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Site-specific cleavage of basement membrane collagen IV during *Drosophila* metamorphosis

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SUMMARY

Breakdown of basement membranes is an important step in the controlled rearrangement of cells during metamorphosis, cell migration, and metastatic spread of tumor cells. One of our two laboratories found a unique collagenous peptide that only appears during metamorphosis of *Drosophila melanogaster*. The other laboratory previously reported that during 20-hydroxyecdysone-induced eversion of *Drosophila* imaginal discs a glycoprotein named gp125 arises (Birr et al., 1990). We show that these two peptides are identical and that they are formed from basement membrane collagen IV. Cleavage occurs at an imperfection of this homotrimeric collagen helix between residues 755/756 in the sequence CALDE/IKMPAK. The peptide is the carboxyl fragment, 100,647 M_r, as derived from the amino acid sequence of the collagen α1(IV) chain. The corresponding amino fragment was also recovered from a disulfide-linked aggregate. This specific cleavage supports the concept of highly targeted, controlled breakdown of basement membranes during metamorphosis. Furthermore, these cuts occur at strategic sites of the predicted supramolecular network of collagen IV molecules of *Drosophila* basement membranes.

Key words: *Drosophila*, collagen IV cleavage, metamorphosis, basement membrane, imaginal discs

INTRODUCTION

The remarkable morphological transformations of metamorphosis require a combination of controlled synthesis of new materials and the precisely targeted removal or modification of existing structures. Recognition of the importance of targeted destruction during metamorphosis of tadpoles led to the discovery of vertebrate collagenases (Gross, 1966). Subsequently, distinct metalloproteinases were found for the removal of fibrous collagens and others that cleave collagen IV, which forms a molecular network component of basement membranes. Secreted collagenases can play an important role in cell migration, and some highly invasive tumor cells migrate through basement membranes with the assistance of collagenase IV (reviewed by Alexander and Werb, 1991 and Liotta et al., 1980). Moreover, it has become increasingly clear that a number of different cell types, including epithelial cells (McGuire and Seeds, 1989), neurons (Pittman et al., 1989), lymphocytes (Simon et al., 1991), and fibroblasts (Hamilton et al., 1991), modify their extracellular environment through the release of proteases, although the endogenous substrates of these enzymes and their role in normal development are largely uncharacterized. Here we report the outcome of two convergent research approaches to the study of changes in extracellular matrix proteins during *Drosophila* development. Our results indicate that collagen IV is specifically cleaved in response to the steroid hormone 20-hydroxyecdysone (ecdysone) at pupariation, suggesting that proteolytic modification of the extracellular matrix plays a role in the cellular events of metamorphosis.

The external structures of the adult fly originate in larvae, from folded epithelial sacs called imaginal discs, each enclosed by a basement membrane. *Drosophila* basement membranes contain laminin, collagen IV, glutactin, the proteoglycan papilin, and several other glycoproteins (Fessler and Fessler, 1989). The morphological changes that imaginal discs undergo during metamorphosis appear to be accompanied by the modification of their enclosing basement membranes, for example, as evidenced by the detachment of cells from the basal matrix during disc morphogenesis (Brower et al., 1987; Fristrom and Fristrom, 1993).

Birr et al. (1990) reported that an ecdysone-dependent proteolysis of a surface glycoprotein may play a role in imaginal disc morphogenesis in *Drosophila*. They found that a glycoprotein, designated gp125 from its apparent

125×10^3 M_r in SDS-PAGE, appeared in imaginal discs under the influence of ecdysone, though concurrently supplied radioactive amino acids were not incorporated into it. As an antigenically similar protein of the same size was produced by trypsin treatment of discs in a hormone-independent manner, they suggested that gp125 is a cleavage product of a pre-existing glycoprotein and that a corresponding ecdysone-dependent proteolytic process was necessary for disc morphogenesis. Antibodies, prepared against partially purified gp125, stained both the apical and basal aspects of imaginal disc epithelia, and gp125 was assigned mostly to an apical site. However, our current studies indicate that the gp125 antigen has a basal site, colocating with collagen IV in imaginal disc basement membranes.

Drosophila basement membrane collagen IV is synthesized predominantly by hemocytes and fat bodies and accumulates during embryonic and larval stages, but decreases with the onset of metamorphosis (Fessler and Fessler, 1989 and unpublished observations). Here we report that, in addition to synthesis of collagen IV for the adult, an apparently new collagen appears only during the prepupal and pupal period. This collagen is smaller than *Drosophila* collagen IV (Blumberg et al., 1988; Lunstrum et al., 1988), and relative to the usual globular protein standards the component chains have an apparent 125×10^3 M_r. We demonstrate the identity of this smaller collagen with the above gp125 protein and by sequence comparison show that gp125 protein is the carboxyl fragment of *Drosophila* collagen IV. As collagen molecules are three stranded, this carboxyl fragment of the collagen IV molecule consists of three chains, each of apparent M_r 125×10^3 (Fig. 1). Correspondingly, the gp125 protein deduced by Birr et al. (1990) is a trimer of the gp125 peptide chains observed in SDS-PAGE.

METHODS

Isolation of proteins

Oregon R *Drosophila* embryos were collected over 2-hour periods and larval cultures were maintained at 25°C on standard media that in some instances were supplemented with 10 mg/ml of β-aminoproprionitrile fumerate (Aldrich Chemical Co.) to reduce the crosslinking of collagen (Murray and Leipzig, 1986). Development was restaged from pupariation. At 25°C, pupation (head emergence) occurs 12 hours after pupariation (AP) and eclosion occurs 96 hours AP. We refer to 0-12 hours AP animals as prepupae. Animals were recovered from the following stages: late 3rd instar (wandering) larvae, very late 3rd instar larvae and 0- to 2-hour prepupae (which do not float on water), 2- to 4-hour prepupae (which float), 10- to 12-hour prepupae, and 2- and 3-day pupae, and pupae close to eclosion. Imaginal discs were isolated from 3rd instar larvae as previously described (Birr et al., 1990). Washed and dechorionated embryos, larvae, pupae and adults were frozen in liquid nitrogen and ground to a fine powder under liquid nitrogen with a mortar and pestle. Equal weights of tissue were homogenized with 10 volumes of 2% SDS, 0.01 M EDTA, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5, plus 0.01 M dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) at 100°C, followed by 5′ heating at 100°C, and sonication. The insoluble residue was removed by sedimentation in a Sorvall SS34 rotor at 8 K for 30 minutes at room temperature. Extracts of imaginal discs were prepared as described (Birr et al., 1990). For quantitative assays, the protein concentration was determined with the BioRad protein assay. Larval and pupal extracts containing equal quantities of protein were electrophoresed on SDS-PAGE and electroblotted onto nitrocellulose in an electroblotter (Hoefer Scientific Instruments) with 20% methanol, 20 mM Tris, 150 mM glycine, 0.02% SDS. The filters were stained with Ponceau Red (Sigma) and the positions of stainable proteins were indicated with ink dots. Western blots were made as follows: the filter was blocked with 4% reconstituted Carnation fat free dried milk in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4 (TBS), washed in TBS plus 0.05% Tween 20 (Fisher Scientific), and 3 mM NaN_3, incubated with primary antibody to collagen IV (Lunstrum et al., 1988) or to gp125 (Birr et al., 1990) overnight at room temperature, washed, incubated with anti-rabbit IgG-alkaline phosphatase (Promega) for 2 hours at room temperature, washed and color developed. Protein molecular mass standards (Gibco BRL) served as size markers and the α1 and α2 chains of type I collagen extracted from chick embryo tendons were good markers for the electrophoretic mobility of gp125. The native proteins from 6 g of 1- to 2-day pupae were prepared as follows. Soluble and membrane proteins were first removed with 30 ml each of hypotonic solutions at 0°C: (1) 0.01 M Tris-HCl, and 0.01 M EDTA, pH 7.4, plus inhibitors: 0.01 M N-ethylmaleimide (NEM), 1 mM PMSF (Sigma), 1 μg/ml each leupeptin, aprotinin (Boehringer Mannheim) and pepstatin A (Sigma); (2) the same buffer plus 1% Triton X-100 was used for two extractions. The precipitates were sedimented in a Ti60 Beckman rotor at 40K for 30 minutes at 4°C. The precipitate was then extracted at 0°C for 18 hours with 1 M NaCl, 0.03 M Tris-HCl, 0.01 M EDTA, pH 7.4, plus protease inhibitors, and centrifuged. The clear extract was dialyzed at 0°C against 0.15 M NaCl, 0.03 M Tris-HCl, 0.01 M EDTA, pH 7.4, plus PMSF and NEM, and the small precipitate that formed was removed by sedimentation. Then the proteins were flocculated during dialysis against 0.03 M Tris-HCl, and 0.01 M EDTA, pH 7.4. This precipitate was sedimented in an SS34 Sorvall rotor at 8 K for 20 minutes, and gently redissolved in 1 M NaCl buffer plus inhibitors. Dialysis against 0.2 M NaCl buffer with EDTA retained the proteins in solution. An aliquot was removed and dialyzed against 0.2 M NaCl, 0.03 M Tris-HCl, pH 7.4, and this was used for enzymatic treatment. The proteins in the main sample were precipitated as above, and the precipitate was dissolved gently in 1 ml 2% SDS, 0.05 M Tris-HCl, 0.01 M EDTA, pH 7.4, and heated at 100°C for 5 minutes. LiCl was added to the clarified sample to 1.0 M and the proteins were separated on a Superose 12 molecular sieve column using the FPLC system of Pharmacia LKB Technologies. 1 ml fractions were collected and aliquots were analyzed on SDS-PAGE either without or with reduction with dithiothreitol. Western blots were developed as above.

The fractions containing the peak of gp125 peptide (fractions 6 and 7, Fig. 6) were concentrated by centrifugation in a Centricon 30 tube (Amicon) and electrophoresed on 5% SDS-PAGE and electrotransferred to a BioRad PVDF membrane. The H_2O washed blot was stained with 0.1% Coomassie blue in 50% methanol and destained in methanol. The amino acid composition (Gharahdaghi et al., 1992) and N-terminal sequence (Hewick et al., 1981) were analyzed on nonreduced gp125 peptide.

The proteins soluble in 0.2 M NaCl buffer plus 0.01 M $CaCl_2$ were incubated at 37°C for 3 hours either without or with purified bacterial collagenase (Worthington CLSPA) which had been further purified by gel filtration, or the sample was acidified by addition of acetic acid to 0.1 M and incubated with 200 μg/ml pepsin (Millipore Corp.) at 4°C overnight. Western blots were developed as above.

Drosophila collagen IV

Kc cell culture medium was treated with protease inhibitors, the proteins were precipitated with 45% saturation $(NH_4)_2SO_4$, the

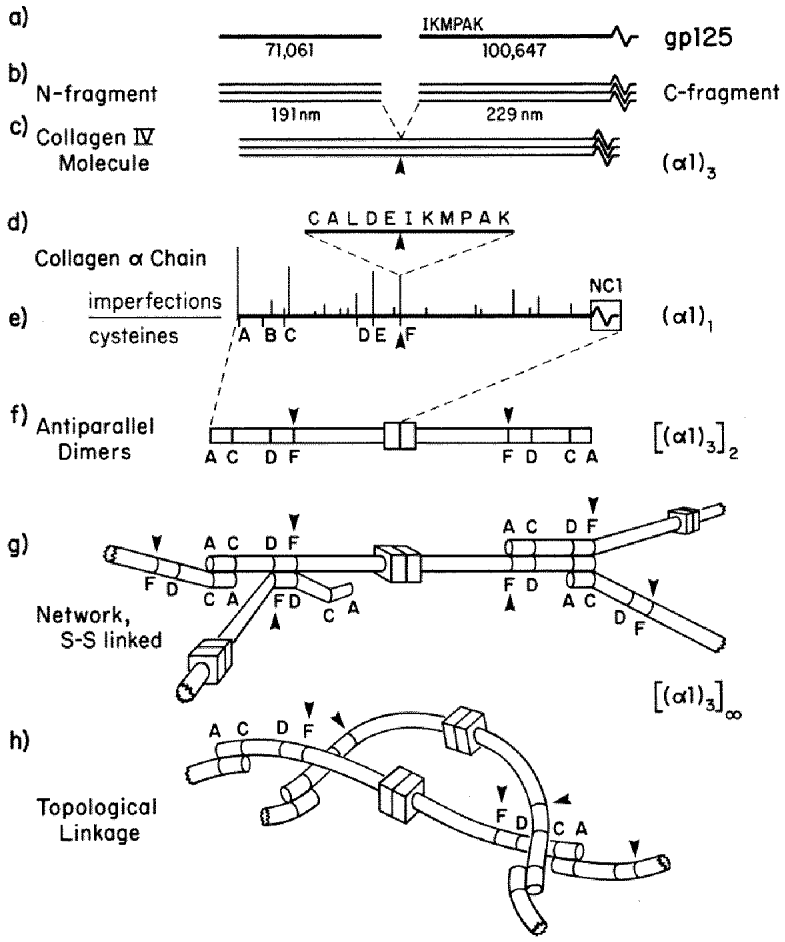

Fig. 1. Relationship of the gp125 peptide to postulated hierarchy of collagen IV in *Drosophila* basement membranes. (a) Shows the previously described gp125 protein to be the M_r 100,647 C-fragment of a *Drosophila* collagen α1(IV) chain, and the complementary N-fragment is also indicated. (b) Shows the triple-chained N- and C-fragments of a *Drosophila* collagen IV, with the length of collagen helix that is calculated to be associated with each fragment. (c) A collagen IV molecule is diagrammed; the (NC1) carboxyl portion of each chain is folded differently from a collagen helix and is indicated by an undulation. Throughout the figure an arrowhead indicates the cleavage site. (d) Amino acid sequence of the imperfection of collagen helix that is cleaved. (e) Compound diagram of one *Drosophila* α1(IV) chain. The thick horizontal line represents the amino acid sequence of the collagen thread, followed by the carboxyl terminal NC1 portion. Vertical lines above the horizontal line indicate the location and relative size of imperfections of collagen helix sequence. Tick marks below the horizontal line and letters A-F indicate locations of cys residues (there are 3 cys residues at location A and 2 cys residues at location D). (f) A pair of antiparallel, triple-chained collagen molecules that are joined through their NC1 domains, diagrammed as boxes. Each of the two potential junctional domains delineated by cys residues A-C and D-F is the equivalent of two back-to-back '7S' domains of vertebrate collagen IV which are linked by intermolecular disulfide bridges. (g) Some of the possible connections between junctional domains of antiparallel dimeric collagen molecules are indicated as part of a network. Junctional domains A-C, D-F, are consolidated by intermolecular disulfide links which could form between any of the apposed cys residues as considered in Blumberg et al., 1988. Cleavage at arrowheads produces clusters of disulfide-linked N-fragments and dimers of C-fragments of collagen IV molecules. (h) Topological linkage is indicated between two portions of separate local networks of collagen IV molecules. Cleavage at arrowheads disrupts this topological linkage. (Supramolecular assemblies are based on Blumberg et al., 1988.)

dissolved proteins were partially separated by velocity sedimentation on a sucrose gradient, and the collagen IV fractions were purified by ion exchange chromatography on a Mono Q column using 0.3 M sucrose, 0.03 M Tris-HCl, pH 8, 0.01 M EDTA, 0.05% Triton X-100, 0.01 M NEM, and 0.1 mM PMSF, and a 0-0.5 M NaCl gradient. Final purification was on a Superose 12 molecular sieve column using 0.3 M sucrose, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0.01 M EDTA, and 0.05% Triton X-100, 0.01 M NEM, and 0.1 mM PMSF in the FPLC system.

Antibodies

Polyclonal antiserum to pure, nonreduced *Drosophila* collagen IV was prepared in rabbits as described, and this recognizes the C-fragment and N-fragment as well as the fragments produced by prolonged pepsin digestion (Lunstrum et al., 1988). Neither the epitopes, nor relative strength of binding of the antibodies to these fragments is known. The antibodies immunostain collagen IV at both intracellular and extracellular locations in embryo whole mounts. The antiserum to gp125 peptide, which had been isolated by HPLC chromatography and SDS-PAGE, was prepared in rabbits as described (Birr et al., 1990). Affinity purification of antibodies was carried out on pure *Drosophila* collagen IV bound to nitrocellulose, elution with 0.1 M glycine buffer, pH 2.3, immediate neutralization and dialysis against PBS.

Immunostaining

Whole-mount embryos were immunostained with either anticollagen IV or anti-gp125 primary antibodies and biotinylated anti-rabbit IgG, avidin-biotinylated peroxidase ABC Vectastain (Vector Labs) as described (Olson et al., 1990). Sections of imaginal discs were immunostained with anti-gp125 serum or affinity purified IgG as described (Patel et al., 1989).

RESULTS

The amount of collagen present was determined throughout development. Collagen IV occurred in minute amounts in early embryos, started to increase in 10-hour embryos, and continued to accumulate until pupariation. The level of collagen IV then decreased, although more collagen IV is made during pupal development and in the adult. In western blots of reduced, SDS extracts prepared at pupariation and during prepupal and pupal development (Fig. 2), antibodies to collagen IV recognize a new prominent antigen with an apparent M_r 125×10^3 and some minor proteins*. These antigens were not detected in extracts of embryos,

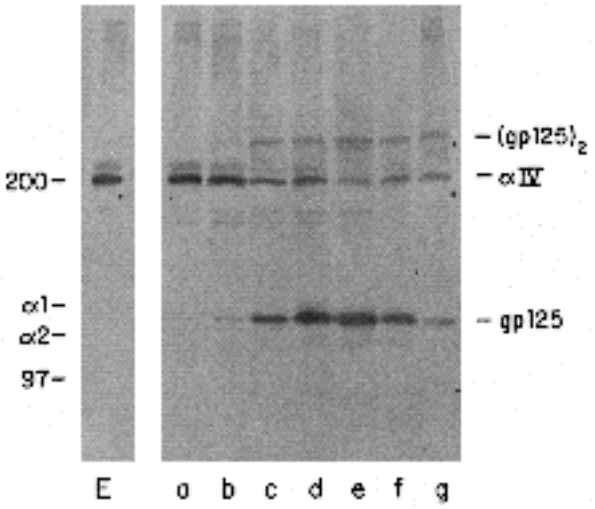

Fig. 2. Antibodies to Collagen IV demonstrate formation of gp125 in extracts of prepupae and pupae. Reduced SDS extracts of the following developmental stages were electrophoresed (5% SDS-PAGE), transferred to nitrocellulose and stained with antibodies against purified *Drosophila* Collagen IV (Lunstrum et al., 1988): Embryos (E), 3rd instar wandering larvae (a), very late 3rd instar larvae and 0- to 2-hour prepupae (b), 2- to 4-hour prepupae (c), 10- to 12-hour prepupae (d), 2-day pupae (e), 3-day pupae (f), pupae near eclosion (g). The protein concentrations of all samples were equal, except for the embryo sample which was three-fold greater. Standard relative molecular masses ($\times 10^{-3}$) and collagen α(I) chain positions are indicated.

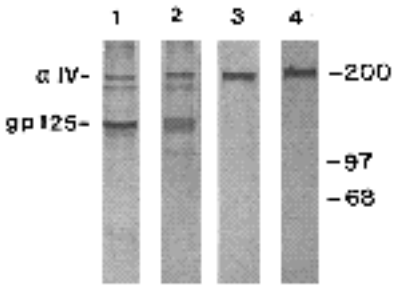

Fig. 3. Comparison of western blots developed with antisera to collagen IV and gp125. An SDS extract of imaginal discs (lanes 1 and 2) and collagen IV purified from KC cell culture (lanes 3 and 4) were electrophoresed and the western blots were developed with anti-gp125 antiserum (lanes 1 and 3) and anticollagen IV antiserum (lanes 2 and 4). Standard relative molecular masses ($\times 10^{-3}$) are indicated.

1st, 2nd, and 3rd instar larvae, or adults. The developmental acquisition of this 125×10^3 M_r antigen could result either from cleavage of collagen IV, from de novo synthesis of a new collagen gene product, or from de novo synthesis of a crossreacting antigen.

The dramatic increase of a glycoprotein, gp125, in imaginal discs cultured in vitro with the steroid hormone ecdysone has been described by Birr et al. (1990). This protein was not synthesized de novo but was derived from an existing macromolecule, gp200, apparently by the action of hormonally controlled proteolysis. An antibody to gp125 recognized reduced proteins of approximately 200, 180, and 125×10^3 M_r (Birr et al., 1990). Our data suggest that gp200 is the α-chain of collagen IV and that gp125 and the 125×10^3 M_r collagenous protein described above are identical. Antibodies to gp125 and to collagen IV both recognize reduced collagen IV in western blots (Fig. 3, lanes 3 and 4). These two antibodies recognize a similar pattern of antigens in western blots of reduced proteins from ecdysone-treated imaginal discs (Fig. 3, lanes 1 and 2). Slight differences in lanes 1 and 2 may be due to recognition of different spectra of epitopes with these two antisera. These initial findings indicated that the antibody to gp125 recognized a portion of the collagen IV molecule. Moreover, gp200 from discs has an apparent molecular mass identical to that of the α-chain of collagen IV. gp180 is recognized by both antibodies in extracts of imaginal discs, but is a minor protein in SDS extracts of pupae. It is likely that gp180 is a minor degradation product produced in imaginal discs in vivo and/or in vitro (Birr et al., 1990).

The antibody to gp125 was used to screen two expression libraries to isolate 26 independent clones that by the following criteria encode collagen IV. Restriction maps of these clones and cross-hybridization showed that they all share identity. The largest cloned insert was used to identify the chromosomal location at 25C, the site of the collagen IV gene. This gp125 cDNA insert hybridized to a 6.5 kb RNA from imaginal discs and larvae, a size consistent with that of collagen IV mRNA (Blumberg et al., 1988). Furthermore, the tissue distribution in embryos and larvae of gp125 transcripts (data not shown) and collagen IV transcripts (Lunstrum et al., 1988) are equivalent. Finally, 380 nucleotides of the largest gp125 insert were sequenced and found to have a sequence identical to that of collagen IV from nucleotides 2395 to 2775 (Blumberg et al., 1988).

Affinity purified antibodies to gp125 and to collagen IV stained hemocytes, fat bodies and basement membranes in whole mounts of embryos (not shown). The temporal and spatial immunostaining patterns with these two antibodies coincided and were fully consistent with the expression and location of collagen IV established previously (Lunstrum et al., 1988); Mirre et al., 1988; Fessler and Fessler, 1989; Le Parco et al., 1989).

*Due to their high glycine content, collagens have a smaller average mass per amino acid residue than proteins in general. Correspondingly, erroneous molecular masses are predicted from the relative electrophoretic mobilities of collagens in SDS-PAGE if the usual, noncollagenous protein molecular weight markers are used as standards (Butkowski et al., 1982). This occurred with the glycoprotein gp125 described by Birr et al., 1990, which we now show is an approximately 101×10^3 M_r carboxyl fragment of the *Drosophila* collagen α1(IV) chain. Similarly, the amino acid sequence mass of the *Drosophila* collagen α1(IV) chain is about 172×10^3 M_r, not allowing for glycosylation, while globular markers would indicate a 190-210$\times10^3$ M_r from the electrophoretic migration of this collagen chain (see also Table 1 of Lunstrum et al., 1988). This corresponds well with the gp200 which Birr et al., 1990, regarded as the precursor from which gp125 was formed by proteolytic cleavage. For simplicity we retain the terms gp125 and gp200, and the corresponding size scale, except where critical analysis is required and is indicated.

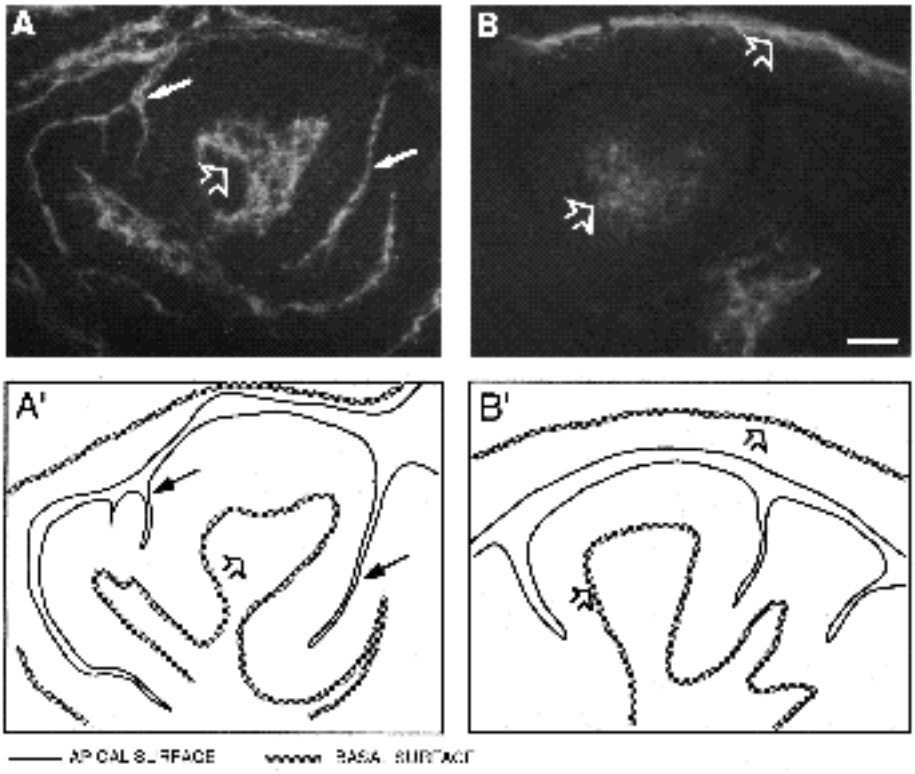

Fig. 4. Basal location of gp125 in imaginal discs. Crude anti-gp125 antiserum was affinity purified on collagen IV. Cryostat median sections of late third instar imaginal leg discs were either stained with crude anti-gp125 (A), or with affinity purified antibodies (B). Although the crude antiserum stained both apical (arrow) and basal (open arrow) regions, only the basal regions (open arrow) reacted with the affinity purified reagent. The diagram outlines the structure of the disc sections. Controls were negative, and as cuticle had not yet formed there was no autofluorescence. Calibration bar, 20 μm.

Immunostaining of sections of imaginal discs from 3rd instar larva with the crude antiserum to gp125 showed both apical and basal staining (Birr et al., 1990). However, following affinity purification of the serum on pure collagen IV, the antibodies recognized exclusively the basal region of the epithelial cell layer (Fig. 4). Thus, the apical staining seen with the crude antiserum was not due to antibodies specific to gp125. The basis of the apical staining, although perhaps due to a contaminant in the partially purified gp125, is unknown. Whether the antigens that are located at the apical side of the epithelia are also increased by the hormone is not known at present.

The above observations suggest that gp125 is a cleavage product derived from collagen IV. To confirm this, we first analyzed the native gp125 protein. The partially purified extract of 1- to 2-day pupae containing native gp125 protein was used to demonstrate that the proteins recognized by the anticollagen IV antibody were sensitive to purified bacterial collagenase and partially resistant to pepsin (Fig. 5). The fact that pepsin yielded a major product of apparent size 100×10^3 M_r on SDS-PAGE was consistent wih the removal of the approx. 26×10^3 M_r pepsin-sensitive carboxyl peptide of collagen α1(IV) (Lunstrum et al., 1988). The appearance of several other bands is consistent with possible cleavage within the imperfections of the collagen Gly-X-Y helix sequence. The highest relative molecular mass band may be derived from a dimer of gp125 peptide, as will be discussed below.

To identify the site of cleavage of collagen IV, we separated the denatured cleavage products on a Superose 12 sizing column and then determined the N-terminal amino acid sequence of the gp125 peptide. Fig. 6 shows a western blot of fractions of either nonreduced or reduced pro-

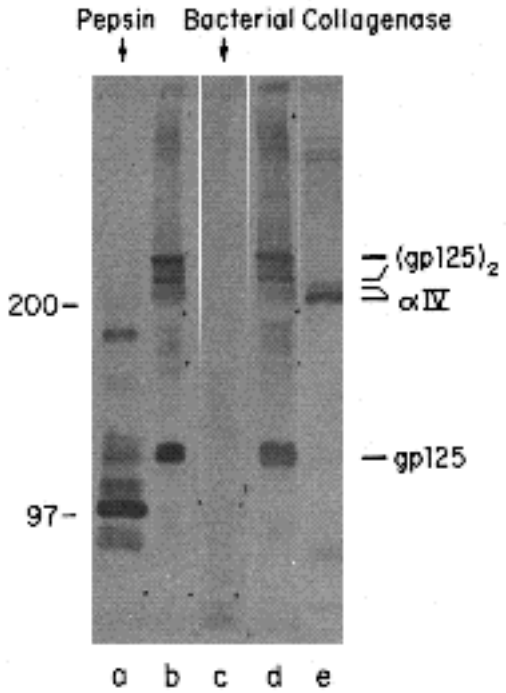

Fig. 5. Differential stability of gp125 to pepsin and bacterial collagenase. Native gp125 in extracts of 1- to 2-day pupae were incubated at 4°C in 0.1 M acetic acid with (a) or without (b) pepsin, and at 37°C with buffer at pH 7.4 in the presence of 0.01 M $CaCl_2$ with (c) or without (d) purified bacterial collagenase. *Drosophila* collagen IV (e) and molecular mass markers were coelectrophoresed and a western blot was developed with antibodies to collagen IV. Standard relative molecular masses ($\times10^{-3}$) are indicated.

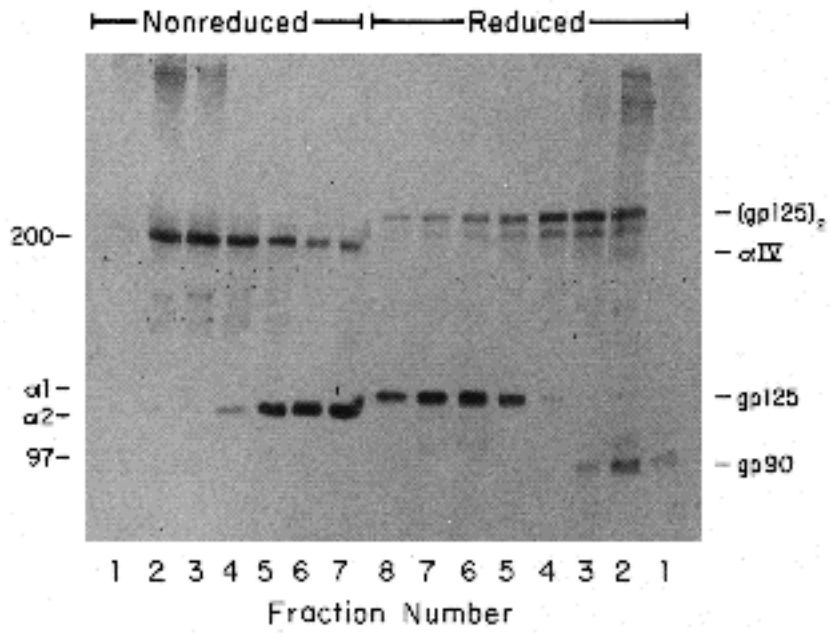

Fig. 6. Separation of gp125 monomers and dimers and disulfide linked aggregates of the N-fragment on a Superose 12 molecular sieve column. The partially purified denatured collagens were separated on a Superose 12 column with a buffer of 2% SDS, 1 M LiCl, 0.03 M Tris-HCl, and 0.01 M EDTA, pH 7.5. Aliquots of nonreduced or reduced fractions were electrophoresed and a western blot was developed with antibodies to collagen IV. Molecular mass standards ($\times 10^{-3}$) and position of the collagen α(I) chains are indicated.

teins separated on a Superose 12 column and immunostained with the antibody to collagen IV. The nonreduced proteins in fraction 2 near the void volume of the column consisted of a single, sharp band in the range of 200×10^3 M_r and of a small amount of a series of very high relative molecular mass bands, which probably transferred poorly during the electroblotting. The single sharp band in the range of 200×10^3 M_r peaked in fractions 2 and 3 and then decreased in quantity. Fractions 6 and 7 contained the peak of gp125 peptide. The electrophoretic mobility of the gp125 peptide decreased slightly on reduction, indicating the opening of intramolecular disulfide bridges. The single larger 200×10^3 M_r band, upon reduction yielded two bands, again with slightly decreased electrophoretic mobilities (apparent size of 215 and 235×10^3 M_r). We postulate that these are dimeric forms of gp125 peptide joined by nonreducible crosslinks in different locations. In addition, after reduction, a single band of approx. 90×10^3 M_r is seen in fraction 2. This might be the amino fragment of collagen IV, which would be expected to exist as a disulfide linked high molecular mass complex derived from association of several triple stranded collagen IV molecules (Fig. 1). Only a small quantity of the amino fragment and of intact collagen IV were extracted with the 1 M NaCl buffer without reducing agents.

N-terminal amino acid sequence of gp125: I K M A P(K)G N – E(P)–Q T....

Amino acid sequence derived from cDNA: G S P A L D E I K M P A K G N K G P E G Q T....

Fig. 7. Determination of collagen IV cleavage site. The N-terminal amino acid sequence was determined by analysis of gp125 peptide purified from pupae. The clearly identified amino acids are indicated. The amino acid sequence derived from the cDNA coding for collagen IV (Blumberg et al., 1988) shows the sequence for the imperfection of the collagen helix (underlined) and the adjacent collagen helical sequences. Parentheses indicate some uncertainty in assignment of amino acid residues. Dash means that no assignment could be made for that residue.

The peak fractions containing gp125 peptide were concentrated, and electrophoresed, either nonreduced or after reduction with dithiothreitol, electroblotted and stained with Coomassie blue. A single stainable band with the expected changing mobilities of nonreduced and reduced gp125 peptide was seen in this size range. N-terminal sequence analysis gave the sequence shown in Fig. 7. The unidentified ninth residue of this sequence could be a post-translationally modified Lys residue, such as hydroxylysine or one of the oxidized forms of Lys. The deduced N-terminal amino acid sequence aligns uniquely with the published sequence of collagen α1(IV), derived from cDNA. The cleavage of collagen IV therefore occurred within a large imperfection of the collagen helix, between amino acid 755 and 756 (Fig. 1). The predicted size of this carboxyl fragment of collagen α1(IV) is 100,647 M_r and of the amino fragment is 71,061 M_r. Since collagen α1(IV) is glycosylated and is predominently a collagenous protein which has aberrant electrophoretic mobility (see footnote on p. 1064), the fragments which were observed with electrophoretic mobilities of 125×10^3 M_r and 90×10^3 M_r are most likely produced by a single cleavage of collagen IV, with each fragment containing intact C- and N-terminal noncollagenous peptides.

Table 1 gives the amino acid composition of gp125 peptide and, for comparison, the amino acid composition calculated from the known sequence of residues 756 to 1775 of the carboxyl fragment of collagen α1(IV). The agreement is good, though not perfect, and is consistent with gp125 being a carboxyl fragment of collagen IV. This protein is highly hydroxylated: 44% of the proline residues and 64% of the lysine residues are hydroxylated (data of Table 1).

Table 1. The animo acid composition of peptide gp125 corresponds to the calculated composition of the C-fragment of collagen IV

	Number of residues from cDNA sequence of collagen IV, 756-1775		Amino acid composition of gp125 peptide: Number of residues per 1019 residues	
Ala	57		63	
Cys	12		–	
Asp	44	77	78	
Asn	33			
Glu	57	100	98	
Gln	43			
Phe	16		22	
Gly	272		238	
His	8		10	
Ileu	37		36	
Lys	48		16	45
OHlys			29	
Leu	61		65	
Met	14		8	
Pro	128		75	133
OH Pro			58	
Arg	54		56	
Ser	38		40	
Thr	35		42	
Val	40		46	
Trp	7		–	
Tyr	16		34	

Off-set numbers are the sums of corresponding pairs of amino acids which cannot be distinguished in the other analysis.

DISCUSSION

We have shown that collagen IV in *Drosophila* prepupae and pupae and imaginal discs is specifically cleaved to generate a carboxyl fragment, which consists of three chains of apparent M_r 125×10^3 (gp125 peptide). Collagen IV is cleaved between residues 755-756 in an eleven amino acid interruption of the collagen Gly-X-Y helix. This cleavage product is not found in embryos, larvae, and adults. In isolated imaginal discs, collagen IV cleavage is dependent on ecdysone. The cleavage product, gp125, appears within two hours of exposure to the hormone (Birr et al., 1990). These results constitute the first demonstration of targeted collagen IV proteolysis during a normal developmental process in vivo. Our data suggests that hormonally regulated modification of the extracellular matrix plays a role in the cellular events of imaginal disc morphogenesis and the remodelling of other tissues that occurs during metamorphosis.

The imaginal discs of *Drosophila* are epithelial sacs. One side of the sac consists of a folded columnar epithelium and the other side a flat, squamous epithelium, the peripodial epithelium. The entire sac is surrounded by a basement membrane containing collagen IV. The initial events in disc morphogenesis, occurring in the first 6 hours after pupariation, result in profound and rapid changes in tissue shape (Fristrom, 1989; Condic et al., 1991). In order for these changes to occur, the basal lamina must be either removed or be extremely extensible. In fact, both these conditions apply. The basal lamina detaches completely from the epithelium in the center of wing and leg discs, regions that will elongate to form the distal parts of the appendages (Fristrom and Fristrom, 1993). This detachment begins before gp125 is detected and is therefore probably independent of site specific cleavage. The separation of the epithelial cells from the basal lamina in these regions appears to be due to the synthesis of new matrix material displacing the original basal lamina (Brower et al., 1987). In contrast, the cells of the peripodial epithelium remain closely associated with the basal lamina during two distinct phases of morphogenesis. First, these cells and their basal laminae become enormously stretched as the appendage-forming regions elongate. It is possible that the cleavage of collagen IV makes the basal lamina more extensible. Then, the cell shape change is reversed. The flat cells contract to become columnar and the area of the peripodial epithelium is greatly reduced (Milner et al., 1984; Fristrom and Fristrom, 1993). During this process, cells apparently ‘slide along’ or migrate on the basal lamina, which remains in its stretched state.

Trypsin treatment accelerates contraction of the peripodial epithelium (Nardi et al., 1987; D. Fristrom, unpublished observation) as well as elongation of appendages (Poodry, 1971; Fekete et al., 1975) in vitro. Ecdysone-dependent serine proteases are produced endogenously in discs (Pino-Heiss and Schubiger, 1989). The elongation and eversion of appendages can be inhibited by exogenous serine protease inhibitors (Pino-Heiss and Schubiger, 1989). These observations indicate that proteolysis is an important aspect of morphogenesis. However, treatment of hormonally naive discs with trypsin does not cause their morophogenesis (Fekete et al., 1975). Digestion of the basal lamina with bacterial collagenase neither causes nor accelerates morphogenesis (D. Fristrom, unpublished observation). We infer from these observations that general matrix proteolysis and destruction of collagen per se are not sufficient for morphogenesis. However, the ecdysone-dependent site-specific cleavage of collagen IV may be a critical aspect of normal disc morphogenesis and tissue remodelling during metamorphosis.

In addition to imaginal discs, the C-fragment we extracted from whole pupae is likely to be derived from other basement membrane-containing structures, e.g. fat body, muscles and larval epidermis, that degenerate at the onset of metamorphosis.

The gp125 protein that we extracted from pupae and discs is a collagen by the criteria of digestibility by highly purified bacterial collagenase, overall resistance to pepsin and an appropriate amino acid composition. The total proline content is characteristically high (130/1000 residues) and 44% of these residues are modified to hydroxyproline, which occurs almost exclusively in collagens. The hydroxylation of two thirds of the lysine residues is also consistent with collagens, particularly with those of basement membranes.

The identification of this new collagen as a fragment of *Drosophila* collagen IV is based on the amino acid sequence identity shown in Fig. 7, and on the agreement of its SDS-PAGE mobility and its amino acid composition with the correspondingly predicted carboxyl fragment of the *Drosophila* α1(IV) chain (Table 1). Furthermore, this sequence is a portion of a unique interruption of the *Drosophila* α1(IV) Gly-X-Y motif, that shows no evident

similarity with helix interruptions of any other published collagen.

The identity of the gp125 glycoprotein of Birr et al., 1990 with this pupal collagen is based on equivalent SDS-PAGE mobility, similar times of appearance in the *Drosophila* life cycle and mutual cross-reactivities of polyclonal antibodies, at both the western blot and the histological levels. This does not exclude recognition of epitopes of other potential *Drosophila* collagens by both anti-gp125 and anti-collagen (IV) antibodies (Natzle et al., 1983; Le Parco et al., 1986). However, it is noteworthy that in an expression screen of two *Drosophila* cDNA libraries the anti-gp125 antibodies recognized 26 clones that all appeared to carry portions of the *Drosophila* α1(IV) cDNA sequence as evidenced by restriction mapping and cross hybridization and sequencing. The chain of logical connections between the new pupal collagen, *Drosophila* collagen IV and gp125 is further strengthened by the previous observation that gp125 is not synthesized de novo but is a proteolytic cleavage product of a gp200 glycoprotein (Birr et al., 1990).

We need to identify the enzyme that generates gp125 from *Drosophila* collagen IV. This enzyme might occur as a zymogen or enzyme-inhibitor complex. As the cleavage site is in a 'noncollagenous' imperfection of the *Drosophila* Gly-X-Y sequence (Blumberg et al., 1988), the enzyme might be a general protease or a homolog of the vertebrate collagenases. Like vertebrate collagenase IV (Fessler et al., 1984), cleavage separates the network of type IV collagen molecules into clusters of N-fragments, disulfide-linked through 7S junctional domains and C-fragments, which are devoid of Cys residues except for those in the NC1, carboxyl domains.

The triple-chained *Drosophila* collagen IV molecules are presumed to be a mixture of homotrimers of the *Drosophila* collagen α1(IV) chain, and of heterotrimers of this chain with a closely related α1′(IV) chain, which may be a post-transcriptional modification of the α1(IV) chain (Lunstrum et al., 1988). The helix imperfection within which the cut occurs is near the middle of the thread-like portion of the collagen IV molecule and coincides with a region of greater molecular flexibility of this thread (Figs 1 and 5 of Lunstrum et al., 1988). Our finding of a single amino acid sequence for the amino end of the C-fragment supports the above homotrimeric model of *Drosophila* collagen IV and suggests that the cleavage occurred cleanly in each of the three chains, as opposed to less specific proteolysis of an unstructured peptide domain. As indicated in Fig. 1, the triple-stranded collagen IV molecule is cut into a triple-stranded N-fragment and a triple-stranded C-fragment. The only Cys residues predicted for the C-fragment of the collagen α1(IV) chain are in the globular NC1 domain. They are known to form disulfide bridges which are entirely intra-peptide (Lunstrum et al., 1988). The properties of the gp125 protein are in accordance with this prediction. First, the gp125 protein dissociates into 125×10^3 M_r chains in SDS buffer without reduction, as shown by SDS-PAGE. Secondly, the electrophoretic mobility of these 125×10^3 M_r chains is slightly slowed by reduction, corresponding to unfolding of the intra-peptide disulfide linked NC1 domain.

Western blots of gp125 (Fig. 6) suggest that a small proportion of the protein may consist of two chains which are connected by one or more covalent links that are not dissociated by reduction. Although the nature of this linkage is unknown, collagens are stabilized by covalent links between modified lysine side chains (Tanzer, 1976).

All 9 Cys residues of each collagen α1(IV) chain which are potential bridgeheads for disulfide links between either adjacent *Drosophila* collagen IV molecules, or between component chains of one triple helical set, are in the N-fragment that is generated simultaneously with the C-fragment by the cleavage of collagen IV. The calculated M_r of each N-fragment peptide is 71×10^3 and this corresponds to an SDS-PAGE mobility equivalent to approximately 90×10^3 M_r. We detected this peptide with our antibodies against *Drosophila* collagen IV in extracts of pupae. As expected, the peptide was in a high molecular mass fraction of the gel filtration chromatogram of the extract and was released from these disulfide-linked complexes by reduction. This is consistent with disulfide-linked complexes of several collagen IV molecules (Lunstrum et al., 1988). It is noteworthy that in nonreduced extracts of pupae the N-fragment of collagen IV was only detected in higher, disulfide linked complexes while the C-fragment was predominantely in the 125×10^3 M_r peptide form which lacks both disulfide and other covalent links to other peptides. Furthermore, the quantity of the N-fragment solubilized without reduction was small, as was the quantity of intact collagen IV. Although reduction was necessary to extract more of these disulfide linked N-fragment complexes, it was not extracted in quantities equivalent to that of the C-fragment. Thus in Fig. 2, when the western blot development was stopped after the signal for gp125 appeared strongly, there was no clear detection of the reduced N-fragment. Following longer immunostaining of such extracts, the N-fragment was also seen specifically with the antibodies to collagen IV.

The network of collagen IV molecules in *Drosophila* basement membranes is presumably linked through a tandem pair of double '7S' junctional domains in the N-fragment and pair-wise, antiparallel associations of the carboxyl ends of the C-fragments (see Fig. 1; Blumberg et al., 1988). We conclude that at least in that portion of the *Drosophila* basement membrane collagen IV network which is cleaved the associations of junctional domains in the N-fragment are mostly stabilized by disulfide bridges and other covalent bonds, but those between the NC1 globules are stabilized to a small extent. This is consistent with the observations of Birr et al. (1990), that while strong denaturants extracted gp125 from ecdysone-induced discs without reduction, the gp200 precursor of gp125 (which is collagen IV) could not be solubilized without reduction.

The physiological cleavage site of *Drosophila* collagen IV (residues 755/756) is at a strategically interesting location. It is just 4 residues from the last Cys (residue 751) of the N-fragment which could participate in disulfide crosslink formation. Electron microscopy of extracted vertebrate basement membranes has elegantly demonstrated a topological linkage between intertwined strands of collagen IV molecules (Yurchenco and Ruben, 1987). Like the topological linkages of knitted fabric, these intertwinements may impart special flexibility properties. Cleavage at this site would allow removal of potential topological linkages

in the C-fragment region of intertwined strands of collagen IV molecules without interfering with the multiple disulfide-linked junctions of the N-fragment regions. This is the equivalent of cutting knitted fabric while protecting against propagation of the lesion through unravelling, i.e. this type of cleavage should allow highly controlled, localized cleavage of an intertwined molecular network.

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