to identify the length of the signal peptide. For this purpose different prediction programs were used. "PrediSi" [27] predicted a signal peptide with a length of 65 amino-acids, an unusual long signal peptide, but such long signal sequences are already described for a protein descending from *Drosophila* [28]. Additionally predictions of secondary structure elements were performed using "PredictProtein" [29] and "Jpred" [30] (Fig. 1A). As already described for Upd [3] the secondary-structure prediction indicates a high α-helical content for Upd3 which is similar to those of IL-6 type cytokines, like IL-6, LIF and CNTF (Fig. 1B and D). One of the predicted α-helices in Upd3 is located at the anticipated "end" of the signal peptide, a region that was recognized as a transmembrane helix by the prediction programs "Phobius" [31] and "SignalP" [32]. Hence, amino-acid T93 which is located directly in front of the second predicted α-helix was chosen to be the first residue in the mature Upd3.

3.2. Expression, purification and structural characterization of recombinant Upd3

By cloning the cDNA of Upd3 (T93-L401) into the bacterial expression vector pET28 the protein was N-terminally fused to a His-tag (Fig. 2A). As shown in Fig. 2B, recombinant Upd3 (38 kDa) can be obtained in high yields but as insoluble inclusion bodies. After Ni^{2+}-affinity purification under denaturing conditions

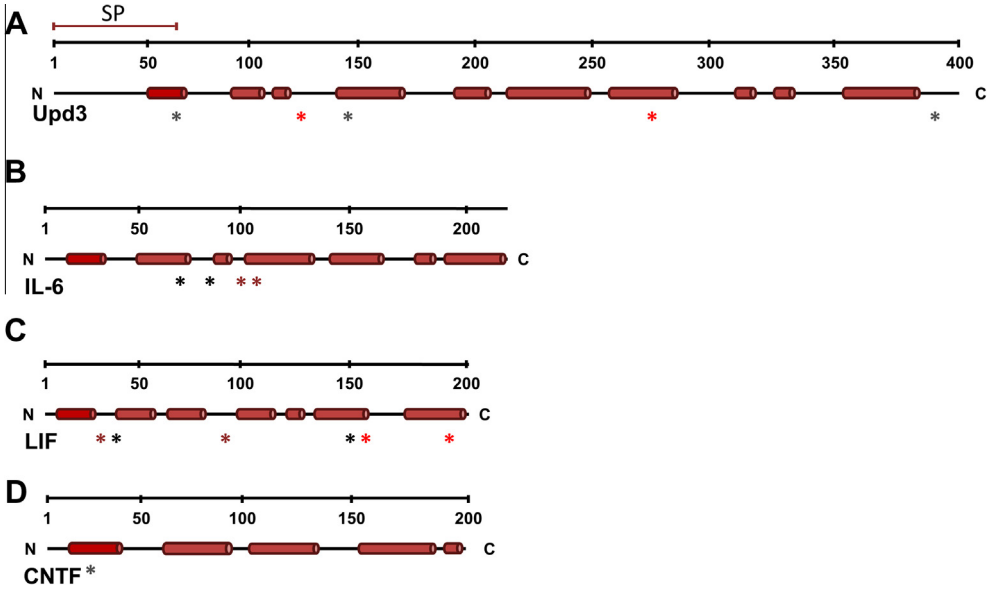

Fig. 1. Schematic drawing of Upd3 and IL-6 type cytokines. The secondary structure elements predicted by "Jpred" [30] are displayed as red cylinders in case of α-helices. The positions of cysteine residues are marked by asterisks. (A) Upd3 contains five cysteine residues (C69, C129, C146, C271, C387) whereby one disulfide-bond was identified (red asterisk). (B) In case of IL-6 two successively disulfide-bounds (black and dark red asterisk) are present. (C) In contrast, LIF contains three not successively disulfide-bonds (dark red, black and red asterisk). (D) CNTF did not contain any disulfide-bond since only one cysteine residue is present within the first α-helix.

the protein was renaturated by dialysis against 20 mM TRIS, 500 mM NaCl at pH 8.0. After size-exclusion chromatography the protein was obtained as a pure and monomeric protein (Fig. 2C). To verify the identity of recombinant Upd3 an in house generated monoclonal antibody raised against Upd3 was used to confirm the protein by western blot (Fig. 2D).

For structural characterization CD-spectroscopy was used. The recorded CD-spectrum is indicative for a native, folded protein with a substantial α-helical content (Fig. 2E). The calculated secondary structure contents are 43% α-helices and 14% β-strands and 16% loops. These experimental data are in good agreement with the secondary structure prediction with 46% (Fig. 1A).

3.3. Recombinant Upd3 is able to activate the JAK/STAT pathway

To analyze the biological activity of Upd3 its ability to activate the JAK/STAT pathway was studied using a luciferase gen-reporter assay [33]. Thereby, *Drosophila* Kc_{167} cells, which express all components of the pathway except the ligand [34–36], were transfected with a reporter 10xSTAT92E-luciferase- and a control Act-*Renilla*-plasmid. One day after transfection cells were incubated with increasing amounts of Upd3 (Fig. 2F). The activity of the luciferase gen-reporter construct showed clearly that Upd3 is able to activate the JAK/STAT pathway resulting in a significant increase in firefly-luciferase activity.

3.4. Identification of the disulfide pattern

Upd3 contains four cysteine residues (C129, C146, C271 and C387). We investigated the oxidation state of Upd3 by proteolytic cleavage followed by (RP)-HPLC coupled online to nanoESI-LTQ-Orbitrap tandem mass spectrometry. The elution profile of the LC-MS/MS run is shown in Fig. 3. Eighty-two peptides (Supplementary Table 1) were identified, leading to an overall sequence coverage of 93%.

Two peptides containing carbamidomethylated cysteine residues were identified (Table 1): peptide 3: $SKAKRC_{146}$(cam)KKRQRIL; peptide 14: C_{387}(cam)KATTHVPKAIQKKL. As alkylation of cysteine residues occurs only at free, non-oxidized sulfhydryls we concluded that C146 and C387 are not involved in disulfide formation in the native Upd3.

Four chymotryptic peptides (73, 76, 78, and 82, Table 1) are built by two peptide chains connected via a single disulfide bind between C129 and C271. The structures of these peptides were confirmed by the high mass accuracy of their MS signals (see Table 1) and MS/MS spectra. Exemplarily, the MS/MS spectra of the triply charged disulfide-linked peptide 76 fragmented by HCD-MS^2/CID-MS^2 and acquired in the Orbitrap MS are shown in Fig. S1. Product ions formed by HCD-MS^2 are complementary to those formed by CID-MS^2. While the detection of b_2(P1), b_3(P1), b_4(P1) and y_4(P1) ions at the low mass region in the HCD-MS^2 spectrum allowed a sequence assignment and in particular the identification of both the N- and the C-terminus of one peptide (in this case P1 was identified), the partial peptide sequence tags [e.g., (1054.46^{2+})[I/L]N(1168.52^{2+}) in Fig. S1B] revealed at the higher mass range in the CID-MS^2 spectrum allowed the identification of the second peptide chain (P2) connected to peptide P1 via the disulfide bond.

The four peptides share the same core region connected by the disulfide, but differ in the number of residues prolonging their particular N- and C-termini due to missed chymotryptic cleavage sites. Interestingly, in both peptides (76, 82), cleavage occurred directly N-terminal aside the disulfide. Thus, the disulfide did not shield the protease cleavage site.

In summary, Upd3 contains one disulfide bond, linking residues C129 and C271. The other two cysteine residues (C146 and C387) are present in the reduced form. It has to be noted, that during the time course no alterations in the oxidation states of the four residues were observed; hence, disulfide scrambling can be ruled out.

3.5. The disulfide bond stabilizes Upd3

To confirm that the two free cysteine residues are not involved in intermolecular disulfide bonds we run an SDS–PAGE under reducing and non-reducing conditions (Figs. 4A and B). As shown in Fig. 4A no bands corresponding to higher molecular weights under non-reducing conditions are present.

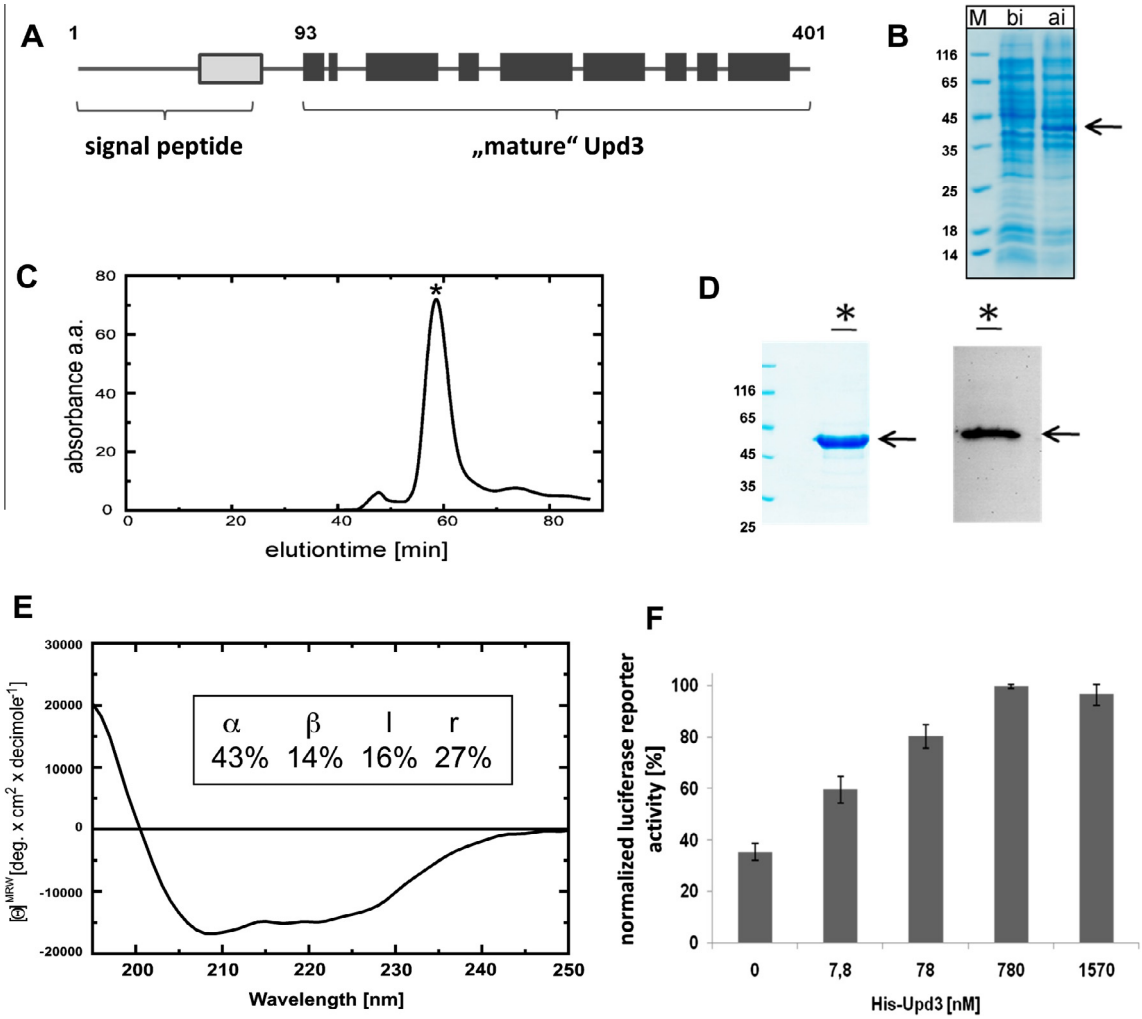

Fig. 2. Expression, purification and structural characterization of Upd3. (A) Schematic presentation of the sequence used for expression of the recombinant Upd3. The predicted α-helical regions are depicted as squares and the region encompassing the cDNA is marked, starting with amino-residue T93 and ending with L401. (B) Analysis of the bacterial expression of Upd3 by SDS–PAGE. (M: molecular weight marker, bi: before induction, ai: after induction.) (C) Size-exclusion chromatogram of the renaturated Upd3. The protein elutes at an elution time corresponding to monomeric Upd3 at approximately 60 ml. (D) SDS–PAGE and western blot analysis of the purified protein. By western blot analysis the protein was detectable by an antibody directed against the "mature" part of Upd3. (E) CD-Spectrum of the monomeric Upd3 is indicative for an α-helical protein. The insert shows the secondary structure content calculated from the CD-spectrum [26]. (F) Biological activity of recombinant Upd3 was analysed using *Drosophila* Kc_{167} cells transfected with 10xSTAT92E-luciferase assay. After 24 h stimulation with increasing amounts of Upd3 the respective cell lysates were analysed in respect to firefly and renilla-luciferase activity. The later one was used for normalization of the firefly luciferase activity and the maximal measured activity set to 100% of activity, which correspond to cells which are stimulated with 780 nM recombinant Upd3.

The impact of the disulfide bond for the stability of Upd3 was analyzed by CD-spectroscopy. Therefore, Upd3 was incubated with an excess of TCEP and the CD-spectrum was recorded (Fig. 4C, dashed line). No changes compared to the untreated sample were observable (Fig. 4C, solid line). However, the melting curve of the reduced sample revealed a decreased thermal stability as manifested by a shift in the Tm from approximately 55 °C to 47 °C (Fig. 4D).

4. Discussion

To our knowledge, we describe for the very first time the expression of an *unpaired*-like protein from *Drosophila*. According to the signal-peptide prediction a bacterial expression construct was designed to express "mature" Upd3. This construct contains four cysteine residues and the secondary structure prediction resulted in nine α-helices. The expression and renaturation of the protein yields a monomeric protein which is dominated by α-helices as judged from CD-spectroscopy. A luciferase gen-reporter assay confirmed that the purified protein is biologically active and able to induce the JAK/STAT pathway in *Drosophila* cells, implicating that it is indeed a cytokine that activates the JAK/STAT pathway directly, most likely via the type-I cytokine receptor *domeless*.

Like cytokines in mammals which bind to type-I cytokine receptors Upd3 displays a strong α-helical character. The contend of about 43% α-helical and 14% strand is in accordance with four-helix bundle cytokines like human IL-6 (67% helix, 15% strand) [37] and human CNTF (43% helix, 15% strand) [38]. This implicates that Upd3 is indeed an orthologue of the "four-helix bundle cytokines", like the IL-6 type cytokines are. This is supported by the fact that Upd3 binds most likely to domeless, the orthologue of the common signal transducer of IL-6 type cytokines gp130 and that Upd3 is able to activate, like IL-6, the expression of acute-phase protein [6].

The analysis of the disulfide-bonds within Upd3 revealed that there is no similarity between those of Upd3 and the four-helix bundle cytokines of the IL-6 type cytokines, like IL-6, LIF or CNTF.

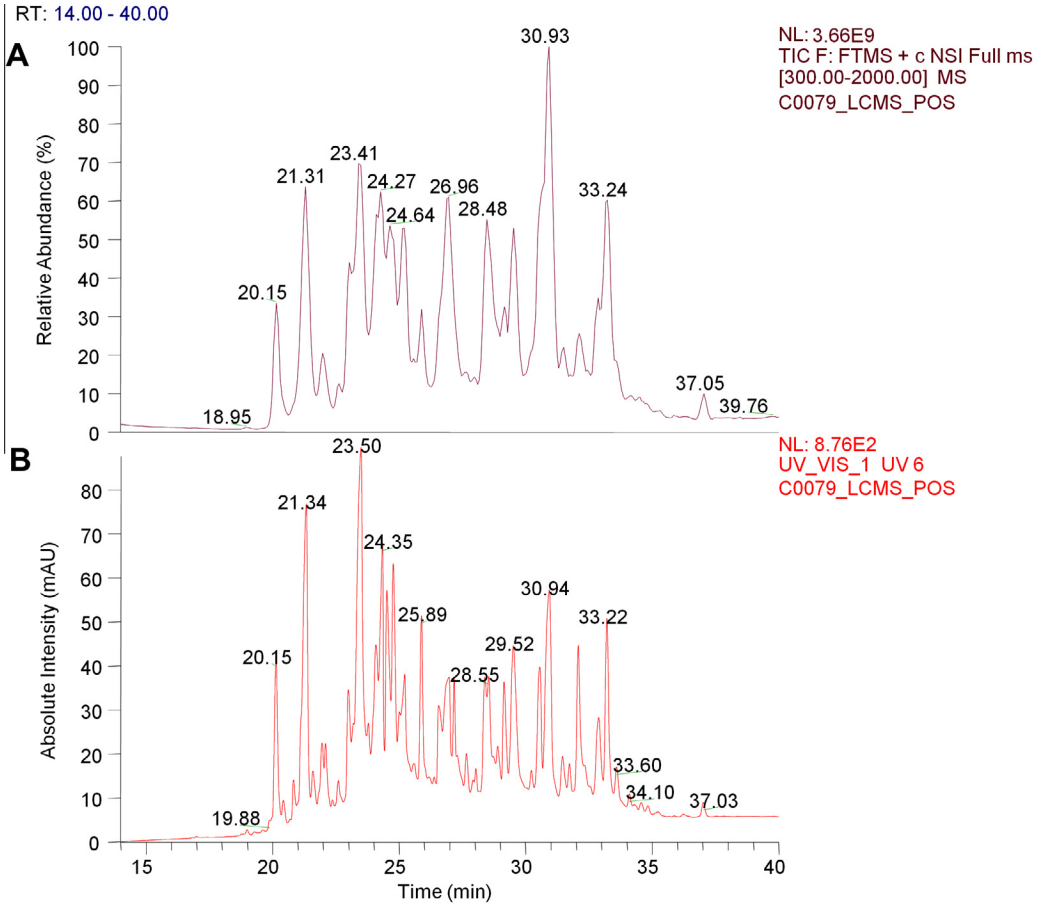

Fig. 3. Determination of the disulfide-bond pattern of recombinant UPD3 by LC-MS/MS. Chromatographic separation of a chymotryptic digest (2 h) of Upd3. (A) MS-trace (total ion current) in electrospray MS, (B) UV-trace (214 nm).

Table 1
Alkylated (carbamidomethylated) cysteine and disulfide bond containing peptides identified in LC-MS/MS analysis of chymotryptic digest of Upd3. Peak numbering refers to Fig. 4 and Supplementary Table S1.

Peak no.	Retention time (min)	Peptide Fragment	Observed ions	Mw_exp (Da)	Mw_theo (Da)	Mass deviation (ppm)
3	20.31	H.SKAKRC_{146}(cam)KKRQRIL.Q	418.763 (4+) 558.013 (3+) 836.518 (2+)	1671.017	1671.0202	−1.812
14	22.70	L.C_{387}(cam)KATTHVPKAIQKKL	431.507 (4+) 575.006 (3+)	1721.999	1721.9974	0.869
73	32.93	L.NASSTHLDWENTC_{129}NLKPTGL.N|M.RKSAREVLC_{271}SVEEAINL.T	823.813 (5+) 1029.514 (4+) 1372.350 (3+)	4114.028	4114.0212	1.804
76	33.24	L.DWENTC_{129}NLKPTGL.N|L.C_{271}SVEEAINL.T	822.382 (3+) 1233.069 (2+)	2464.124	2464.1250	−0.336
78	34.39	L.NASSTHLDWENTC_{129}NLKPTGL.N|R.MRKSAREVLC_{271}SVEEAINL.T	850.019 (5+) 1062.273 (4+) 1416.030 (3+)	4245.059	4245.0617	−0.726
82	34.66	L.DWENTC_{129}NLKPTGL.N|L.C_{271}SVEEAINL**M**.T	866.062 (3+)	2595.164	2595.1655	−0.512

C(cam): carbamidomethylated cysteine.

Since there is no congeneric pattern within the family of IL-6 type cytokines itself this could be expected (Fig. 1).

Remarkably, the biologically active Upd3 contains only one disulfide bond and two additional free cysteine residues, but the cytokine remains as a monomer in solution as shown by size-exclusion chromatography (Fig. 2C) and no intermolecular disulfide bonds are formed (Table 1 and Fig. 4A). The presence of free cysteine residues in an α-helical cytokine is not that unusual. CNTF

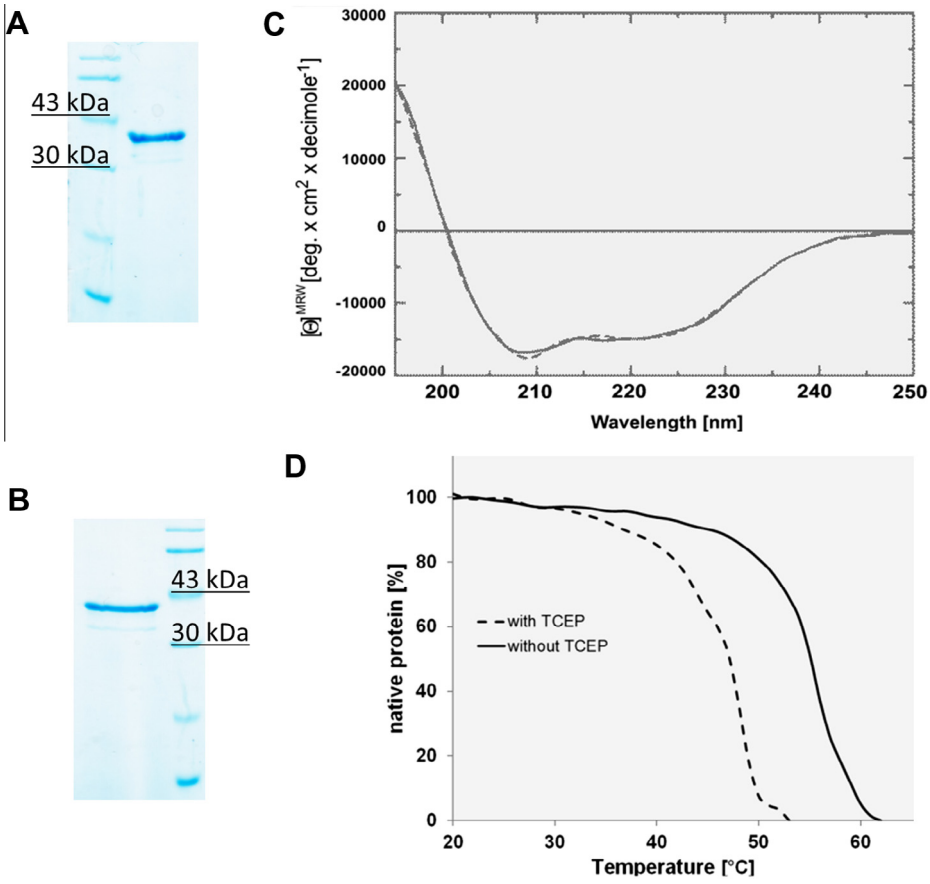

Fig. 4. The disulfide bond within recombinant Upd3 is needed for its stability. (A) No intermolecular disulfide bonds are formed within Upd3, as shown by SDS–PAGE. Purified recombinant Upd3 was loaded under non-reducing. (B) As well as reducing condition onto a SDS page. After coomassie staining no bands higher than the monomeric Upd3 were visible in both samples, indicating that Upd3 forms no interresidual cysteine bonds. (C) To test if the disulfide bond within Upd3 is essential for its stability 0.4 mM TCEP was added to about 0.9 μM of recombinant protein. Afterwards the sample were analysed by CD-spectroscopy (dashed line) corresponding to the non-reduced sample (solid line). (D) To further proof if the disulfide bond is essential for the temperature stability of the cytokine from melting curve from both samples was recorded. Thereby the T_m of the non-reduced sample (solid line) was shifted from approximately 55 °C to 47 °C under reducing conditions (dashed line).

holds only one unpaired cysteine residue, but this is involved in dimer formation at high concentrations [39].

Obviously, Upd3 is about twice as big as the IL-6 type cytokines and this discrepancy can not be solved by creating a smaller molecule, since the most N- and penultimate C-terminally located cysteine residues form a disulfide bond which is necessary for the stability of Upd3. It will be exciting to solve the three-dimensional structure of Upd3 in future work and to see whether Upd3 resembles the typical up–up–down–down topology of four-helix bundle cytokines.

Acknowledgments

This work was supported by the cluster of excellence "Inflammation at Interfaces" and SFB877 project A6.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.107.

References

[1] S. Brown, N. Hu, J.C. Hombria, Identification of the first invertebrate interleukin JAK/STAT receptor, the Drosophila gene domeless, Curr. Biol. 11 (2001) 1700–1705.
[2] H.W. Chen, X. Chen, S.W. Oh, M.J. Marinissen, J.S. Gutkind, S.X. Hou, Mom identifies a receptor for the Drosophila JAK/STAT signal transduction pathway and encodes a protein distantly related to the mammalian cytokine receptor family, Genes Dev. 16 (2002) 388–398.
[3] D.A. Harrison, P.E. McCoon, R. Binari, M. Gilman, N. Perrimon, Drosophila unpaired encodes a secreted protein that activates the JAK signaling pathway, Genes Dev. 12 (1998) 3252–3263.
[4] J.C. Hombría, S. Brown, S. Häder, M.P. Zeidler, Characterisation of Upd2, a Drosophila JAK/STAT pathway ligand, Dev. Biol. 288 (2005) 420–433.
[5] M.M. Gilbert, B.K. Weaver, J.P. Gergen, N.C. Reich, A novel functional activator of the Drosophila JAK/STAT pathway, unpaired2, is revealed by an in vivo reporter of pathway activation, Mech. Dev. 122 (2005) 939–948.
[6] H. Agaisse, U.M. Petersen, M. Boutros, B. Mathey-Prevot, N. Perrimon, Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury, Dev. Cell 5 (2003) 441–450.
[7] J. Grötzinger, G. Kurapkat, A. Wollmer, M. Kalai, S. Rose-John, The family of the IL-6-type cytokines: specificity and promiscuity of the receptor complexes, proteins: structure, Funct. Genet. 27 (1997) 96–109.
[8] B. Giese, C. Roderburg, M. Sommerauer, S.B. Wortmann, S. Metz, P.C. Heinrich, G. Müller-Newen, Dimerization of the cytokine receptors gp130 and LIFR analysed in single cells, J. Cell Sci. 118 (2005) 5129–5140.
[9] S. Tenhumberg, B. Schuster, L. Zhu, M. Kovaleva, J. Scheller, K.J. Kallen, S. Rose-John, Gp130 Dimerization in the absence of ligand: preformed cytokine receptor complexes, Biochem. Biophys. Res. Commun. 346 (2006) 649–657.
[10] S. Brown, N. Hu, J.C. Hombria, Novel level of signalling control in the JAK/STAT pathway revealed by in situ visualisation of protein–protein interaction during Drosophila development, Development 130 (2003) 3077–3084.
[11] P.C. Heinrich, I. Behrmann, S. Haan, H.M. Hermanns, G. Müller-Newen, F. Schaper, Principles of interleukin (IL)-6-type cytokine signalling and its regulation, Biochem. J. 374 (2003) 1–20.
[12] M.A. Febbraio, Gp130 receptor ligands as potential therapeutic targets for obesity, J. Clin. Invest. 117 (2007) 841–849.
[13] A. Chalaris, C. Garbers, B. Rabe, S. Rose-John, J. Scheller, The soluble Interleukin 6 receptor: generation and role in inflammation and cancer, Eur. J. Cell Biol. 90 (2011) 484–494.

[14] S. Rose-John, G.H. Waetzig, J. Scheller, J. Grötzinger, D. Seegert, The IL-6/sIL-6R complex as a novel target for therapeutic approaches, Expert Opin. Ther. Targets 11 (2007) 613–624.
[15] H. Luo, W.P. Hanratty, C.R. Dearolf, An amino acid substitution in the Drosophila hopTum-l Jak kinase causes leukemia-like hematopoietic defects, EMBO J. 14 (1995) 1412–1420.
[16] H. Luo, P. Rose, D. Barber, W.P. Hanratty, S. Lee, T.M. Roberts, A.D. D'Andrea, C.R. Dearolf, Mutation in the Jak kinase JH2 domain hyperactivates Drosophila and mammalian Jak-Stat pathways, Mol. Cell Biol. 17 (1997) 1562–1571.
[17] J. Grötzinger, T. Kernebeck, K.-J. Kallen, S. Rose-John, IL-6 type cytokine receptor complexes: hexamer or tetramer or both?, Biol Chem. 380 (1999) 803–813.
[18] P.D. Sun, D.R. Davies, The cystine-knot growth-factor superfamily, Annu. Rev. Biophys. Biomol. Struct. 24 (1995) 269–291.
[19] N.Q. McDonald, R. Lapatto, J. Murray-Rust, J. Gunning, A. Wlodawer, T.L. Blundell, New protein fold revealed by a 2.3-A resolution crystal structure of nerve growth factor, Nature 354 (1991) 411–414.
[20] A.G. Murzin, A.M. Lesk, C. Chothia, Beta-trefoil fold. Patterns of structure and sequence in the Kunitz inhibitors interleukins-1 beta and 1 alpha and fibroblast growth factors, J. Mol. Biol. 223 (1992) 531–543.
[21] X. Zhu, H. Komiya, A. Chirino, S. Faham, G.M. Fox, T. Arakawa, B.T. Hsu, D.C. Rees, Three-dimensional structures of acidic and basic fibroblast growth factors, Science 251 (1991) 90–93.
[22] H.T. Idriss, J.H. Naismith, TNF alpha and the TNF receptor superfamily: structure-function relationship(s), Microsc. Res. Tech. 50 (2000) 184–195.
[23] J.L. Boulay, J.J. O'Shea, W.E. Paul, Molecular phylogeny within type I cytokines and their cognate receptors, Immunity 19 (2003) 159–163.
[24] J.F. Bazan, Haemopoietic receptors and helical cytokines, Immunol. Today 11 (1990) 350–354.
[25] G.C. Chen, J.T. Yang, Two-point calibration of circular dichrometer with d-10-camphorsulfonic acid, Anal. Lett. 10 (1977) 1195–1207.
[26] S.W. Provencher, J. Glockner, Estimation of globular protein secondary structure from circular dichroism, Biochemistry 20 (1981) 33–37.
[27] K. Hiller, A. Grote, M. Scheer, R. Munch, D. Jahn, PrediSi: prediction of signal peptides and their cleavage positions, Nucleic Acids Res. 32 (2004) W375–W379.
[28] A. Kilic, S. Klose, B. Dobberstein, E. Knust, K. Kapp, The Drosophila crumbs signal peptide is unusually long and is a substrate for signal peptide peptidase, Eur. J. Cell Biol. 89 (2010) 449–461.
[29] B. Rost, G. Yachdav, J. Liu, The predictprotein server, Nucleic Acids Res. 32 (2004) W321–W326.
[30] C. Cole, J.D. Barber, G.J. Barton, The Jpred 3 secondary structure prediction server, Nucleic Acids Res. 36 (2008) W197–W201.
[31] L. Käll, A. Krogh, E.L. Sonnhammer, Advantages of combined transmembrane topology and signal peptide prediction – the Phobius web server, Nucleic Acids Res. 35 (2007) W429–W432.
[32] T.N. Petersen, S. Brunak, G. von Heijne, H. Nielsen, SignalP 4.0: discriminating signal peptides from transmembrane regions, Nat. Methods 8 (2011) 785–786.
[33] G.H. Baeg, R. Zhou, N. Perrimon, Genome-wide RNAi analysis of JAK/STAT signaling components in Drosophila, Genes Dev. 19 (2005) 1861–1870.
[34] P. Müller, D. Kuttenkeuler, V. Gesellchen, M.P. Zeidler, M. Boutros, Identification of JAK/STAT signalling components by genome-wide RNA interference, Nature 436 (2005) 871–875.
[35] O.M. Vidal, W. Stec, N. Bausek, E. Smythe, M.P. Zeidler, Negative regulation of Drosophila JAK-STAT signalling by endocytic trafficking, J. Cell Sci. 123 (2010) 3457–3466.
[36] V.M. Wright, K.L. Vogt, E. Smythe, M.P. Zeidler, Differential activities of the Drosophila JAK/STAT pathway ligands Upd, Upd2 and Upd3, Cell Signal 23 (2011) 920–927.
[37] A. Krüttgen, S. Rose-John, C. Moller, B. Wroblowski, A. Wollmer, J. Müllberg, T. Hirano, T. Kishimoto, P.C. Heinrich, Structure-function analysis of human interleukin-6. Evidence for the involvement of the carboxy-terminus in function, FEBS Lett. 262 (1990) 323–326.
[38] A. Krüttgen, J. Grötzinger, G. Kurapkat, J. Weis, R. Simon, M. Thier, M. Schröder, P. Heinrich, A. Wollmer, M. Comeau, J. Müllberg, S. Rose-John, Human ciliary neurotrophic factor: a structure-function analysis, Biochem. J. 309 (1995) 215–220.
[39] N.Q. McDonald, N. Panayotatos, W.A. Hendrickson, Crystal structure of dimeric human ciliary neurotrophic factor determined by MAD phasing, EMBO J. 14 (1995) 2689–2699.