A *Drosophila* haemocyte-specific protein, hemolectin, similar to human von Willebrand factor

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We identified a novel *Drosophila* protein of ≈ 400 kDa, hemolectin (*d*-Hml), secreted from haemocyte-derived Kc167 cells. Its 11.7 kbp cDNA contains an open reading frame of 3843 amino acid residues, with conserved domains in von Willebrand factor (VWF), coagulation factor V/VIII and complement factors. The *d-hml* gene is located on the third chromosome (position 70C1-5) and consists of 26 exons. The major part of *d*-Hml consists of well-known motifs with the organization: CP1-EG1-CP2-EG2-CP3-VD1-VD2-VD′-VD3-VC1-VD″-VD‴-FC1-FC2-VC2-LA1-VD4-VD5-VC3-VB1-VB2-VC4-VC5-CK1 (CP, complement-control protein domain; EG, epidermal-growth-factor-like domain; VB, VC, VD, VWF type B-, C- and D-like domains; VD′, VD″, VD‴, truncated C-terminal VDs; FC, coagulation factor V/VIII type C domain; LA, low-density-lipoprotein-receptor class A domain; CK, cysteine knot domain). The organization of VD1-VD2-VD′-VD3, essential for VWF to be processed by furin, to bind to coagulation factor VIII and to form interchain disulphide linkages, is conserved. The 400 kDa form of *d*-Hml was sensitive to acidic cleavage near the boundary between VD2 and VD′, where the cleavage site of pro-VWF is located. Agarose-gel electrophoresis of metabolically radio-labelled *d*-Hml suggested that it is secreted from Kc167 cells mainly as dimers. Resembling VWF, 7.9% (305 residues) of cysteine residues on the *d*-Hml sequence had well-conserved positions in each motif. Coinciding with the development of phagocytic haemocytes, *d-hml* transcript was detected in late embryos and larvae. Its low-level expression in adult flies was induced by injury at any position on the body.

Key words: coagulation factor, cysteine knot, disulphide bond, haemocytin, haemostasis.

INTRODUCTION

von Willebrand factor (VWF) is a large multimeric blood glycoprotein essential for haemostasis and its deficiency causes von Willebrand disease, which is the most common bleeding disorder inherited in an autosomal dominant manner [1]. VWF was first purified in the early 1970s as a multimeric protein complex consisting of 2050 amino acid residues (reviewed in [2]), but its complete sequence based on cDNA cloning revealed that human VWF has a putative signal peptide of 22 residues and a prosequence of 741 residues in addition to mature VWF sequence [3]. The pro-VWF is a highly repetitive protein which consists of two duplicated B and C, a triplicated A, a quadruplicated D and a partially duplicated D′ domains in the order D1-D2-(N-terminus of the mature VWF)D′-D3-A1-A2-A3-D4-B1-B2-C1-C2. To the C-terminus, the cysteine knot domain (CK) homologous to that found in nerve growth factor, transforming growth factor β, platelet-derived growth factor and Norrie disease protein [4–6] is added for 'tail-to-tail' dimerization by disulphide linkage formed in the endoplasmic reticulum [7]. Tail-to-tail pro-VWF dimers are transported to the Golgi apparatus and additional 'head-to-head' disulphide bonds are formed within the D3 domain [8], yielding multimers that may exceed 20000 kDa in size. The majority of such multimers are constitutively secreted, whereas the remainder are stored in granules called Weibel–Palade bodies, which are specific for VWF-producing cells [9]. However, megakaryocytes that do not contain Weibel–Palade bodies are also known to secrete VWF. By expressing pro-VWF cDNA in various cell lines, packaging into storage granules was shown to require the prosequence D1-D2 and to be the general potential of hormone-secreting cells [10]. Additional modifications in the Golgi apparatus include the proteolytic removal of the propeptide, possibly by the propeptide-processing enzyme PACE (furin) [11]. VWF performs two essential functions in haemostasis: firstly it mediates the adhesion of platelets to sub-endothelial connective tissues and secondly it binds and stabilizes blood coagulation factor VIII. Multimerization by tail-to-tail and head-to-head disulphide linkages is crucial for the first function. The binding sites to fibrillar collagens in connective tissues are mapped in domains A1 and A3 [12–14]. The binding site to platelet glycoprotein GPIb has been localized in the A1 domain [15]. The RGDS motif near the C-terminal end of the C1 domain may also contribute to the interaction with platelets through a GPIIβ–IIIα complex (αIIbβ3 integrin) [16]. The N-terminal end of mature VWF is responsible for the second function, and the site of binding to coagulation factor VIII is localized within the N-terminal 272 amino acid residues (namely within the D′-D3 domains) [17].

Abbreviations used: *b*-Hmc, *Bombyx mori* haemocytin; *d*-Hml, *Drosophila* hemolectin; RT-PCR, reverse transcriptase PCR; TCA, trichloroacetic acid; VWF, von Willebrand factor; CK, cysteine knot domain; CP, complement-control protein domain; VD, VWF type D-like domain; VD′, VD″ and VD‴, truncated C-terminal VDs; VC, VWF type C-like domain; EG, epidermal-growth-factor-like domain; LA, low-density-lipoprotein receptor class A domain.

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The nucleotide sequence data reported here are deposited in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AB035891 and in the Berkeley *Drosophila* Genome Projects database with Gadfly number CG7002.

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Several C-type (Ca^{2+}-dependent) humoral lectins have been identified in insect larval haemolymph. From the American cockroach *Periplaneta americana*, regenectin [18], a 26 kDa lectin [19], *Periplaneta* lectin [20] and a lipopolysaccharide-binding protein [21] have been characterized. PCR using degenerate primers based on the partial amino acid sequences detected many mRNAs encoding molecules that were structurally related to *Periplaneta* lectin [22]. Database searches revealed several putative C-type lectins within the *Drosophila* genome [23]. Other C-type lectins such as *Sarcophaga* lectin [24] have been found in the flesh fly. However, their monomeric sizes range between 30 and 40 kDa. Not many lectin-like molecules that are comparable in size with VWF have been demonstrated in insects.

Here we report the cloning of a cDNA encoding a large *Drosophila* protein secreted from the haemocyte-derived cell line Kc167. It conserves the domain organization D1-D2-D′-D3 of VWF, which is the site for packaging into Weibel–Palade bodies, for cleavage by processing enzyme PACE (furin), for binding to coagulation factor VIII and for head-to-head disulphide linkage. A striking similarity of the *Drosophila* protein to VWF was that it was sensitive to cleavage around the boundary between the D2 and D′ domains. The C-terminal two-thirds of the sequence shared similarity with *Bombyx mori* haemocytin (*b*-Hmc) [25], a haemolymph protein identified in the silkworm *B. mori* as a 280 kDa protein resulting from a 340 kDa precursor upon post-translational processing [26]. Since the N-terminal sequence of $\approx$ 1200 residues is not found in *b*-Hmc, however, we will refer to this novel *Drosophila* protein as hemolectin (*d*-Hml) and describe its full-length amino acid sequence, domain organization, chromosome mapping and developmental expression.

MATERIALS AND METHODS

Cell culture, and identification and partial amino acid sequencing of *d*-Hml

The *Drosophila* haemocyte-derived cell line Kc167 was maintained with HyQ CCM3 medium (HyClone Laboratories) at 25 °C under air.

We identified *d*-Hml as a protein that migrates as a $\approx$ 400 kDa band in SDS/PAGE of the medium from Kc167 culture under reducing conditions, whereas it migrates as a $\approx$ 300 kDa band after trichloroacetic acid (TCA) precipitation from the medium. For partial amino acid sequencing, proteins in the medium were concentrated by adjusting to 10% TCA and separated by SDS/PAGE under reducing conditions. After staining the gel with Coomassie Brilliant Blue, the $\approx$ 300 kDa band was cut out and digested by trypsin. Peptide fragments were separated by reversed-phase HPLC on a SMART SYSTEM (Amersham Pharmacia Biotech) and partially sequenced from the N-terminus [27].

cDNA cloning of *d*-Hml

A cDNA fragment corresponding to nt 6532–7260 (729 bp) was first obtained by reverse transcriptase PCR (RT-PCR) using a 16-fold redundant forward primer of 5′-AWGAYCARATGC-ARTA-3′ based on the peptide sequence $NDQMQY^{2129}$, and a 128-fold redundant reverse primer 5′-TTRTCRTGRTTNGC-YTCRAA-3′ based on the peptide sequence $FEANHD^{2366}$. Superscript™ II reverse transcriptase (Gibco BRL) was used for transcription of total RNA from Kc167 cells. PCR of the cDNA sequence was run in a TaKaRa PCR Thermal Cycler MP (TaKaRa, Tokyo, Japan) with the following program: after denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min were followed by final extension at 72 °C for 10 min. With a similar strategy, two additional fragments corresponding to nt 4608–6819 (2212 bp) and 6819–8679 (1861 bp) were obtained. These fragments were labelled with $[^{32}P]$dCTP (Amersham Pharmacia Biotech) using a Multiprime DNA labelling system (Amersham Pharmacia Biotech), and a *Drosophila* larvae cDNA library in a λgt11 vector (Clontech) and an adult genomic library were screened. Plaque hybridization on Colony/Plaque Screen nylon membranes (NEM, Tokyo, Japan) was carried out at 65 °C in 6×SSC (where 1×SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.5% SDS, 5× Denhardt's solution, 50 mM Tris/HCl (pH 7.5) and 0.1 mg/ml denatured salmon sperm DNA for 16 h. Membranes were washed twice with 2×SSC containing 0.1% SDS at room temperature and twice with 0.2×SSC containing 0.1% SDS at 65 °C for 30 min. Positive plaques were selected after re-screening. DNA was extracted from plate lysate or using the Lambda Midi kit (Qiagen), digested with *Eco*RI, subcloned into the pBluescript vector and sequenced using the Thermo Sequenase fluorescently labelled primer cycle sequencing kit (Amersham Pharmacia Biotech) and a model 4000 DNA sequencer (LI-COR). After sequential screening, ten cDNA clones and one genomic clone covering 11791 bp were isolated (see Figure 2, below). To cover a gap of 450 bp at nt 5773–6222 among cDNA clones, five cDNA clones with identical sequences were obtained by RT-PCR of Kc167 total RNA and PCR of a genomic clone.

Sequence analysis

General sequence analysis was performed by MacDNAsis version 3.7 (Hitachi Software). BLAST 2.0 [28] was used for similarity searches against the non-redundant GenBank database (June 1999). The deduced amino acid sequence was checked for common motifs by PROSITE [29]. Motifs were compared with those in the Pfam (version 4.4) domain database [30] using Clustal W [31] for multiple sequence alignments.

Preparation of antibodies

A monoclonal antibody recognizing *d*-Hml was screened from a library raised against heparin affinity-purified proteins from conditioned medium of Kc167 cells [32]. Antiserum recognizing either the N-terminal CP2 domain (complement-control protein domain 2) or the C-terminal VD4 domain (VWF type D-like domain 4) was raised in a rabbit against a recombinant peptide corresponding to ^{236}I–^{294}S or ^{2850}D–^{2920}N, respectively, expressed in *Escherichia coli* as a fusion protein with glutathione S-transferase using pGEX-2T. The antiserum against *Drosophila* laminin β chain was obtained as described in [33].

SDS/PAGE and Western blotting

SDS/PAGE was performed using 5% polyacrylamide gels as described in [34]. The migration positions of endogenous laminin α (400 kDa), β (220 kDa) and γ (200 kDa) chains [35] were detected as the size markers for the electrophoresis. For Western blotting of *d*-Hml secreted from Kc167 cells, the media were separated before or after treating with 10% TCA solution. Separated proteins in the gel were transferred to a nitrocellulose membrane. The membrane was blocked with 5% dried skimmed milk and 0.1% Tween-20 in PBS for 1 h, reacted with the antibodies for 16 h at 4 °C, washed and reacted with 1000-fold-diluted horseradish peroxidase-conjugated anti-mouse or anti-rabbit Ig for 1 h. The ECL Western-blotting system (Amersham Pharmacia Biotech) was used for detection.

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SDS/agarose-gel electrophoresis of metabolically radiolabelled *d*-Hml

Potential multimerization of *d*-Hml by interchain disulphide linkage was examined by SDS/agarose-gel electrophoresis of metabolically radiolabelled *d*-Hml after immunoprecipitation. Metabolic labelling of Kc167 cells with [^{35}S]methionine/[^{35}S]cysteine and immunoprecipitation were carried out as described in [33] with minor changes. Briefly, confluent Kc167 cells pre-attached to 35 mm culture dishes (Nunc) in the methionine-free M3 medium (Sigma) for 30 min were labelled with 0.4 mCi of [^{35}S]Pro-mix (Amersham Pharmacia Biotech) for 22 h at 25 °C. The radiolabelled conditioned medium was adjusted to 1 × NET gel buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1 % Nonidet P-40, 1 mM EDTA, 0.25 % gelatin and 0.02 % sodium azide, incubated with the *d*-Hml monoclonal antibody or laminin γ-chain-specific polyclonal antibody [33] for 3 h at 4 °C and followed by incubation with 50 μl of 10 % (w/v) Protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) for 1.5 h at 4 °C. The beads were washed three times with 700 μl of 1 × NET gel buffer for 5 min at 4 °C and resuspended in SDS sample buffer. After boiling for 3 min, the samples were separated by SDS/PAGE (4 % gel) or SDS/agarose-gel electrophoresis (1.5 % gel). The SDS/agarose-gel electrophoresis was carried out as described [36], except that a horizontally placed gel (150 mm long, 135 mm wide and 6 mm thick) was used and the electrophoresis was carried out for 18 h at a 10 mA constant current. After fixation with a solution containing 10 % (w/v) TCA, 30 % (v/v) methanol and 10 % (v/v) acetic acid for 15 min, the gel was dried, exposed to an imaging plate and analysed with a Fuji Film BAS 2000 Image Analyser.

Northern blotting and RT-PCR

Total RNA was isolated from Kc167 cells by the guanidine thiocyanate/phenol/chloroform method [37]. Poly(A)$^+$ RNA from different stages of wild-type *Drosophila* (Oregon-R strain) was prepared using the QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech). For Northern blotting, 10 μg of total RNA or 5 μg of poly(A)$^+$ RNA was separated on a 1 % agarose/formaldehyde gel, blotted to Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech) and hybridized to a ^{32}P-labelled RT-PCR cDNA probe (nt 6532–7260) at 42 °C in 5 × SSPE (standard saline phosphate/EDTA) containing 0.5 % SDS, 5 × Denhardt's solution, 50 % formamide and 0.1 mg/ml denatured salmon sperm DNA for 16 h. The membrane was washed twice with 2 × SSC containing 0.1 % SDS at room temperature and twice with 0.1 × SSC containing 0.1 % SDS at 55 °C for 30 min, and analysed with a Fuji Film BAS 2000 Image Analyser.

To identify *d-hml* gene expression for all stages of *Drosophila* development, total RNAs from various stages of *Drosophila* were isolated, and RT-PCR was performed to amplify *d-hml* (nt 6819–7061) and ribosomal protein Rp49, using forward primer 5′-TGTGGAGCTCCTGATCTGTG-3′ and reverse primer 5′-CAAGCTAGTGGTGTCGATAG-3′, and forward primer 5′-GACAACAGAGTCGGTCGC-3′ and reverse primer 5′-GTTGTGCACCAGGAACTT-3′, respectively. Then, 24 h after pricking the posterior region of adult flies with a fine tungsten needle, which had or had not been dipped into an *E. coli* (DH5α) suspension, mRNAs were isolated by QuickPrep mRNA Purification Kit. RT-PCR was also performed to amplify *d-hml* (nt 9241–9945) and 5C actin using forward primer 5′-CGCG-GATTCATATCCGAGTGTAAAGACTGT-3′ (added using *Bam*HI) and reverse primer 5′-CGCGAATTCTAGGACACT-TGCCAAATAATTCAGC-3′ (added using *Eco*RI), and forward primer 5′-AAGGCCGGATTTGCCGGAGACGA -3′ and reverse primer 5′-ACGACGAAGCCAGGATGGAGCCA-3′, respectively.

Mapping of *d-hml* on polytene chromosomes

In situ hybridization of *d-hml* cDNA probe to salivary gland chromosomes was carried out as described in [38]. Non-radioactive probes were prepared from the cDNA clone (nt 4608–6819) using DIG DNA labelling mixture (Boehringer Mannheim), and the signal was detected with alkaline phosphatase-conjugated anti-digoxigenin Fab fragment (Boehringer Mannheim) and 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim).

RESULTS AND DISCUSSION

Characterization of a novel ≈ 400 kDa protein secreted from Kc167 cells

Drosophila embryonic haemocyte-derived Kc167 cells secrete large amounts of extracellular matrix proteins such as laminin, type IV collagen, glutactin and peroxidasin [33,35,39]. SDS/PAGE of the conditioned medium under reducing conditions showed an unidentified protein of ≈ 400 kDa. A curious feature of this protein's behaviour was migration as a ≈ 300 kDa band after precipitation of proteins by adjusting the medium to 10 % TCA (Figure 1A). Identification of the ≈ 300 kDa protein as a fragment of the ≈ 400 kDa protein was confirmed by Western blotting with a monoclonal antibody screened from a library raised against heparin affinity-purified proteins from Kc167 medium (Figure 1B). Antiserum raised against a recombinant N-terminal sequence (the sequence from ^{236}I to ^{294}S in Figure 3, see below) detected doublet bands of ≈ 100 kDa in the TCA-treated conditioned medium (Figure 1C), whereas antiserum against a recombinant C-terminal sequence (the sequence from ^{2850}D to ^{2920}N in Figure 3) recognized the ≈ 300 kDa protein (Figure 1D). Thus the ≈ 400 kDa protein appeared to be cleaved at two sites near the boundary between VD2 and VD′ (see below). The cleavage occurred even in 100 mM glycine/HCl buffer at pH 2.5 (results not shown). The N-terminus of the ≈ 300 kDa fragment was modified into a form resistant to Edman degradation for sequencing. It was possible that the proteolytic cleavage of the proform ≈ 400 kDa protein was completed intracellularly but the ≈ 100 and ≈ 300 kDa fragments were linked by bond(s) resistant to the components of SDS sample buffer (SDS and 2-mercaptoethanol). We will refer to this novel protein as *Drosophila* hemolectin (*d*-Hml), since the amino acid sequence deduced from cDNA of the protein has many repeats of C-type lectin-like motifs (see below).

cDNA cloning

In order to design PCR primers for amplification of cDNA fragments, the ≈ 300 kDa band (instead of the ≈ 400 kDa band to avoid contamination of the laminin α chain) was cut out and subjected to micro-sequencing after digestion with trypsin. Eight partial peptide sequences of 5–21 residues in length could be determined, and they were later found within the C-terminal half of the sequence deduced from cDNA clones. This confirmed again that the ≈ 300 kDa fragment corresponded to the C-terminal part of the ≈ 400 kDa protein.

In the strategy summarized in Figure 2 and detailed in the Materials and methods section, we isolated ten cDNA clones covering a 11 791 bp full-length cDNA sequence except for a gap of 450 bp corresponding to nt 5773–6222, which was determined

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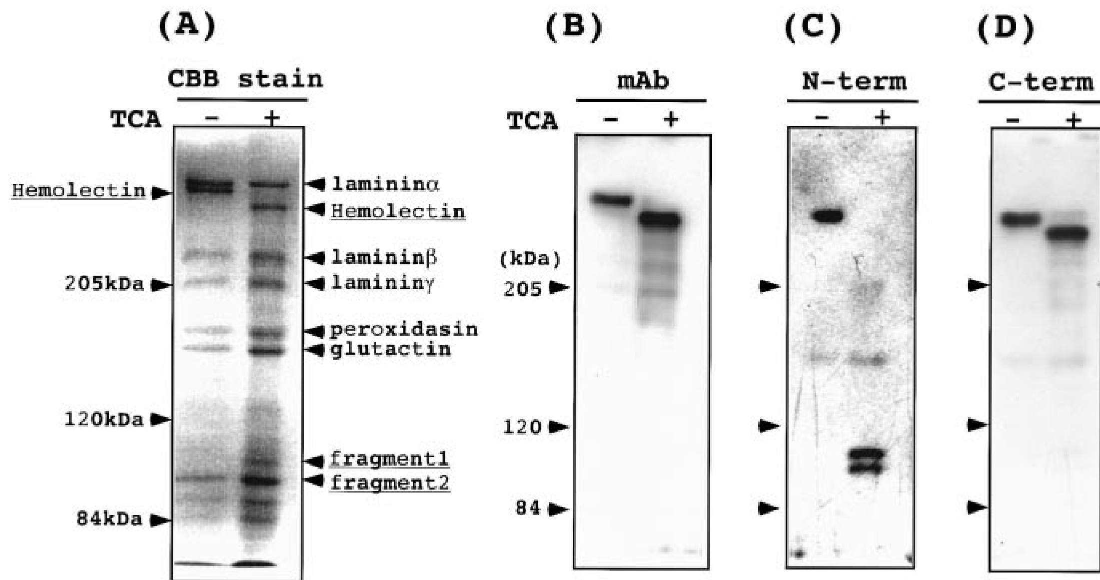

Figure 1 Identification of *Drosophila* hemolectin (*d*-Hml) among proteins secreted from Kc167 cells and its cleavage under acidic conditions

Proteins in the conditioned medium of Kc167 cells before (−) or after (+) precipitation in 10% TCA were separated by SDS/PAGE (5% gel) under reducing conditions and stained with Coomassie Brilliant Blue (CBB, **A**) or Western blotted with monoclonal antibody (mAb) against *d*-Hml screened from a library against conditioned medium of Kc167 cells (**B**) or rabbit antiserum against N-terminal (**C**) or C-terminal (**D**) domains of *d*-Hml. The migration positions of size markers are indicated on the left. Endogenous laminin α (400 kDa), β (220 kDa) and γ (200 kDa) chains and other known proteins also served as size markers.

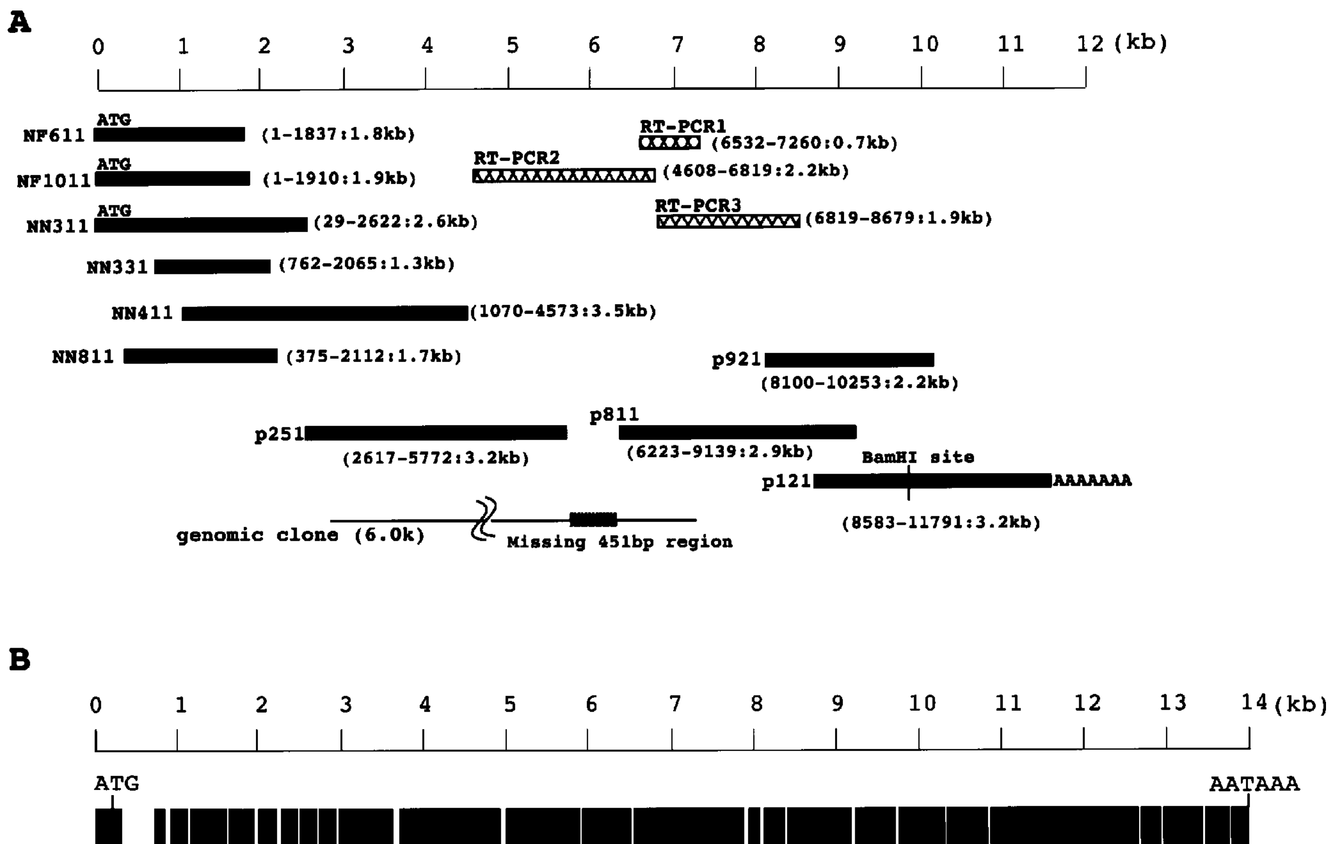

Figure 2 cDNA cloning strategy of *d*-Hml and exon/intron organization of *d-hml*

(**A**) Positions of RT-PCR fragments obtained with redundant primer sets designed based on partial peptide sequences, screened cDNA clones and the sequence determined from a genomic clone are summarized. (**B**) Exon/intron organization of *d-hml*.

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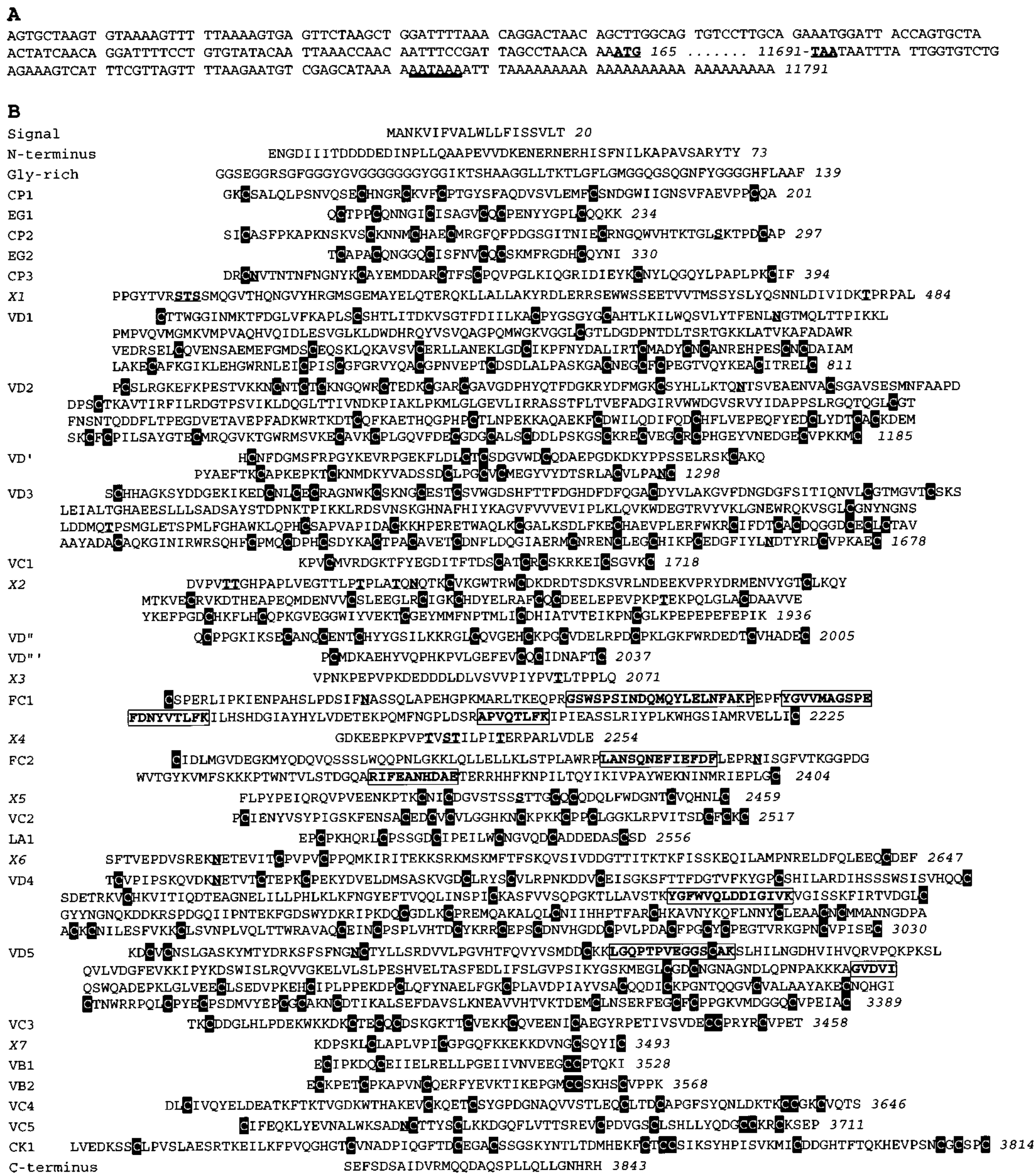

Figure 3 Sequence of *d*-Hml

(**A**) The 5′ and 3′ ends of the cDNA sequence are shown. The translation-initiation and termination codons are underlined. The polyadenylation signal is double underlined. (**B**) The cDNA-derived amino acid sequence is arranged in segments corresponding to the domains given on the left. X, no similarity to currently known motifs; other abbreviations for domains are given in the text. The same domains are distinguished by numbering from the N- to C-terminus. All cysteine residues are printed as white on black. Asparagine and serine/threonine residues that are predicted as putative acceptor sites for N- and O-linked glycosylation, respectively, are underlined and printed in bold. The partial peptide sequences determined directly from the ≈ 300 kDa band are shown in boxes. Residue numbers are given on the right.

by RT-PCR of Kc167 total RNA and PCR of the genomic clone. The overlapping regions of some cDNA clones showed the sites of sequence polymorphism, all resulting in synonymous codon change or the in-frame replacement of amino acids. For such sites, we obtained at least five independent cDNA clones after RT-PCR amplification of RNA extracted from the larvae to determine the most frequent sequence. An open reading frame encoding 3843 amino acid residues with a predicted molecular mass of 426 kDa and an isoelectric point of 5.65 started at nt 163, which followed an upstream sequence containing nonsense codons in all reading frames. A termination codon (TAA) was found at nt 11692 followed by 97 bp of 3′ non-coding sequence, including a polyadenylation signal at nt 11754. Recently, the Berkeley *Drosophila* genome projects sequenced around the *d-hml* region (Gadfly number CG7002) and comparison with the cDNA sequence (DDBJ/EMBL/GenBank accession number AB035891) revealed that it consists of 26 exons, as depicted in Figure 2(B). It was discovered to have a compact exon/intron structure covering 14 kb.

Mapping of *d-hml* on polytene chromosomes

In situ hybridization of polytene chromosomes with a *d-hml* cDNA fragment gave a single signal localized to the left arm of

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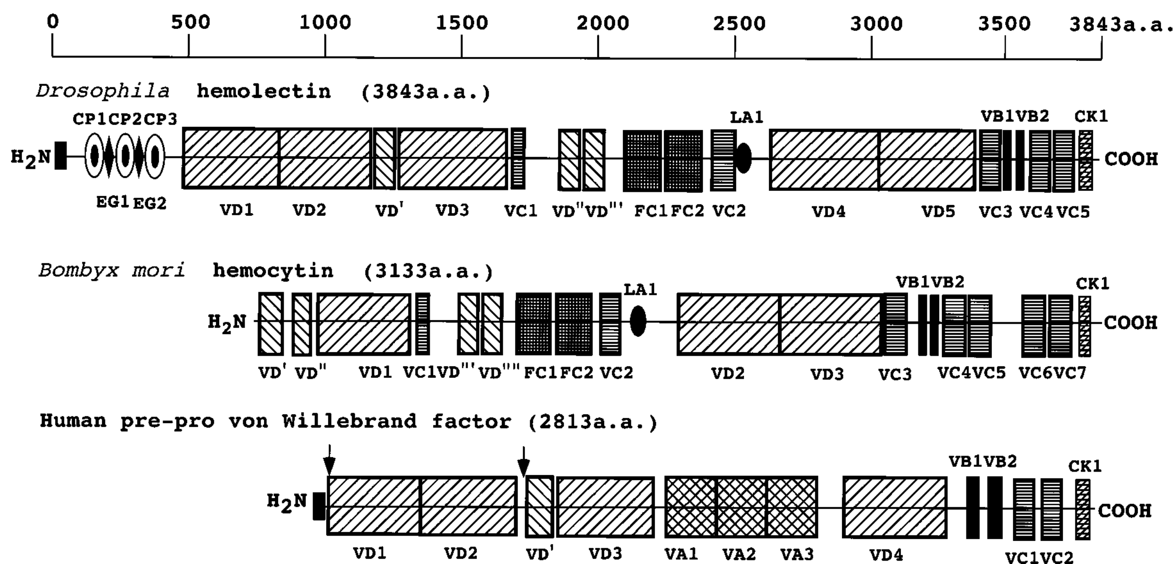

Figure 4 Domain organization of *d*-Hml compared with *b*-Hmc and human pre-pro-VWF

Black boxes at the N-terminus indicate putative signal sequences. Domain names are as summarized in the text. The arrows indicate the processing sites of pre-pro-VWF resulting in the signal sequence, the 741-residue VWF antigen II and mature VWF.

the third chromosome at position 70C1-5 (results not shown). This localization was identical with that of P1 phage genomic clones (DS03618, DS06405 and DS06476), which contain partial sequence of *d-hml* cDNA.

Domain organization of *d*-Hml

The cDNA-derived amino acid sequence of 3843 residues is arranged in Figure 3 in segments corresponding to the motifs discussed below. The predicted protein starts with a stretch of ≈ 20 amino acids rich in basic and hydrophobic residues. The programs SignalP [40] and PSORT II [41] predicted this region to be a signal peptide with ^{21}E as the first residue of the mature protein. The major part of the protein sequence consists of the motifs found in VWF and proteins of the blood coagulation system. In the nomenclature suggested by Bork et al. [42], the sequence can be depicted as: CP1-EG1-CP2-EG2-CP3-VD1-VD2-VD′-VD3-VC1-VD″-VD‴-FC1-FC2-VC2-LA1-VD4-VD5-VC3-VB1-VB2-VC4-VC5-CK1 (EG, epidermal-growth-factor-like domain; VB, VC and VD, VWF type B-, C- and D-like domains; VD′, VD″ and VD‴, truncated C-terminal VDs; FC, coagulation factor V/VIII type C domain; LA, low-density-lipoprotein receptor class A domain). Some of exon boundaries of *d-hml* coincided with the domain boundaries but no general correlation was found between the exon/intron organization of *d-hml* (Figure 2B) and the domain organization of *d*-Hml (Figure 3).

When the domain organization of *d*-Hml was compared with *b*-Hmc and human VWF (Figure 4), the C-terminal 75 % of *d*-Hml was similar to *b*-Hmc except that the repeated VC6-VC7 domains were truncated. An additional similarity that we discovered between *d*-Hml and *b*-Hmc was the motif resembling the LA domain (^{2518}E–^{2556}D and ^{1383}E–^{1420}T, respectively), which is a cysteine-rich module binding to many lipoproteins [43,44]. The *d*-Hml sequence had an extended N-terminal region that is absent in *b*-Hmc. These contained the VD1-VD2 domain to form the domain organization of VD1-VD2-VD′-VD3, which is essential for VWF to be packed into Weibel–Palade bodies, to be cleaved by processing enzyme PACE (furin), to bind to coagulation factor VIII and to form head-to-head disulphide linkages. It was intriguing that the cleavage sites of the ≈ 400 kDa form under acidic conditions fell between D2 and D′, based on the size of N-terminal ≈ 100 kDa fragments. The N-terminal region of *d*-Hml has mutual repeats of three Sushi domains (also known as SCR, small consensus repeat, and CCP, complement-control protein domain) [45,46] and two epidermal-growth-factor-like domains [47], both of which are contained in complement factors and other proteins involved in haemostasis [48].

Sequence comparison of the domains in *d*-Hml

Figure 5 compares the sequences of various domains repeated in *d*-Hml with each other, with the corresponding sequence of *b*-Hmc and human VWF, and with those selected proteins that were established to have characteristics of that motif. This comparison allowed us to assign most cysteines to intrachain disulphide linkages.

Two EG domains (Figure 5A) repeated in the N-terminal region belong to the non-calcium-binding EG domain. They are rather compact in the sequence between Cys-1 to Cys-4 and the first Cys follows the last Cys of the upstream CP domain, which is only three residues away. Out of 3016 Pfam entries, human transforming growth factor β1-binding protein (hTGFβ1) has the most similar EG domain, and the first EG module of human factor IX (hFACTOR IX in Figure 5A) is the most similar motif for which a high-resolution structure is known. As underlined in Figure 5(A), the EG2 domain contains the sequence RGD between Cys-5 and Cys-6. Such putative cell-binding sites are also found in a similar position in nine other EG modules [48]. In the case of *d*-Hml, the shorter distance between Cys-5 and Cys-6 might restrict the mobility of this RGD and interfere with cell binding.

Three CP domains contain the usually observed four cysteine residues and one aromatic residue (most frequently tryptophan),

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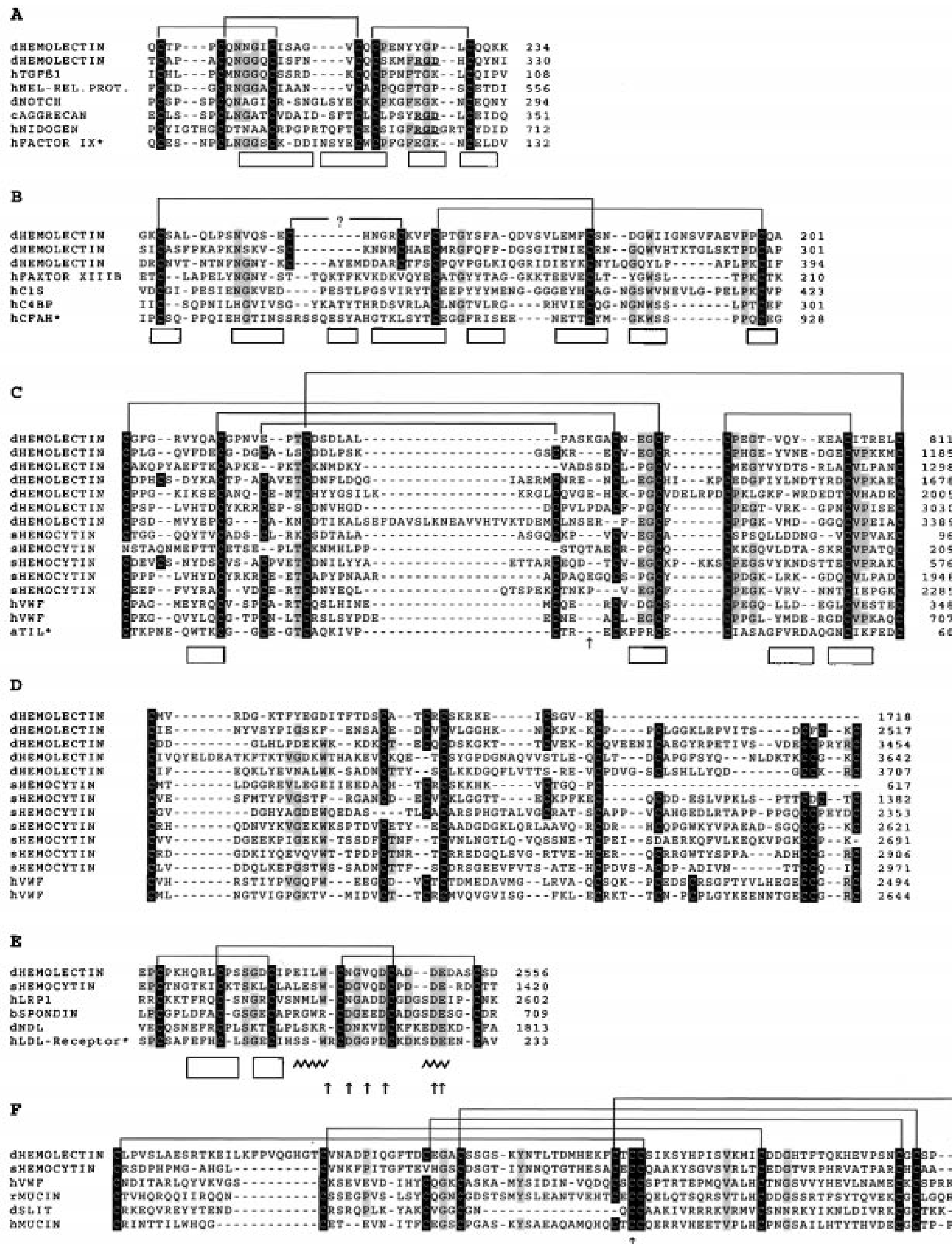

Figure 5 Sequence comparison of selected domains in *d*-Hml

Selected sequence regions of *d*-Hml (see Figures 3 and 4) are aligned with each other, to the corresponding parts of *b*-Hmc, and to some proteins that are established to show the characteristics of this type of sequence motif. Cysteine residues are printed as white on black. Other conserved residues are highlighted with a grey background. The disulphide-linkage pattern established for one or more members of the motif family is schematically shown by lines above the sequences. Proteins or motifs for which a high-resolution structure is known are marked with an asterisk, and their secondary structure is depicted below the sequence (open box, β-strand; zig-zag line, 3-10 helix). Residue numbers are shown on the right. Species origin of the different proteins is denoted by the initial lower-case letter (b, bovine; c, dog; d, *Drosophila*; h, human; s, silkworm; r, rat). (**A**) EG1 and EG2 show highest similarity with transforming growth factor β1 (TGFβ1). RGD sites are printed in bold. (**B**) CP1–CP3 contain two cysteine residues that are absent in most related CP domains and might form a separate disulphide-stabilized loop. (**C**) The C-terminal region of VD1–VD5 and VD′/VD′′ are aligned with the trypsin inhibitor-like protein of *Ascaris suum* (aTIL), of which disulphide-linkage pattern is shown. The arrow denotes the scissile peptide bond of aTIL. (**D**) VC1–VC5 are mainly characterized by a number of conserved cysteine residues and VC1 lacks the C-terminal region. (**E**) LA1 is highly similar to other LA domains. The arrows denote residues which form an octahedral Ca^{2+}-co-ordination centre in the low-density-lipoprotein (LDL) receptor. (**F**) CK1 contains an odd number of cysteine residues. Recent determination of the cysteine residues for interchain disulphide connections in human VWF suggests Cys^{3778} (arrow) of *d*-Hml to be involved in interchain disulphide formation.

but have an additional pair of cysteines following Cys-1 (Figure 5B). A total of six Cys-containing CP domains were also found in P-, E- and L-selectins, although the additional ones are at different positions. Among the CP domains for which a high-resolution structure is known, one in human complement factor H (hCFAH in Figure 5B) is most similar to those of *d*-Hml. This consists of several anti-parallel β-sheet-forming strands and is stabilized by two disulphide bonds, Cys-1–Cys-5 and Cys-4–Cys-

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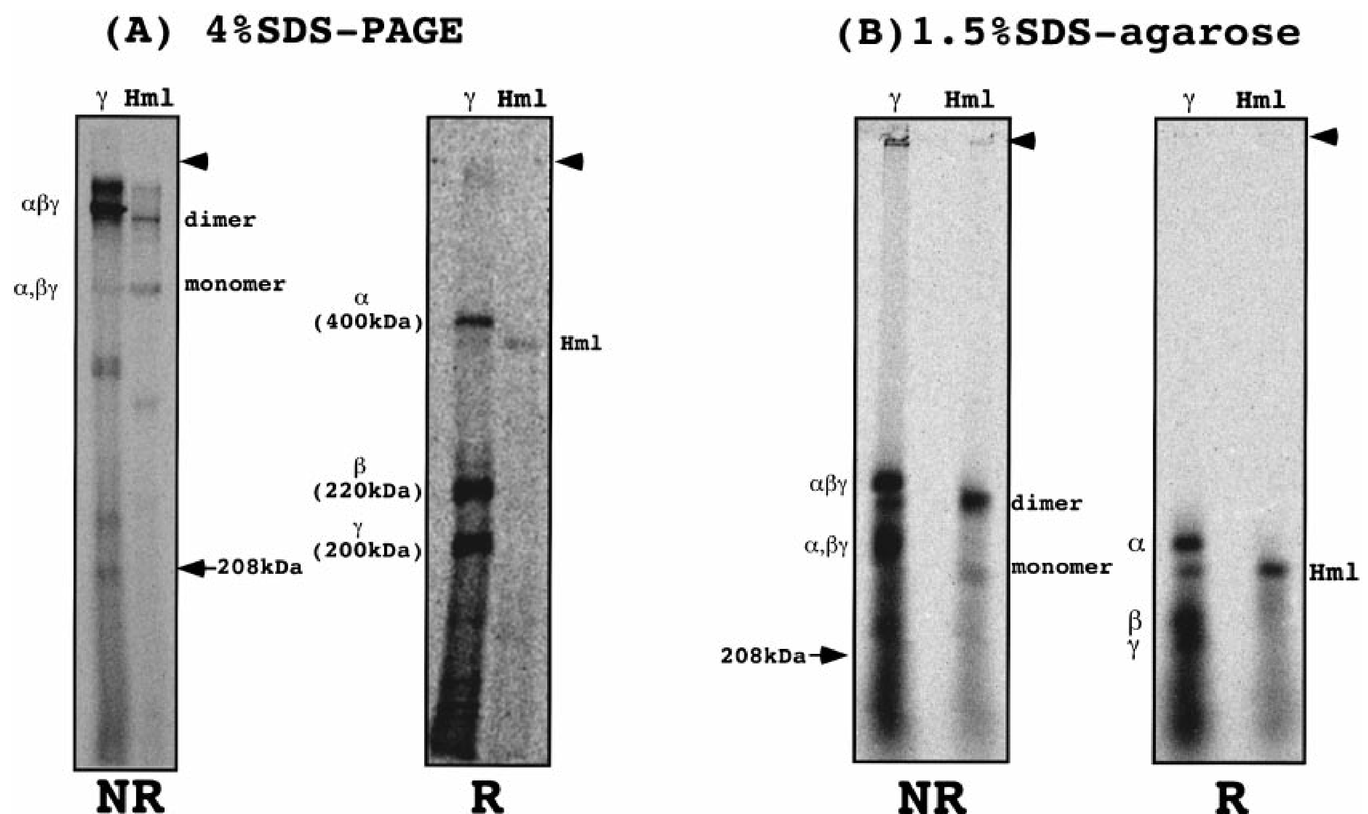

Figure 6 Disulphide linkages of *d*-Hml secreted from Kc167 cells

The conditioned medium from metabolically labelled Kc167 cells with [^{35}S]methionine/[^{35}S]cysteine was immunoprecipitated with polyclonal antibody against laminin γ chain (γ) or monoclonal antibody against *d*-Hml, the precipitates were separated by SDS/PAGE (4% gel; **A**) or SDS/agarose-gel electrophoresis (1.5% gel; **B**) under non-reducing (NR) or reducing (R) conditions, and the fluorogram was taken. Arrowheads indicate the origins of the gels. Migration of endogenous laminin α (400 kDa), β (220 kDa) and γ (200 kDa) chains served as size makers.

6 (Figure 5B). The CP domains of *d*-Hml lack the residues corresponding to the third β-strand. A possible loss of stability might be compensated by an additional disulphide linkage of Cys-2–Cys-4, which could connect the end of strand 2 to the middle of strand 4.

As in *b*-Hmc and human VWF, the C-terminal regions of VD1–VD5 and VD′/VD″ in *d*-Hml contain a 10-cysteine pattern resembling that of the *Ascaris suum* trypsin inhibitor for which a high-resolution structure has been determined [49]. Figure 5(C) aligns disulphide linkages of Cys-1–Cys-7, Cys-2–Cys-6, Cys-3–Cys-5, Cys-4–Cys-10 and Cys-8–Cys-9 of these regions. These disulphide linkage patterns are consistent with that reported for the VD2 domain of VWF with modest differences in the length between Cys-2 and Cys-3 [8]. The VC1 domain of both *d*-Hml and *b*-Hmc lack the C-terminal region. VC4 and VC5 contain only eight instead of the more frequently observed ten cysteine residues (Figure 5D). Both *d*-Hml and *b*-Hmc contain an LA domain (Figure 5E), although this has not been noticed earlier [25]. Comparison with the LA module in human low-density-lipoprotein receptor, of which structure has been solved by X-ray and NMR analyses, suggests two β-strands and two short helices (Figure 5E). Notably, the residues forming an octahedral Ca^{2+}-co-ordination centre in the human low-density-lipoprotein receptor are conserved. CK1 contains an odd number of cysteine residues (Figure 5F). The interchain disulphide connection of human VWF at CK has been determined recently [50]. The result suggests that Cys^{3778} of *d*-Hml is involved in interchain disulphide-bond formation.

Interchain disulphide linkages of *d*-Hml

To examine the multimerization of *d*-Hml by interchain disulphide linkages, Kc167 cells were metabolically labelled with [^{35}S]methionine/[^{35}S]cysteine and the conditioned medium was immunoprecipitated with *d*-Hml-specific monoclonal antibody. Laminin α, β and γ were also precipitated with γ-chain-specific polyclonal antibody as size markers. When the precipitates were separated by SDS/PAGE (4% gel) under non-reducing conditions, the dimeric form was detected as a major band migrating faster than the laminin $\alpha\beta\gamma$ trimer ($\approx$ 800 kDa), and the monomeric form was detected as a minor band co-migrating with the laminin α chain ($\approx$ 400 kDa) and disulphide-linked $\beta\gamma$ dimer ($\approx$ 400 kDa; Figure 6A, left-hand panel). Under reducing conditions, one band migrating faster than the laminin α chain was detected (Figure 6A, right-hand panel). Since the weak smeared band observed close to the origin of the SDS/PAGE under non-reducing conditions (Figure 6A, left-hand panel) could suggest further multimerization of *d*-Hml, we separated the same samples by SDS/agarose-gel electrophoresis (1.5% gel). However, we detected only dimeric and monomeric froms (Figure 6B).

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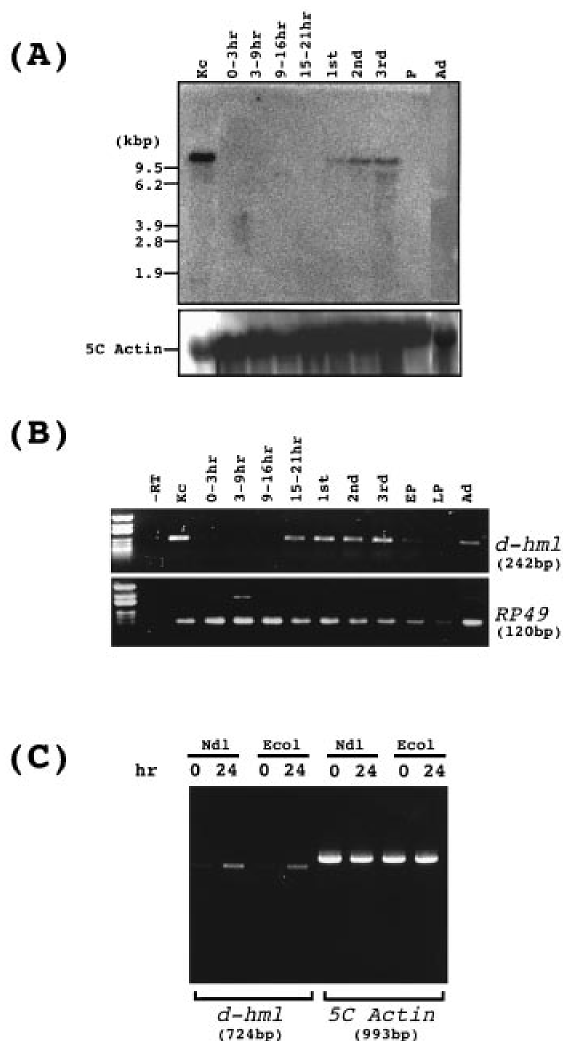

Figure 7 Developmental and injury-induced expression of *d-hml*

(**A**) Total RNA (10 μg) of Kc167 cells (Kc) and poly(A)$^+$ RNA (5 μg) from *Drosophila* at different developmental stages [0–3 h, 3–9 h, 9–16 h and 15–21 h embryos, first-, second- and third-instar larvae, pupae (P), early-stage pupae (EP), late-stage pupae (LP) and adults (Ad)] were hybridized with a radiolabelled 769 bp cDNA probe. The lower panel shows the result of hybridization of the same filter with *Drosophila* 5C actin cDNA. (**B**) Developmental expression of *d-hml* was also investigated by RT-PCR using *d-hml*- and Rp49-specific primers. The ribosomal protein Rp49 was used as an internal control. —RT indicates no reverse-transcription reaction to eliminate the possibility of the contamination of genomic DNA. (**C**) Adult flies were pricked in the posterior region with a fine tungsten needle with (Ecol) or without (Ndl) *E. coli*-dipped suspension. mRNAs were isolated 24 h later, and RT-PCR was performed using *d-hml*- and 5C actin-specific primers.

The majority of the constitutively secreted VWF from human umbilical vein endothelial cells in culture was the dimeric form, but very large and biologically more potent multimers were released from Weibel–Palade bodies when these cells were stimulated with the calcium ionophore A23187, purine nucleotides or thrombin [51,52]. Challenging Kc167 cells with A23187, however, gave essentially the same result as in Figure 6 (results not shown), suggesting that secretion of *d*-Hml from Kc167 is not through the regulatory pathway. Although we cannot exclude the possibility of secretion of multimeric *d*-Hml *in vivo*, our analysis of cultured *Drosophila* cell lines so far has showed that *d*-Hml is secreted mainly in the disulphide-linked dimeric form.

Expression of *d-hml* during *Drosophila* development

Northern blotting of *Drosophila* mRNA prepared from different developmental stages showed a single band of $\approx$ 12 kb for larval stages, the intensity of which gradually increased up to the third instar stage (Figure 7A). A cDNA probe covering nt 6532–7260 was used for this blot, although other probes detected the same band. The more sensitive method of RT-PCR also indicated the presence of the mRNA in late-stage embryos, very-early-stage pupae and adults (Figure 7B). Thus the mRNA expression pattern coincides well with the appearance of phagocytic haemocytes [53].

Suggesting a potential role of *d*-Hml in haemostasis, the low level of RT-PCR product in adult flies was elevated by injuring the flies with a fine needle (Figure 7C). The induction was independent of the site of injury. A similar extent of response was observed even if the needle was dipped in *E. coli* suspension (Figure 7C), suggesting the signalling mechanism to be independent of bacterial infection.

Conclusions

d-Hml identified and characterized in this study has similarity to VWF in the following ways. (i) *d*-Hml shares many domains with VWF. (ii) *d*-Hml conserves the domain organization of D1-D2-D′-D3, which is essential for VWF to be packed into Weibel–Palade bodies, to be processed by furin, to bind to coagulation factor VIII and to form interchain disulphide linkages. (iii) *d*-Hml is sensitive to acidic cleavage at sites near the boundary between VD2 and VD′, where pro-VWF is processed into VWF antigen II and mature VWF. (iv) *d*-Hml is secreted from Kc167 cells mainly in the form of a disulphide-linked dimer in the same way as VWF is constitutively secreted from many cultured cell lines. (v) Expression of *d-hml* in adult flies appears to be enhanced in response to injury at any position on the body. Such similarity of *d*-Hml to VWF suggests a mechanism of haemostasis that is evolutionarily conserved from insects to mammals.

Since *Drosophila* has no platelets, thrombin, fibrinogen or fibrillar collagens, the mechanism of haemostasis can be quite different from that of mammals. Actually, *d*-Hml lacks A-domains, which is critical for VWF to bind to fibrillar collagens and platelet glycoprotein GPIb [12,15]. This suggests that *d*-Hml may exert its activity in a mechanism independent of platelets and fibrillar collagens. Considering the powerful genetic methods available for *Drosophila*, further study of the function of *d*-Hml may reveal a new function for VWF in mammals.

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