A novel, tissue-specific integrin subunit, $\beta_\nu$, expressed in the midgut of
*Drosophila melanogaster*

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SUMMARY

The integrins are a family of cell surface receptors for extracellular matrix proteins and counter-receptors on other cells. We have used the polymerase chain reaction to identify a novel integrin receptor $\beta$ subunit in *Drosophila melanogaster*. The deduced amino acid sequence of this subunit, which we have termed $\beta_\nu$ (beta-neu), indicates that it has several unusual properties. The $\beta_\nu$ subunit is roughly 33% identical with each of the previously sequenced vertebrate and *Drosophila* $\beta$ subunits and is lacking four of the 56 cysteine residues characteristic of most members of this protein family. The expression of the $\beta_\nu$ gene is strikingly restricted. It is temporally regulated, with maximal expression occurring at 12-15 hours of embryonic development. In situ hybridization analyses and antibody localization on whole-mount embryos reveal that $\beta_\nu$ expression is tissue-specific and confined to the developing midgut endoderm and its precursors during embryogenesis. Tissue specificity of expression is maintained through later stages of development as $\beta_\nu$ transcripts are found exclusively in the larval midgut. Within this structure, $\beta_\nu$ transcripts are especially concentrated in the cells of the midgut imaginal islands which give rise to the adult midgut.

Key words: cell adhesion, *Drosophila*, integrin, midgut

INTRODUCTION

The interactions of cells with each other as well as with the surrounding extracellular matrix are known to be important in a variety of biological processes such as embryogenesis, hemostasis, wound healing, immune recognition, inflammation, and oncogenic transformation (Hynes and Lander, 1992). Crucial to the interactions of cells with counter-receptors on other cells and with extracellular matrix components such as fibronectins, laminins, and collagens is a family of cell surface receptors termed the integrins (reviewed by Albelda and Buck, 1990; Hemler, 1990; Reichardt and Tomaselli, 1991; Hynes, 1992).

Integrins are large transmembrane glycoproteins composed of non-covalently associated $\alpha$ and $\beta$ subunits. In addition to their interactions with proteins outside cells, integrins interact with cytoskeletal components and thus provide a link between the exterior and interior of cells. In vertebrates, eight $\beta$ subunits and 14 $\alpha$ subunits are currently known and these can associate in various combinations to produce at least 20 distinct $\alpha\beta$ combinations. The ligand-binding specificity of individual integrins depends on both the $\alpha$ and the $\beta$ subunit (Hynes, 1992). The multiplicity of $\alpha$ and $\beta$ subunits in vertebrates accounts for the functional heterogeneity observed within this family of molecules and likely reflects the diverse roles that these protein complexes play in many different biological processes.

Given both the complexity and diversity of integrin functions, it would be advantageous to apply genetic methods to elucidating the roles that these molecules play in different biological processes. Toward this end, one family of integrins has been characterized in *Drosophila melanogaster*. *Drosophila* integrins were originally identified in a monoclonal antibody screen for developmentally regulated cell surface antigens in imaginal discs and were termed position-specific (PS) antigens, based on their distinctive spatial expression patterns (Wilcox et al., 1981; Brower et al., 1984; Wilcox and Leptin, 1985). Further biochemical work showed that the PS3 antigen resembled vertebrate $\beta$ subunits and could complex with either the PS1 or PS2 antigens, which were presumably $\alpha$ subunits (Brower et al. 1984; Wilcox et al., 1984; Leptin et al., 1987). Subsequent isolation of cDNA clones encoding the PS2 and PS3 antigens confirmed the identity of these proteins as *Drosophila* integrin $\alpha$ and $\beta$ subunits (Bogaert et al. 1987; MacKrell et al., 1988).

Mutations in two of these genes have been identified. The *inflated* (*if*) mutation results from alteration of the gene encoding the PS2$\alpha$ subunit (Wilcox et al., 1989), while the *lethal (l)* myospheroid (*mys*) mutation is the result of disruption of the gene encoding the common PS3$\beta$ subunit (which will be referred to in this paper as $\beta$s; MacKrell et al., 1988; Leptin et al., 1989). *Drosophila* embryos that are homozygous for loss of function *mys* mutations develop in
a relatively, though not completely, normal fashion during early embryogenesis. However, when the first muscular contractions occur, embryonic muscles detach from their sites of attachment and become spheroidal and the dorsal suture opens leading to herniation. These defects contribute to embryonic lethality (Wright, 1960; Newman and Wright, 1981). Determination of the requirements for mys in the development of adult structures by mosaic analysis has revealed roles for this gene in the development of wings and eyes. In particular, X-ray-induced mys mitotic recombination clones show blisters and vein defects in wings and ommatidial disorganization in developing eyes (Brower and Jaffe, 1989; Zusman et al., 1990). Gynandromorph analysis of flies mosaic for mys indicated a widespread requirement for this gene in the development of many adult structures (Zusman et al., 1990).

While the β subunit encoded by the mys gene and its associated α subunits are clearly required for both embryogenesis and the formation of many adult structures in the fly, many other morphogenetic processes appear to proceed normally in the absence of this gene. In particular, there are examples of tissues which show high level mys+ gene expression but which show no obvious phenotype when this gene is disrupted. For example, despite the widespread distribution of βPS during early embryogenesis, the phenotypes observed in mys mutant embryos occur relatively late in development. In the morphogenesis of the eye, βPS expression is observed in the eye disc at the third larval instar stage of development; however, the phenotypic consequences of the absence of this gene in somatic clones are not evident until late pupal stages (Zusman et al., unpublished data). Thus, many morphogenetic processes can occur relatively normally in the absence of this gene, despite the widespread distribution of its product. Such results suggest the possibility that there are other adhesion systems in Drosophila that may compensate for the loss of βPS function in some situations. We are particularly interested in determining whether such cell adhesion systems might involve the participation of previously uncharacterized integrins.

To explore this possibility, we have used the polymerase chain reaction to identify additional members of the integrin receptor family. In this paper, we report the complete cDNA sequence and characteristics of the deduced protein sequence of a new integrin β subunit in Drosophila and describe the temporal and spatial expression pattern of this gene during development.

**MATERIALS AND METHODS**

**PCR amplification of integrin β subunit sequences**

Oligonucleotide primers for the polymerase chain reaction (PCR) were designed based on conserved amino acid sequence motifs found in the sequences of human β1,3, chicken β1, Xenopus β1, and Drosophila βPS (Argraves et al., 1987; Kishimoto et al., 1987; Law et al., 1987; Fitzgerald et al., 1987; Rosa et al., 1988; Tamkun et al., 1986; DeSimone and Hynes, 1988; MacKrell et al., 1988). Degenerate primers, 30 nucleotides in length, were synthesized with a maximum of 25% inosine substitution in order to maintain the degeneracy of each primer at approx. 500-fold or lower. The forward PCR primer 5’TACTA(C/T)/(C/T)TATGA(C/T)-ITI(A/T)(G/C)/(G/C)/(A/T)/(A/T)/(A/T)/(G/C)IATG3’ (512-fold degenerate) includes all the possible codons encoding the amino acid sequence YLYMDLS(Y/F/K/R)SM. The reverse PCR primer was 5’-T/CTGATATIGC/G/AI/TCIAG/G/AI/CTCCI(T/C)TCIG3’ (16-fold degenerate), whose complement encodes the peptide sequence PEGGF/L/D/A/I/MMQ. A third primer 5’-IR(G/A)IT/C/G/(T/C)ITTTTCIAC(A/G)AAI/C/G/BBCC/G/AI/AACICIAA-CIA3’ (96-fold degenerate) was designed, based on the amino acid sequence (L/G/F/G/S/A/F/V/E/D/K/T/T/B/V) which is internal to the first two sequences; this primer was used as a Southern blotting probe to verify the identity of PCR products. The forward and reverse PCR primers had the recognition sequences for EcoRI and HindIII as well as four additional bases (AGCT) appended to their 5’-ends to facilitate subcloning of PCR products. The size of products resulting from amplification using the forward and reverse primers is predicted to be about 300 bp when cDNA templates are used.

Single-stranded cDNA templates for PCR were synthesized from total 0-24 hour Canton-S embryonic RNA using random primers and first-strand synthesis reagents commercially available (Boehringer-Mannheim). 30 μg of total RNA was used in 20 μl reactions under conditions specified by the manufacturers. For PCR amplification, 1/20 of the cDNA synthesis reaction was combined with 1 μg of each of the forward and reverse PCR primers in a 100 μl reaction as previously described (Saiki et al., 1988). The amplifications were performed with 40 thermal cycles of 94°C for 1 minute, 50°C for 2 minutes, and 72°C for 3 minutes, followed by a 10 minute final incubation at 72°C.

To determine whether PCR had generated products distinct from βPS, the PCR reaction was treated with the restriction enzymes XhoI and HindIII, which both cut within the amplified βPS sequence. The PCR products and fragments resulting from restriction enzyme digestion were fractionated on 2% agarose gels. To confirm that the restriction enzyme-resistant material of the correct size contained β subunit-related sequences, the gel was blotted onto nitrocellulose and hybridized with 32P-labelled internal oligonucleotide under low stringency conditions as described (Ausubel et al., 1989).

To subclone PCR products for further analysis, the reactions were precipitated, redissolved in the appropriate restriction enzyme buffer to which 1 mM dNTPs and 5 units of Klenow enzyme were added. The mixture was incubated at 37°C for 15 minutes, followed by a 10 minute final incubation at 72°C. The resulting sequence data was inserted into pBluescript SK+(−) vectors using the restriction enzyme EcoRI. The sequence of these enzymes XhoI, HindIII and EcoRI. Following fractionation of the digest on a 2% agarose gel, the XhoI- and HindIII-resistant band of approx. 300 bp was isolated from the gel using Gene Clean (Bio 101), and the eluted DNA ligated into the EcoRI/HindIII site in pBluescript SK(−) (Stratagene). The ligation reactions were used to transform competent E. coli strain BB4 (Stratagene).

**Library screening and DNA sequencing**

The PCR clone corresponding to βV was used to screen a 0-24 hour embryonic library in the vector λ-Zap (Stratagene) using standard methods. Three clones that represented partial sequences were isolated from this library, and the clone that extended the farthest in the 5’ direction was used to select longer clones from a 9-12 hour embryonic library in λgt11 (Zinn et al., 1988). The partial clones and a 3.5 kb clone that was subsequently found to contain the entirety of the βV coding sequence were completely sequenced. DNA sequencing was performed by the DNA sequencing method (Sanger et al., 1977) using Sequenase (U.S. Biochemical) Single-stranded templates for PCR clones contained in pBluescript SK(−) vectors were made by single strand DNA rescue using the filamentous helper phage M13. The sequence analysis of library clones for βV was performed using m13mp18 templates containing random fragments of the cDNA obtained through sonication of plasmid DNA. The resulting sequence data
were compiled and assembled using the Staden computer package (Staden, 1986). Further analysis of the data was performed using the UWGCG computer package.

**RNA isolation and RNase protection analysis**

Total RNA from different stages was isolated by extraction with guanidinium thiocyanate followed by acidic phenol/chloroform extraction as described by Chomczynski and Sacchi (1987) with the exception that the RNA was precipitated by adding 1/50 volume of 1 M acetic acid followed by 1/2 volume of ethanol. RNase protection analysis was performed as described in Norton and Hynes (1990). Equal amounts of total cellular RNA (10-20 µg) from each stage were hybridized at 37°C with uniformly labelled RNA probes prepared by in vitro transcription of linearized plasmid DNA templates. Following overnight incubation, unhybridized probe was removed by digestion with 20 µg/ml RNase A and 2 µg/ml RNase T1 at 25°C for 30 minutes. The samples were then treated with proteinase K in 0.5% SDS for 15 minutes at 37°C, extracted with phenol/chloroform, and ethanol-precipitated prior to electrophoresis through 4% polyacrylamide denaturing gels. Some experiments were normalized by comparison with the hybridization signal obtained with a probe encoding ribosomal protein 49 (RP49; O'Connell and Rosbash, 1984). Controls showed that DNAse digestion of the RNA samples did not alter the pattern of expression observed.

**In situ hybridization**

In situ hybridization of DNA probes to whole-mount embryos was performed essentially as described by Tautz and Pfeifle (1989) using the Genus kit (Boehringer-Mannheim), except that hybridization was performed at 50°C. Single-stranded anti-sense and sense DNA probes labeled with digoxigenin were synthesized by PCR using pBluescript SK(−) plasmids linearized at the appropriate ends of inserts as templates and either the T7 or T3 promoter primers (Perkins et al., 1990). The average size of the resulting single-stranded DNA molecules was reduced by heating the probe for 1 hour at approximately 100°C in a water bath prior to hybridization. Following hybridization, the embryos were washed with solutions of decreasing concentration of hybridization buffer at 50°C until the samples were returned to PBS containing 0.1% Tween-20. After development of the alkaline phosphatase-mediated color reaction, the embryos were dehydrated in an ethanol series, cleared with xylene and mounted in Permount.

For sectioning, embryos which had undergone the whole-mount in situ hybridization procedure were embedded in JB-4 medium (Polysciences), and 10 µm microtome sections were cut. The resulting sections were mounted in DPX medium (Fluka) and viewed using Nomarski optics.

The larval digestive tract was hand-dissected from wandering third instar larvae by pulling the head away from the body with small forceps. This method of dissection allowed the isolation of the intact digestive system consisting of the foregut, midgut, and hindgut. These preparations also contained the larval brain and the anterior-most imaginal discs. These gut preparations were then subjected to whole-mount in situ hybridization with βν probes as described above for embryos. Following overnight development of the color reaction, cell nuclei were marked by incubating the samples in 0.1 µg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) for 5 minutes. Samples were then mounted in glycerol for microscopy. Photographs were taken with both the fluorescence and bright-field light sources on to allow the nuclei and in situ hybridization signals to be viewed simultaneously.

**Antibody production, in vitro translation of βν cDNA, and western immunoblotting**

A peptide corresponding to the last 23 amino acid residues of the βν cytoplasmic domain with an added N-terminal cysteine residue was coupled to keyhole limpet hemocyanin (KLH; Calbiochem) using m-maleimidobenzoylsulfo succinimide ester (Pierce Chemical) as described previously (Marcantonio and Hynes, 1988). The resulting peptide conjugate was used to raise polyclonal antibodies in rabbits.

In vitro transcription and translation of the βν cDNA using rabbit reticulocyte lysate in the presence of canine microsomal membranes was performed using commercially available reagents and the protocols provided by the manufacturers (Promega). The products of in vitro translation reactions done in the presence of L-[35S]methionine were analysed by SDS-PAGE and fluorography. Immunoprecipitation of the in vitro translation products with βν antibodies directed against the cytoplasmic domain was performed using protein A sepharose as described previously (Hynes et al., 1989).

Lysates for western blot analysis were prepared from staged, dechorionated embryos by homogenization in reducing SDS gel sample buffer (Hynes et al., 1989). Approximately 100 µg of protein were loaded into the lanes of 7% SDS gels. SDS-PAGE was performed as described previously (Hynes et al., 1989). The separated proteins were then electroblotted onto nitrocellulose filters. The filters were blocked for 1 hour in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% PVP 360, 1% BSA, and 0.25% Tween-20. The primary antibody was added at a 1:100 dilution and allowed to incubate with the filters in the presence or absence of 100 µg/ml peptide competitor overnight. After thorough washing with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, the filters were incubated with 50 µCi/ml 125I-labelled protein A in the blocking buffer for 2 hours and then given a final wash prior to autoradiography.

**Antibody localization in whole-mount embryos**

Staged embryos were dechorionated in 50% chlorox solution and fixed for 30 minutes at room temperature in a mixture of 1:1 heptane and 4% paraformaldehyde in PBS. Vitelline membranes were removed by the heptane method (Mitchison and Sedat, 1983). Whole-mount antibody staining using horseradish peroxidase (HRP) detection was performed as described by Zusman and Wieschaus (1987). A 1:300 dilution of the primary antibody was incubated with embryos for 24 hours at 4°C. Control incubations included 100 µg/ml peptide competitor. Visualization of antibody binding was accomplished using the Vectastain HRP kit (Vector Laboratories). The biotinylated secondary antibody was diluted 1:1500 and incubated with the embryos for 24 hours at 4°C. After a final incubation with avidin-conjugated HRP for 30 minutes at room temperature, the color reaction was developed by the addition of H2O2 to 0.03% and diaminobenzidine to 0.05 mg/ml. Embryos were mounted in 90% glycerol for viewing using Nomarski optics. Alternatively, embryos were incubated with FITC-conjugated avidin after the biotinylated secondary antibody step and processed for fluorescence microscopy.

**RESULTS**

**Isolation of cDNA clones for a novel integrin β subunit (βν)**

Degenerate oligonucleotide primers for the polymerase chain reaction (PCR) were designed based on conserved amino acid sequences found in the ligand-binding domains of a number of different integrin β subunits from different species (DeSimone and Hynes, 1988; MacKrell et al., 1988). A third oligonucleotide, internal to the two used for PCR was designed for use as a Southern blotting probe to confirm the identity of PCR products (see Materials and
Methods for details). Fig. 2B shows the location of these primers within the sequences of different integrin β subunits. These PCR primers were used to amplify integrin β subunit-related sequences from cDNA templates synthesized from 0-24 hour *Drosophila* embryonic RNAs (see Materials and Methods). A 327 bp band of the predicted size was the major reaction product visualized by agarose gel electrophoresis (data not shown). To eliminate *mys* gene sequences from the further analysis of the PCR products, the reaction mixture was digested with restriction enzymes (XhoI, HindIII) for which sites are present in the relevant segment of βPs. This treatment resulted in the conversion of approximately half of the 327 bp fragment to lower molecular mass bands of the sizes predicted for βPs. The gel was Southern blotted and probed with the internal oligonucleotide. The remaining material of 327 bp, which was unaffected by restriction enzyme treatment, hybridized to this internal probe, suggesting that it contained integrin β subunit-related sequences which were distinct from βPs (data not shown).

After treatment with XhoI and HindIII, the 327 bp band was isolated from the gel and the DNA subcloned into a plasmid vector and multiple clones were obtained. Sequence analysis of a number of clones revealed two interesting classes of PCR clones. The first of these represented a novel integrin β subunit, which we term βν (beta-neu), and the second class of interest corresponded to a previously unknown alternatively spliced exon in βPs. This second class of clones will be described elsewhere (manuscript in preparation). Other integrin β subunits were not detected with the PCR primers and cDNAs used.

To isolate longer cDNA clones for the βν subunit, we used the subcloned βν PCR fragment to screen 0-24 hour and 9-12 hour embryonic cDNA libraries. A 3.5 kb clone obtained from the 9-12 hour library was completely sequenced. Its sequence was in agreement with sequences obtained from various partial clones. The sequence obtained is shown in Fig. 1. The 3.5 kb clone contained a single open reading frame which predicted a protein with significant homology to all integrin β subunits sequenced thus far.

**Sequence of the βν cDNA and the deduced protein sequence**

The 3552 base pair (bp) cDNA sequence shown in Fig. 1 contains a 309 bp untranslated 5′ leader sequence followed by a single open reading frame of 2397 bp encoding a protein of 799 amino acids. The 3′ untranslated region following the coding region lacks a polyadenylation signal. The first ATG not followed by a termination codon is followed by a sequence rich in hydrophobic residues (underlined in Fig. 1) which is likely to represent a signal sequence. Calculation of the probable site of signal peptide cleavage (cf von Heijne, 1986) strongly predicts the aspartate residue at position 27 as the first amino acid in the mature protein. This site of proposed signal peptide cleavage for the βν subunit is consistent with the positions of the experimentally determined cleavage sites for β1,5 subunits of vertebrate integrins.

The proposed mature βν protein consists of an extracellular domain of 696 amino acids followed by a single transmembrane segment of 27 amino acids (double underline in Fig. 1). The presumptive cytoplasmic domain of this protein is composed of 50 amino acids. In the extracellular domain, there are five sites of potential N-glycosylation as indicated in Fig. 1.

**Comparison of the protein sequence of the βν subunit with other β subunit sequences**

Table 1 indicates that the protein sequence of the βν subunit is approximately 33% identical with each of the other β subunits listed. Thus, the βν subunit is no more related to any particular vertebrate β subunit than it is to *Drosophila* βPs. In contrast, the percentage identities between βPs and each of the vertebrate β subunits are higher, ranging from 36-46%, and βPs appears most similar to β1.

The seven cysteine residues present in the first approx. 150 amino acid residues of all β subunits characterized to date are all conserved in βν, with respect to both number and position in the sequence (see Figs 1, 2A). Fig. 2B shows an alignment of the sequence of the ligand-binding domain of βν with the sequences of this domain from other integrin β subunits. As indicated in this figure, this stretch of approx. 250 amino acids is highly conserved among all known β subunits; the βν sequence is approx. 50% identical with each of the other β subunits in this region of the molecule. Segments that are underlined in Fig. 2B represent protein sequences used in the design of PCR primers. The βν sequence shows especially high similarity to the other β subunits within these segments. Furthermore, the four cysteine residues (numbers 8-11) in this region, which are conserved among different vertebrate β subunits, are also present in βν. The aspartate residue which is mutated in Glanzmann’s thrombasthenia (solid circles) and thought to be involved in ligand binding (Loftus et al., 1990) is conserved, as are the leucine and glycine residues (open circles) mutated in human β2 in two kindreds of leukocyte adhesion deficiency patients whose β2 subunits fail to dimerize (Warldlaw et al., 1990).

In the cysteine repeat region which follows the ligand-binding domain (Fig. 2A), the identity of the βν subunit with each of the other β subunit sequences is approximately 25%, which is low compared with that in the ligand-binding domains. Furthermore, the βν subunit lacks four of the 56 cysteine residues present in most other integrin β subunits. In particular, βν is lacking cysteine residues 14, 15, 47, and 48 (see Fig. 2A and Discussion).

The cytoplasmic domain of 50 amino acids is most similar in length to the cytoplasmic domains observed in the β5, β6, and β5 subunits in vertebrates (see Fig. 2C). The sequence of the cytoplasmic domain of the βν subunit is highly charged and contains several proline residues suggestive of numerous turns in this part of the molecule. Four tyrosine residues are present in this domain including the one found in the conserved NPIY sequence motif, which has been proposed as an internalization signal (Chen et al., 1990) and as a site for phosphorylation (Tamkun et al., 1986; Tapley et al., 1989).

**Temporal expression pattern of βν transcripts during development**

The temporal expression pattern of the βν gene during development is shown in Fig. 3. Because of the low
Novel integrin subunit L13305

Fig. 1. Nucleotide sequence of cDNA and deduced amino acid sequence is indicated by a dashed underline. This sequence has been submitted to the GenBank Database (Accession Number: M23456).

The putative signal peptide that follows the first usable ATG codon is underlined. The signal peptide cleavage site was predicted as described in von Heijne (1986). Cysteine residues in the sequence are conserved in other β subunits, and at positions at which β lacks cysteine residues conserved in other β subunits are indicated by a number in parenthesis. The five potential sites for N-glycosylation are indicated by asterisks underneath the N residues.
abundance of the $\beta_\nu$ message, an RNase protection assay was employed to detect the presence of this message in populations of total RNA. As shown in Fig. 3, the $\beta_{PS}$ gene is abundantly expressed throughout embryogenesis. Maximal expression of $\beta_{PS}$ is observed between 12-15 hours of embryogenesis. Postembryonically, the expression of this gene decreases to levels maintained through later stages of development. Levels of expression of the $\beta_\nu$ gene were observed to be significantly lower than those seen for $\beta_{PS}$. The data obtained using a probe encoding the ligand-binding domain (amino acids 118-346) indicate that expression of $\beta_\nu$ is low during early embryogenesis but increases after 6 hours of development. Maximal expression is observed at 12-15 hours of development after which point expression diminishes to a level maintained for the rest of development.

**Localization of $\beta_\nu$ transcripts during embryogenesis**

To examine the spatial distribution of $\beta_\nu$ expression, in situ hybridization analyses were performed on whole-mount embryos using digoxigenin-labelled cDNA probes. The data presented in Fig. 4 demonstrate that the $\beta_\nu$ gene is expressed exclusively in the developing midgut and its precursors. Significant expression of the $\beta_\nu$ gene is first detected in late stage 11 embryos (approximately 6-7 hours of development) where message localization is seen in the anterior and posterior midgut primordia (Fig. 4A). As germ band retraction proceeds in stage 12 (approx. 7-9 hours), these primordia are brought together resulting in lateral fusion of these two structures to form an open tube surrounding the yolk during stage 13 (9.5 hours). Concurrent with this migration and fusion process, an increase in $\beta_\nu$ expression in the midgut is observed (Fig. 4B, C). Fig. 4D shows a dorsal view of a stage 14 (11 hours) embryo which has undergone ventral closure of the midgut while it is still open dorsally. This view shows that the expression of the $\beta_\nu$ gene is localized specifically to the cells composing the midgut endoderm. No expression is seen in other structures that will contribute to the gut. In particular, no hybridization is observed in the foregut or hindgut, or in the cells that make up the visceral mesoderm. This is confirmed by analysis of longitudinal and cross sections of similar embryos (see Fig. 5).

After completion of midgut closure, the midgut tube develops four constrictions along its length (between 13-16 hours), which give this organ its characteristically convoluted appearance. The expression of the $\beta_\nu$ gene during these stages of development changes in magnitude as well as distribution. Fig. 4E shows an embryo that has developed the initial constrictions in the midgut. Although expression of the $\beta_\nu$ gene is observed throughout the midgut endoderm, expression is no longer uniform. Rather, expression is higher at the posterior end of this structure than it is anteriorly. As further midgut constrictions develop, the difference between anterior and posterior expression of the $\beta_\nu$ gene becomes even more pronounced. Expression of the $\beta_\nu$ gene at this later stage is confined to the posterior end of the midgut; expression is barely detectable in the anterior two lobes of the midgut (Fig. 4F). The expression pattern of the $\beta_\nu$ gene as determined from in situ analysis agrees well with the results obtained from the analysis of staged RNAs presented in Fig. 3.

**Analysis of the $\beta_\nu$ protein and its localization during embryogenesis**

In vitro transcription and translation of the $\beta_\nu$ cDNA was performed as shown in Fig. 6A. Translation in the presence of microsomal membranes resulted in the conversion of the $110 \times 10^3 M_r$ product seen in the absence of microsomal membranes to one of $130 \times 10^3 M_r$. This shift in molecular mass is likely to result from utilization of some or all of the five potential sites of glycosylation present in the deduced amino acid sequence of the $\beta_\nu$ subunit. The $130 \times 10^3 M_r$ product is specifically immunoprecipitated by an antibody raised against a peptide corresponding to the last 23 amino acids of the $\beta_\nu$ cytoplasmic domain (Fig. 6A, lanes 5-7). When this antibody is used to probe a western blot of embryonic lysates, an approx. $130 \times 10^3 M_r$ protein is specifically detected in 12-15 hour embryonic lysates but not in 0-3 hour embryonic lysates (Fig. 6B). The molecular mass of this band agrees with the result obtained from the in vitro translation experiment and is consistent with the size expected, based on the amino sequence of the $\beta_\nu$ subunit. Furthermore, the presence of the immunoreactive band in the 12-15 hour but not in the 0-3 hour embryo lysate is consistent with the expression pattern seen at the RNA level (Fig. 3).

The antibody raised against the $\beta_\nu$ cytoplasmic domain was used to determine the spatial distribution of $\beta_\nu$ protein in whole-mount embryos. The data presented in Fig. 7 show

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The percentage identities of integrin $\beta$ subunits were computed using the BESTFIT program (UWCG). The number of cysteine residues in each $\beta$ subunit is indicated. Only the extracellular domain of the $\beta_4$ subunit was used in these computations.
Fig. 2. (A) Structure of the βν subunit. The positions of the 56 cysteine residues conserved in many β subunits are indicated by the vertical bars. Cysteine residues absent from the βν subunit are indicated by open triangles. Hatched triangles indicate cysteine residues that are absent from both the βν and β4 subunits (see text). The stippled box indicates the location of the transmembrane domain.

(B) Alignment of the ligand-binding region (residues 133-375; see Fig. 1) and (C) of the cytoplasmic domain of βν with other integrin β subunits. The corresponding amino acid sequences of human β1 (Argraves et al., 1987), β2 (Kishimoto et al., 1987; Law et al., 1987), β3 (Fitzgerald et al., 1987; Rosa et al., 1988), β4 (Suzuki and Naitoh, 1990; Hogervorst et al., 1990), β5 (Ramaswamy and Hemler, 1990), β6 (Sheppard et al., 1990), β7 (Erle et al., 1991), β8 (Moyle et al., 1991), and of the Drosophila myo gene product (MacKrell et al., 1988) are shown in comparison with the βν protein sequence. For clarity, the sequence of the unrelated β4 cytoplasmic domain has been omitted in C. Gaps have been introduced to maximize alignment, and the positions of cysteine residues are indicated by numbers at the top of a column as well as by asterisks at the bottom of a column. Amino acid residues which are identical in six or more of the sequences have been shaded. The positions of amino acid residues altered in Glanzmann’s thrombasthenia (solid circle) and leukocyte adhesion deficiency (open circles) are indicated. The regions of sequence homology among all β subunits that were used to design the PCR primers are underlined in B. The position of the NPXY sequence motif is marked by thick underlining in C.
that βν protein is found exclusively in the developing midgut endoderm. Immunoreactivity was blocked by addition of the cytoplasmic domain peptide (data not shown). βν protein is observed in the anterior and posterior midgut primordia of stage 12-13 embryos, which are undergoing migration and fusion of these precursors to form an open midgut tube (Fig. 7A). As midgut development progresses with ventral closure (Fig. 7B) and then dorsal closure (Fig. 7C) expression of βν protein in the midgut endoderm is evident and is notably absent from the foregut, hindgut, and visceral mesoderm. βν protein expression persists in the midgut endoderm as constrictions form along the length of the midgut (Fig. 7D-F).

Fig. 7F illustrates more clearly the three dimensionality of the midgut and shows that the βν protein is found along all surfaces of this structure. Unlike the mRNA (Fig. 4), βν protein does not show a gradient of expression along the midgut.

Localization of βν transcripts in the larval gut
Given the distinct spatial pattern of βν expression observed during embryogenesis, we employed in situ hybridization analyses on whole-mount preparations of dissected larval guts to determine whether the tissue specificity of βν expression was maintained postembryonically. The third

Fig. 4. Localization of transcripts for βν by whole-mount in situ hybridization using digoxigenin-labelled DNA probes. Hybridization signal is observed in the developing midgut. (A) Lateral view of a late stage 11 embryo prior to germ band retraction. Hybridization signal is seen in the anterior and posterior midgut anlagen. (B) Lateral view of a stage 12 embryo during shortening of germ band. Hybridization signal in the two midgut precursors appears to increase as they move toward each other. (C) Lateral view of an early stage 13 embryo. Germ band is shortened and anterior and posterior midgut anlagen are about to fuse laterally around the yolk. Hybridization signal is very strong in these structures which will develop into the midgut. (D) Dorsal view of a stage 14 embryo after ventral closure of the midgut.

Hybridization signal is confined to the midgut endoderm. No signal is observed in either the fore- or hindgut or in the surrounding mesodermal layer of cells. (E) Lateral view of a stage 15 embryo after formation of the first midgut constrictions. Hybridization signal is seen along the midgut endoderm but is stronger at the posterior end. (F) Lateral view of a stage 16 embryo after completion of midgut constrictions. Hybridization signal is largely confined to the posterior end of the midgut. Little or no expression is detected in the anterior lobes. All embryos are shown with anterior to the left. Staging is according to Campos-Ortega and Hartenstein, 1985.
Novel integrin subunit larval instar midgut is composed of two types of cells as shown in Fig. 8A. The larval endoderm is composed of epithelial cells with large polytene nuclei. Embedded among these larval cells are groups of smaller cells termed the midgut imaginal islands that possess smaller nuclei and which will give rise to the adult midgut following subsequent morphogenesis. The data presented in Fig. 8B-D show that expression of the $\beta_\nu$ gene is confined to the midgut portion of the larval digestive tract. Little expression of the gene is seen in either the foregut (Fig. 8B) or in the hindgut (Fig. 8D). Note that there is abundant expression of the $\beta_\nu$ gene seen in the region of the hindgut imaginal ring which is found at the junction of the midgut and the hindgut. Within the midgut proper, especially high levels of $\beta_\nu$ transcripts are observed surrounding the nuclei of cells of the midgut imaginal islands (Fig. 8C), although transcripts are also found surrounding the polytene nuclei of the large, larval endodermal cells. Expression of the $\beta_\nu$ gene is also found in the gastric caeca which extend from the anterior portion of the midgut. Low levels of $\beta_\nu$ expression are seen in the proventriculus. No detectable expression of the $\beta_\nu$ gene was observed in either the larval brain or in any of the

Fig. 5. Localization of transcripts for $\beta_\nu$ in sections of embryos. Hybridization signal is observed in the midgut endoderm but not in the visceral mesoderm (marked by arrowheads). (A) A longitudinal section through a stage 14 embryo. (B) A cross section through a stage 14 embryo.

Fig. 6. Analysis of the $\beta_\nu$ protein by in vitro translation and western blotting. (A) Products of the in vitro translation of the linearized $\beta_\nu$ cDNA performed in the absence (lane 1) or in the presence of canine microsomal membranes (lane 2). Control translations using antisense templates (lanes 3 and 4). Immunoprecipitation of the in vitro translation products shown in lane 2 were performed with either preimmune serum (lane 5) immune serum (lane 6) or immune serum plus the peptide to which the antibody was raised (lane 7). (B) Western blots of 0-3 and 12-15 hour embryo extracts reacted with either immune serum (1) or immune serum plus the peptide to which the antibody was raised (2). The sizes of the molecular mass markers are 200, 97, 68, and $43 \times 10^3 M_r$. 
Fig. 7. Localization of $\beta$ν protein by whole-mount antibody staining. The protein is found in the developing midgut. (A) Lateral view of a stage 13 embryo. Protein is expressed in the anterior and posterior midgut anlagen as they undergo fusion. (B) Dorsolateral view of a stage 14 embryo. Protein is confined to the midgut endoderm. No staining is observed in the fore- or hindgut or in the cell layer comprising the visceral mesoderm. (C) Lateral view of a late stage 14 embryo after dorsal closure of the midgut. (D) Lateral view of a stage 15 embryo after formation of the first midgut constriction. (E) Lateral view of a stage 16 embryo after formation of multiple midgut constrictions. (F) Antibody staining of a stage 15 embryo performed with FITC-linked secondary antibody to show that $\beta$ν protein is found along all surfaces of the midgut. Note that the $\beta$ν protein is found throughout the midgut endoderm in all these stages.

Fig. 8. Localization of $\beta$ν transcripts in the third larval instar gut. Hybridization is confined to the larval midgut. (A) DAPI staining of a whole-mount midgut showing the location of cell nuclei. Note the two classes of nuclei: the large polytene nuclei of the larval endodermal cells and the smaller nuclei of the larval endodermal cells. (B) Whole-mount midgut at the junction of the foregut (f) and midgut (m). Hybridization signal is seen in the midgut and at a lower level in the proventriculus (p) but not in the foregut. (C) Enlarged view of hybridization signal in the midgut. Note the especially high levels of $\beta$ν transcript seen surrounding the nuclei of the midgut imaginal island cells. (D) Whole-mount midgut at the junction of the midgut (m) and the hindgut (h). Note that $\beta$ν expression is observed in the midgut and hindgut imaginal ring (r) but not in the hindgut proper. Magnifications: 200× A and B, and 400× C and D.

Note the especially high levels of $\beta$ν transcript seen surrounding the nuclei of the midgut imaginal island cells. (D) Whole-mount midgut at the junction of the midgut (m) and the hindgut (h). Note that $\beta$ν expression is observed in the midgut and hindgut imaginal ring (r) but not in the hindgut proper. Magnifications: 200× A and B, and 400× C and D.
anteriorly located imaginal discs which were accessible by our dissection methods (data not shown).

**DISCUSSION**

Our data indicate that $\beta_\nu$ is a novel member of the integrin receptor $\beta$ subunit family which is expressed in a markedly tissue-specific and temporally and spatially restricted manner during *Drosophila* development.

**Structure of the $\beta_\nu$ protein**

As shown in Table 1, the sequence of $\beta_\nu$ is equally related to each of the previously identified integrin $\beta$ subunits (approx. 33% identity) when these sequences are compared along their entire lengths. Thus, $\beta_\nu$ does not represent the *Drosophila* homologue of any of the known vertebrate $\beta$ subunits. The only other integrin $\beta$ subunits which show a comparably low degree of relatedness to other members of this family are the extracellular domain of $\beta_4$ and the recently identified $\beta_8$ subunit. The $\beta_1$ subunit is uniformly approx. 36% identical to the other vertebrate $\beta$ subunits and is 29% identical to $\beta_\nu$. The human $\beta_8$ subunit is 31-37% identical to the other vertebrate $\beta$ subunits (Moyle et al., 1991) and is 30% identical to $\beta_\nu$. Thus, from the standpoint of overall sequence homology, $\beta_\nu$, $\beta_4$, and $\beta_8$ represent the most divergent members of the integrin receptor $\beta$ subunit family. In contrast, $\beta_\nu$, the only other integrin $\beta$ subunit previously identified in *Drosophila*, is most related to vertebrate $\beta_1$ (46% identity); $\beta_\nu$ appears to be expressed in a wide variety of cells in *Drosophila* embryos (Leptin et al., 1989; Zusman et al., 1990), similar to the widespread distribution of $\beta_1$ in vertebrates (see reviews cited in Introduction).

The sequence of the ligand-binding domain of $\beta_\nu$ (amino acids 133-374) shows the highest degree of identity to the corresponding sequences in other integrin $\beta$ subunits (Fig. 2B). This high degree of sequence similarity presumably reflects common mechanisms that underlie the functions of all integrin $\beta$ subunits. Notably, the conserved aspartate residue which is mutated in Glanzmann’s thrombasthenia is present in $\beta_\nu$ (Fig. 2B). This amino acid is present in all other integrin $\beta$ subunits and has been proposed to form part of an EF hand metal-binding loop. The oxygenated amino acid residues which have been suggested (Loftus et al., 1990) to provide coordination sites for divalent cations (DxSxSxxxxDxxNorS) are conserved in all $\beta$ subunits including $\beta_\nu$.

As indicated in Table 1 and Fig. 2A, another notable feature of the $\beta_\nu$ protein sequence is its deviation from the pattern of 56 conserved cysteine residues observed in most integrin $\beta$ subunits, with the exception of $\beta_4$, $\beta_7$, and $\beta_8$. $\beta_\nu$ is lacking four of the conserved 56 cysteines at positions 14, 15, 47, and 48. Interestingly, $\beta_4$ also lacks cysteine residues at positions 47 and 48. These observations suggest that cysteines 47 and 48 are disulfide-bonded, if one makes the assumption that linked cysteines are lost in pairs in order to avoid the introduction of extracellular free sulfhydryl groups into the protein. Thus, we can argue that cysteines 14 and 15, which are absent from $\beta_\nu$, are probably disulfide-bonded as are cysteines 55 and 56, the only cysteine residues absent from the sequence of $\beta_7$ (Erle et al., 1991). Cysteine residues 55 and 56 are among the six that are absent from the sequence of the $\beta_8$ subunit (Moyle et al., 1991).

The pattern of disulfide-bonding predicted here, based on sequence comparisons, is in partial agreement with the results presented recently by Calvete et al. (1991), who used protein chemical methods to attempt an assignment of the disulfide bonds in human platelet GPIIa ($\beta_3$). Our assignment of a disulfide bond between cysteines 47 and 48 based on their absence in both $\beta_4$ and $\beta_7$ is consistent with the results in Calvete et al. (1991) since these authors suggest that cysteines 47, 48, 49, 50, and 51 form two disulfide bonds among themselves leaving the unpaired cysteine residue to form a bond with cysteine 56. However, while Calvete et al. (1991) propose that cysteine 14, midway through the $\beta$ subunit protein, forms a long-range disulfide bond with cysteine 54, near the transmembrane domain, inspection of the $\beta_\nu$ protein sequence reveals that cysteine 14 is absent, while cysteine 54 is present. Clearly, the discordance of the protein chemical data with conclusions that can be derived from the sequences of $\beta$ subunits having fewer than 56 cysteine residues points to the need for further experimental work to ascertain the definitive disulfide-bonding pattern of integrin $\beta$ subunits. In particular, since many of the disulfide bonds have been tentatively assigned, specific protease cleavages based on sequence information can be used to test the validity of specific assignments.

**Developmentally regulated and tissue-specific $\beta_\nu$ expression**

The developmental regulation and tissue specificity of $\beta_\nu$ gene expression contrasts sharply with the widespread distribution of $\beta_\nu$ and of many of the $\beta$ subunits found in vertebrates. Our results demonstrate that the embryonic expression of the $\beta_\nu$ gene is confined to the developing midgut and its precursors. No other $\beta$ subunit with a distribution as restricted as $\beta_\nu$ has been reported in vertebrates or invertebrates. The midgut in *Drosophila* results from the fusion of two precursor structures, the anterior and posterior midgut primordia. As germ band retraction occurs, these two primordia migrate toward one another and fuse laterally alongside the yolk mass to generate a midgut tube that is open dorsally and ventrally. Ventral closure of this tube occurs first, followed by dorsal closure so that a continuous midgut tube is formed. Four constrictions later form along the length of this smooth tube to give the midgut a convoluted appearance at the end of embryogenesis.

Expression of the $\beta_\nu$ gene in the cells of the midgut endoderm as revealed by our in situ hybridization and antibody localization experiments appears to be correlated with the fusion, closure, and folding processes that are associated with the morphogenesis of this organ. The expression of the $\beta_\nu$ gene is first observed in the anterior and posterior midgut primordia at the extended germ band stage of development. At this stage, these two primordia have acquired the appearance of two finger-like projections within the embryo, but they have yet to begin migrating toward each other. $\beta_\nu$ transcripts accumulate as these two structures approach one another, and abundant expression of this gene is observed in the cells of the midgut endoderm as fusion and closure occurs. As the initial constriction develops in the now closed
midgut tube, expression of the $\beta_V$ mRNA is at first uniform. As further constrictions form, expression of the $\beta_V$ mRNA is diminished anteriorly while posterior expression is maintained.

In general, the distribution of the $\beta_V$ protein in embryos coincides with the expression pattern exhibited by its transcript. The protein is found exclusively in the developing midgut endoderm and is absent from the foregut, hindgut, and surrounding visceral mesoderm. One dissimilarity in the two expression patterns lies in the observation that while $\beta_V$ RNA expression is diminished anteriorly as midgut constrictions form, $\beta_V$ protein persists throughout the midgut endoderm.

Efforts to use the antiserum to identify $\alpha$ subunits associated with $\beta_V$ in either embryos or in larval midguts have so far been unsuccessful, apparently because of its rather restricted distribution in embryos and low overall level of expression which renders biochemical and immunological analyses difficult.

The tissue specificity of $\beta_V$ gene expression in the midgut is maintained postembryonically as revealed by whole-mount in situ hybridization analyses on dissected larval guts. The larval midgut is composed of large, polytene epithelial cells and clusters of smaller cells, the midgut imaginal islands. During prepupal and pupal stages of development, the larger epithelial cells undergo histolysis and are eventually sloughed off, while the midgut imaginal islands and imaginal rings undergo further proliferation and differentiation to give rise to the endoderm of the adult abdomen (Bodenstein, 1950). Thus, through a series of balanced degenerative and proliferative processes, the larval midgut is remodeled for its adult functions. Within the larval midgut, expression of the $\beta_V$ gene is seen in both cell types but is especially abundant in the midgut imaginal island cells and in the cells of the hindgut imaginal ring.

What does the expression pattern described above suggest about the possible role of the $\beta_V$ gene in the morphogenesis of the embryonic midgut? There are a number of cellular events in this process that might require the participation of adhesive molecules including $\beta_V$ together with any ligands and/or counter-receptors. For instance, the $\beta_V$ protein might be required for the proper movement of the anterior and posterior midgut primordia as they are brought into apposition for fusion. Alternatively, one could envision a role for the $\beta_V$ protein in holding the cells of each of the two midgut primordia together so that they move as a unit. Similarly, during midgut closure, sheets of epithelial cells, which form the midgut walls, are moved ventrally and then fused, followed by similar cellular movements dorsally. Once again, the $\beta_V$ protein could be functioning in movement or in holding together or fusing the migrating cell sheets. Furthermore, a role for the $\beta_V$ gene in the formation of midgut constrictions is also possible given the continued expression of this gene during these stages of midgut development. Finally, $\beta_V$ could play a role in the structure, polarity, and function of the midgut endoderm. $\beta_P$ is also expressed in the midgut (Leptin et al., 1989; Zusman et al., 1990) and in $mvs$ mutants there are major defects in midgut morphogenesis (Wright, 1960; Newman and Wright, 1981). Given the overlapping expression of the $\beta_V$ and $\beta_P$ subunits in the midgut, it will be of interest to determine the extent to which these two proteins act in concert to effect the morphogenesis of this structure.

Similarly, questions might be asked concerning the involvement of the $\beta_V$ gene during larval development. The abundance of $\beta_V$ transcripts in the cells of the midgut imaginal islands and in the hindgut imaginal ring are suggestive of potential roles for $\beta_V$ integrin complexes in the morphogenesis of adult abdominal structures. Obviously, detailed studies of the localization of the protein encoded by the $\beta_V$ gene using antibodies and the analysis of mutants will be necessary to determine the role that this gene plays during development. The gene for $\beta_V$ is located at 39B and does not correspond with any previously described mutations (S. E. Paine-Saunders, and R. O. Hynes, unpublished data). Further genetic analyses are underway.

The restriction of $\beta_V$ gene expression to the endodermal component of the midgut raises the question of how this tissue-specificity is established. Recent work has revealed some elements of a regulatory cascade which involves inductive interactions between the visceral mesoderm and the underlying endoderm that are required for midgut development (Immerglück et al., 1990; Reuter et al., 1990; Pangianban et al., 1990). In particular, a regulatory pathway has been formulated wherein the homeotic genes Ultrabithorax (Ubx) and abdominal-A (abd-A), which are expressed in cells of the visceral mesoderm, activate in these same cells the expression of the genes decapentaplegic (dpp) and wingless (wg) respectively. The dpp and wg genes both encode putative extracellular proteins, which activate the expression of the homeotic gene labial (lab) in the adjoining midgut endoderm by the binding of these proteins to surface receptors on the target cells. Such an inductive process has been proposed to allow the transfer of positional information from the highly segmented mesoderm to the apparently less segmented endoderm. It will be of obvious interest to determine if the $\beta_V$ gene is a downstream target of such regulatory circuits, which determine the identity of cells in the midgut. Furthermore, it will also be of interest to determine whether a $\beta_V$ integrin complex might play a role in adhesion of the two germ layers to each other so that such inductive processes can occur.

We have identified an additional member of the integrin receptor family in Drosophila which is expressed in a tissue-specific and developmentally regulated manner. Further work will be directed toward understanding the role of $\beta_V$-containing integrin complexes in midgut development and in elucidating the mechanisms that control the endoderm-specific expression of this gene. Cell biological and biochemical studies will be required to identify the $\alpha$ subunits that associate with the $\beta_V$ subunit and to determine the ligand-binding specificities of the resulting complexes. Genetic analysis should afford a means to dissect $\beta_V$ function in vivo.

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