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The lethal myospheroid gene of *Drosophila* encodes a membrane protein homologous to vertebrate integrin β subunits

(receptors/extracellular matrix/muscle/development/basement membranes)

ALBERT J. MACKRELL*, BRUCE BLUMBERG†‡, SUSAN R. HAYNES§, AND JOHN H. FESSLER*†¶

*Molecular Biology Institute and †Department of Biology, University of California, Los Angeles, CA 90024; and §Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **A mutant of *Drosophila melanogaster* carrying the lethal(1) myospheroid mutation [*l(1)mys*] has a defective musculature and a phenotype that suggests a defect of basement membranes. The genomic region that is interrupted by an insertion in a mutant carrying *l(1)mys* was used to isolate cDNA clones, and their sequences are presented here. The cDNA sequence predicts a cysteine-rich integral membrane protein that displays 45% sequence identity to chicken integrin and the human fibronectin receptor β subunit and much greater similarity over localized segments. These similarities extend to other vertebrate integrin β subunits, and we conclude that the myospheroid protein is an integrin β subunit of *Drosophila*. This implies evolutionary conservation of a group of transmembrane proteins that are receptors for extracellular matrix and, coupled with the myospheroid phenotype, indicates an important role for the interaction of cells with extracellular matrix during development.**

Interaction of vertebrate cells with extracellular matrix is important in development (1) and in maintenance of the differentiated state of cells (2). Receptors at cell surfaces are also likely to influence the assembly of newly synthesized macromolecules into specialized, adjacent extracellular matrix, such as basement membranes. To study these processes this laboratory has turned to the *Drosophila* experimental system, characterized several extracellular matrix macromolecules, and concluded that key components of basement membranes have been evolutionarily conserved (3, 4).

In vertebrates, some of the interactions of cells with extracellular matrix proteins are mediated by members of the integrin family. Integrins are heterodimeric integral membrane proteins, comprised of α and β subunits, that form a linkage between the extracellular matrix and cytoskeleton (5, 6). These receptors bind to ligands that usually contain the sequence Arg-Gly-Asp-$^{Ser}_{Thr}$ and form the RGD receptor group (6). It has been possible to disrupt these interactions with peptides containing this tetrapeptide sequence or with monoclonal antibodies selected for their ability to inhibit adhesion. These antibodies immunoprecipitate a group of proteins with similar properties. It appears that a multitude of receptors is produced by the association of many different α subunits with a small number of β chains. The β subunits have many cysteine residues at strictly conserved locations. The electrophoretic mobility of the β subunits is characteristically decreased by reduction, indicating intrapeptide disulfide linkages. The α subunits are variable in molecular weight and often dissociate into two peptides upon reduction. Dimeric molecules with similar electrophoretic properties were isolated from *Drosophila* using "position-specific" monoclonal antibodies (7). At least three different α-like chains are immunoprecipitated by antibodies directed against a protein with properties similar to vertebrate integrin β chains. NH_2-terminal sequences from one of these peptides were shown to be homologous to the NH_2 termini of vertebrate integrin α-subunits, and these antigens appear to be related to the vertebrate integrins (8).

The embryonic lethal myospheroid mutation of *Drosophila*, *l(1)mys*, at chromosome 1 location 7D1-5 (9) produces embryos that appear normal until the first muscular contractions, when gross abnormalities arise. Notably, the embryos rupture at their site of dorsal closure (the dorsal suture), and the embryonic muscles retract from their sites of attachment and become spheroidal (10). Although detailed study of these embryos showed fairly normal initial muscle development and indicated that the defect lay in basement membrane synthesis or assembly (10, 11), cell culture investigations showed a lack of fusion of myoblasts into myotubes, and an underlying lesion in cellular differentiation and/or cell interactions was suggested (12).

Recently, genomic clones from the region of the *l(1)mys* locus were isolated "on a walk along the X chromosome" (also known as chromosome 1). A mutant carrying *l(1)mys* has been shown to have a transposable element inserted in this region, and a transcription unit encompassing the site of insertion was identified. Correlation of the molecular and genetic data regarding the locations of *l(1)mys*, female sterile *fs(1)h*, and the deficiency $Df(1)sn^{c128}$ strongly suggests that this transcription unit represents the *l(1)mys* gene (13). Using probes made from the wild-type genomic fragment that is interrupted by the transposable element in the *l(1)mys* mutant, we have isolated cDNA clones containing the complete sequence of the protein encoded by this gene. Sequence analysis of these clones revealed a putative membrane protein with striking homology to the vertebrate integrin β subunits.‖

MATERIALS AND METHODS

Isolation of cDNA Clones. A 3.3-kilobase (kb) *Bgl* II fragment of the Charon 30 clone λφ8 (13) was subcloned into the plasmid vector pBluescribe (pBS, Stratagene Cloning Systems, La Jolla, CA). This vector is a derivative of pUC19 in which the multiple cloning sites are flanked by promoters for bacteriophage T3 and T7 RNA polymerases. RNA probes complementary to the distal *Bgl* II–*Hind*III fragment, into which the *B104* transposable element was inserted in the *l(1)mys* gene, were synthesized as described (4) and used to

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Abbreviation: myospheroid protein, product of the lethal(1) myospheroid locus *l(1)mys*.

‡Present address: Department of Medicine, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, 475 Hoes Lane, Piscataway, NJ 08854.

¶To whom reprint requests should be addressed at: Molecular Biology Institute, University of California, 405 Hilgard Ave., Los Angeles, CA 90024.

‖This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03251).

probe a cDNA library in λgt10 (4) made from RNA isolated from the *Drosophila* K_c cell line (14). The *Eco*RI inserts were subcloned into pBS and mapped with restriction enzymes. RNA probes complementary to the 5′ terminus of the existing clones were synthesized to isolate clones that extended further toward the 5′ end of the message. Seven of 42 clones isolated were chosen for further study.

DNA Sequence Analysis. Selected restriction fragments were subcloned into M13mp18, M13mp19 (15), or Phagescript (Stratagene). The cDNA sequence was determined by using the chain-termination technique (16) with adenosine 5′-[α-(^{35}S)thio]triphosphate (ATP[S]) and either avian myeloblastosis virus reverse transcriptase (Seikagaku, Tokyo) (4) or modified bacteriophage T7 DNA polymerase (Sequenase, United States Biochemicals, Cleveland) (17). The sequence was determined on both strands, across all restriction sites used in cloning, and, with the exception of the 5′-most 118 nucleotides, from at least two independent cDNA clones. The sequencing database was managed by using the DBSYSTEM (18), and analyses were performed with ANALYSEQ (19). Sequence alignments were performed with the programs SEQHP (20) and ALIGN (21) using the mutation data matrix.

RESULTS

Isolation and Characterization of cDNA Clones. A λgt10 cDNA library was screened with sequences from the *l(1)mys* gene. Forty-two clones were isolated of which seven were selected for further study and restriction-enzyme mapping (Fig. 1). This revealed that clone 1 contained introns. This library was made from total RNA, and a small number of clones with introns have been recovered from it (22).

Sequence Analysis of cDNA. From the cDNA clones shown in Fig. 1, the sequence of 3909 nucleotides of the *l(1)mys* message, extending from the 5′ end of clone 29 to the consensus polyadenylylation signal (23) and poly(A) tail were determined. This size agrees well with the 4.4-kilobase (kb) major message reported for this gene (13). The 5′-most 2880 nucleotides of this sequence, containing the entire protein-encoding region, are shown in Fig. 2.

The predicted initiation codon, as shown in Fig. 2, is the first ATG codon of a long open reading frame. This ATG is preceded by a stop codon in the same reading frame and lies within sequences that agree well with the consensus that has been determined for the initiation of translation in eukaryotes (24). The sequence of 23 amino acids beginning with this methionine is an efficient signal sequence (25), with a consensus cleavage site (26) shown by the arrow in Fig. 2. The sequence contains only one other hydrophobic region (27) of 23 amino acids, which is the membrane-spanning domain (28). The sequence predicts a transmembrane protein of 90 kDa following removal of the signal peptide. There are six consensus sites for the glycosylation of asparagine residues (29, 30), which are indicated by triangles.

Homology of *l(1)mys* Gene Product to Integrin β Subunits. A striking feature of the product of the lethal(1) myospheroid locus *l(1)mys* (myospheroid protein) is the abundance of cysteine residues. Thirty of them occur as four repeated units, where they constitute 18% of the amino acids and are arranged in a motif that is characteristic of vertebrate integrin β subunits (5). The substantial degree of identity with the chicken integrin β (31) is shown in Fig. 3. The sequences are essentially colinear, with the exception of a serine-rich region in the *Drosophila* protein. Of the 57 cysteine residues in the myospheroid protein, 56 are shown aligned. While the level of sequence conservation is not as great in the region on the NH_2-terminal side of the serine-rich region, the similarity of the proteins in this region is significant to 7 SD. The spacing of these cysteines is similar but not identical, and at least 6 of the 7 cysteines in this region are flanked by other identical and conservatively substituted amino acids. On the carboxyl side of the serine-rich region, the proteins show substantial similarity, including 49 conservatively located cysteine residues. We conclude that the myospheroid protein is evolutionarily related to the integrin family of extracellular matrix receptors.

Detailed comparisons were made of the myospheroid protein sequence with the following members of the integrin β family of cell-surface molecules: chicken integrin β, the human platelet membrane protein IIIa (32) and the human β subunit of leukocyte adhesion proteins (LAβ) (33, 34). Over its entire length, the myospheroid protein is 45% identical to chicken integrin β (351/779), 41% identical to the human platelet membrane protein IIIa (312/762), and 39% identical to the β subunit of leukocyte adhesion proteins (294/757). The human fibronectin β subunit is the human homologue of

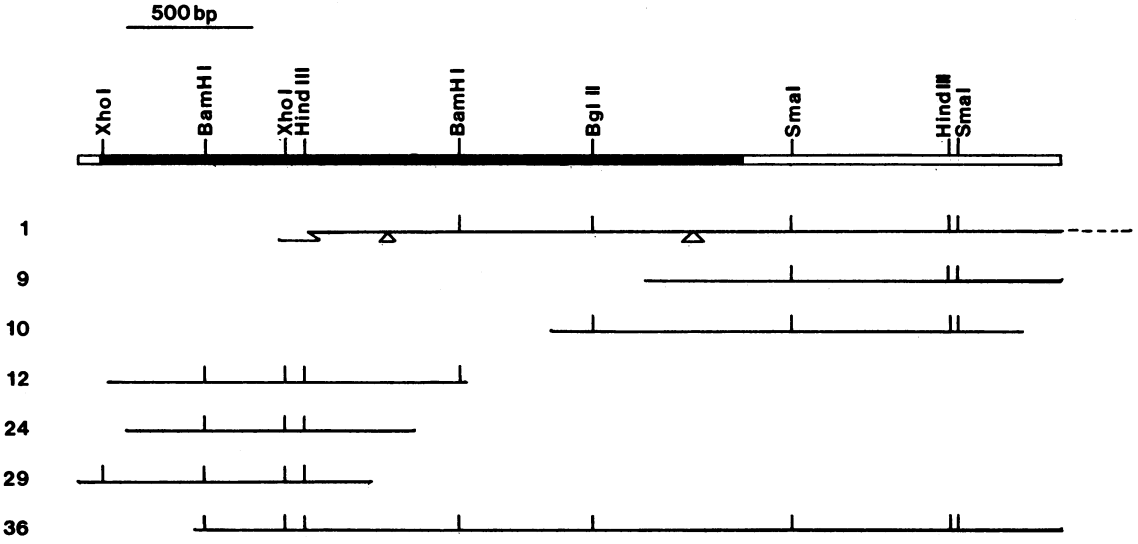

FIG. 1. Structure of *l(1)mys* cDNA clones. The seven cDNA clones that were used in the sequence analysis are shown as fine horizontal lines. The composite cDNA extending from the 5′ end of clone 29 to the poly(A) tail, which is contained in clone 9, is shown as a box, with the black area representing the protein coding sequences. A partial restriction map of this composite is shown along with the locations of these sites in the individual clones. Clone 1 appears to derive from unprocessed nuclear RNA, as it contains intronic sequences. The location and size of the introns are depicted by the triangles, while the sequences extending beyond the poly(A) tail are indicated by the dashed line.

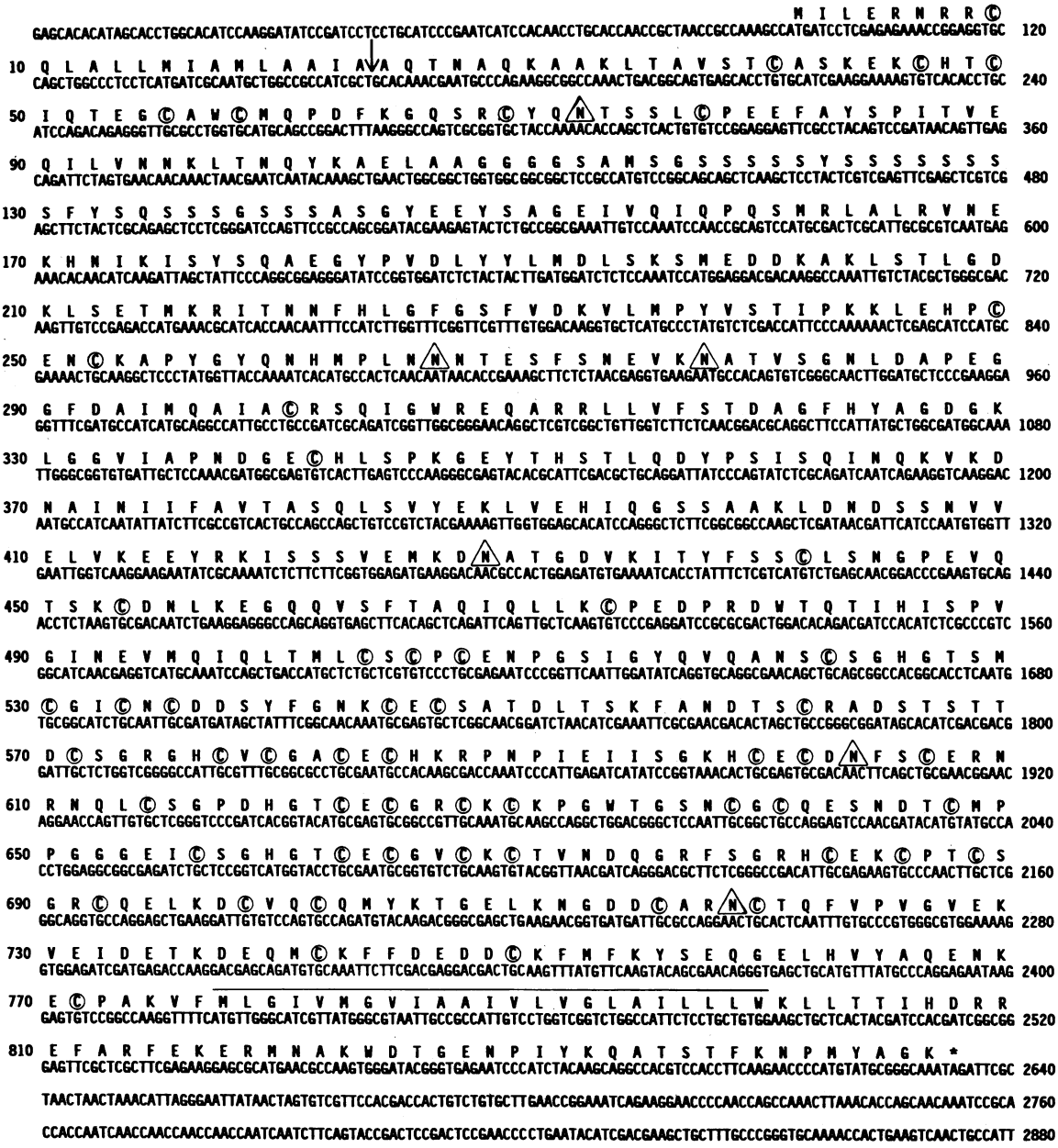

FIG. 2. Nucleotide sequence and deduced protein sequence of myospheroid protein. Amino acids are shown in the single-letter code; 2280 nucleotides of the *l(1)mys* cDNA beginning with the 5′ end of clone 29 and containing the entire protein-encoding sequence are shown along with the translation of a long open reading frame. The signal cleavage site is marked by the vertical arrow, while the predicted transmembrane domain is marked by a line over the sequence. Cysteine residues are circled. Consensus sequences for glycosylation at asparagine residues are marked by triangles, and additional potential glycosylation sites are at residues 403, 557, and 644. Amino acid residue numbers are indicated at the left margin of the figure, and nucleotide sequence numbers are shown on the right.

chicken integrin β, and their sequences show 85.5% identity (35). Correspondingly, the identities between the myospheroid protein and this very recently published sequence are highly similar to those shown in Fig. 3. All 56 cysteine residues in the mature myospheroid protein have counterparts in analogous positions in all four vertebrate proteins.

Over localized regions of the protein sequence, these identities are even more pronounced. The intracellular domains of the myospheroid protein and the three vertebrate β subunits are shown aligned in Fig. 4*A*. The first 33 amino acids of this domain are 88% identical between myospheroid protein and chicken integrin β, and two of the four mismatches are conservative substitutions of lysine for arginine. There is substantial identity in this region between myospheroid protein and the platelet membrane protein IIIa (73%), while the leukocyte protein is substantially less similar in this region to any of the other proteins (42% identical with myospheroid protein). Of particular interest in this regard is the tyrosine residue at position 831 in the myospheroid protein sequence (marked with an asterisk in Fig. 4*A*). The sequences surrounding this residue in three of the proteins shown agree well with the consensus for substrates of protein-tyrosine kinases (31), and it has been shown that the cytoplasmic domain of chicken integrin is phosphorylated on tyrosine in transformed cells (36). Also, all four cytoplasmic domains are nearly identical in length (46 or 47 amino acids), suggesting that the size of this domain plays some important role in its normal function.

Another region of the myospheroid protein that shows substantial identity with the vertebrate integrin β chains is found in the extracellular portion of the molecule. Overall, the extracellular portions of the myospheroid protein and the

FIG. 3. Alignment of the myospheroid protein sequence with chicken integrin β. The amino acid sequences of the myospheroid protein and chicken integrin β were compared and aligned using the program ALIGN. The sequences are essentially colinear over their entire length, with the exception of a 40-amino acid serine-rich region of the myospheroid sequence. The 56 conserved cysteine residues are indicated by the vertical lines, while other identities are marked by colons between the sequences. For reference, the membrane spanning region is indicated by a line over the sequence. The amino acid position in the myospheroid sequence is indicated by the numbers in the right margin. Sequences are in the single-letter amino acid code.

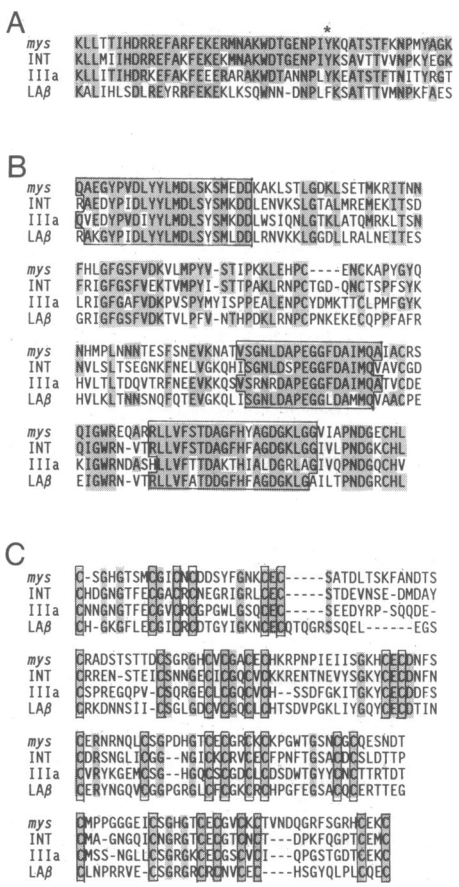

FIG. 4. Local regions of homology of myospheroid protein with integrin β subunits. The amino acid sequences of the myospheroid protein (mys), chicken integrin β (INT), β subunit of human platelet membrane protein IIIA (IIIa), and β subunit of human leukocyte adhesion protein (LAβ) were aligned. Amino acids identical between myospheroid protein and any of the other three proteins are indicated by shading in *A* and *B*. (*A*) Complete intracellular domains, beginning with the lysine that marks the end of the transmembrane domain (myospheroid protein residues 800–846). The tyrosine, which is a consensus substrate for protein-tyrosine kinases, is shown by the asterisk. (*B*) A highly conserved region of the extracellular domain of the chicken integrin β subunits extending from amino acids 179 to 343 of the myospheroid protein sequence. The three most highly conserved regions are boxed. (*C*) Sequences of the four cysteine-rich pseudorepeats are shown, one repeat per line, with the conserved cysteine residues boxed (myospheroid protein residues 522–685). All amino acids that are conserved in all four proteins are shaded.

chicken integrin β chain are 42% identical. The region of these two molecules shown in Fig. 4*B* is 57% identical. This similarity is concentrated largely in three highly conserved segments, which are boxed in Fig. 4*B*. The first boxed region of the myospheroid protein, which extends from amino acid 179 to 200, displays 81% identity to chicken integrin β and the leukocyte protein (17 of 21 residues) and 77% identity to platelet membrane protein IIIa (17 of 22 residues). In the central portion of this box, 11 of 12 amino acids of the myospheroid protein are identical with each of the three vertebrate molecules. A larger highly conserved region spans 63 amino acids from positions 280 to 332 in the myospheroid sequence. This region is 76% identical in myospheroid protein and chicken integrin β and is bounded by two highly conserved domains, which are boxed. The first boxed region of myospheroid protein is 94% identical (15 of 16 residues) to chicken integrin β, 89% identical (16 of 18 residues) to platelet protein IIIa, and 88% identical (14 of 16 residues) to the leukocyte protein. In the third boxed region of Fig. 4*B*, myospheroid protein is 95% identical to chicken integrin β (20 of 21 residues) and 85% identical to the leukocyte protein (17 of 20 residues). There is substantially less identity to the platelet protein (65%, 13 of 20 residues).

The cysteine-rich repeat regions of these four proteins are shown aligned in Fig. 4*C*. While the locations of the 30 cysteine residues have been preserved, the amino acid sequences of the regions separating the cysteines are not highly conserved. In the alignment shown in Fig. 4*C*, only 18 noncysteine amino acids are in identical positions in all four proteins. Of these 18 absolutely conserved residues, 12 are glycines. The apparent absolute requirement for glycine at several positions in this region implies a requirement for small side chains, perhaps due to a highly compact, convoluted folding pattern.

DISCUSSION

From the preceding comparisons of the amino acid sequence derived from the cloned cDNA from the region of the lethal(1) myospheroid locus of *Drosophila*, with members of the integrin family of extracellular matrix receptors, we

conclude that this cDNA encodes an integrin β subunit. This conclusion correlates extremely well with the known phenotype of *l(1)mys*, further supporting the previous identification of this transcription unit as the *l(1)mys* gene (13). The homology of this *Drosophila* protein with the integrin family is notable with respect to the cysteine residues; all 56 cysteine residues of the mature protein, which may contribute to a compact, disulfide-linked structure that causes these proteins to migrate more slowly in $NaDodSO_4$/polyacrylamide gel electrophoresis after reduction, have been conserved. The protein recognized by antibodies to position-specific antigen 3 is a candidate as a *Drosophila* integrin β subunit and has a similar change of electrophoretic migration upon reduction (7).

The intracellular domain of the myospheroid protein exhibits substantial homology to some of the vertebrate integrin β chains. The length of this domain is highly conserved, and the amino acid sequence differences that exist represent conservative substitutions. This suggests a conservation of functional interactions of this domain with adjacent molecules, possibly components of the cytoskeleton or integrin α-subunits.

The extracellular domains of the β subunits contain regions of substantial sequence identity. This region may be involved in conserved functions of these proteins (e.g., interaction with the α subunits and formation of the binding site for the Arg-Gly-Asp-containing segments of their ligands). However, much of the extracellular domain, including the sequences separating the blocks of greatest similarity, is rather divergent in these proteins. This could reflect either a randomization of these sequences or the formation of β subunits with divergent properties. These differences could influence the repertoire of α subunits with which a given β subunit can interact. Different β subunits, in combination with a single α subunit, could generate receptors with different specificities or different affinities for a given ligand.

The earlier observations on the mutant phenotype of *l(1)mys* (10, 11) and on the cells of these embryos in culture (12) can now be accounted for in an interesting way by a defective extracellular matrix receptor. The prime mechanical failure is at muscle insertions; thus, the attachment of cells to adjacent extracellular matrix is defective. The presence of receptors for extracellular matrix proteins may dramatically accelerate their incorporation into supramolecular assemblies. For instance, stimulation of the expression of fibronectin receptors leads to a greater-than-proportional increase in the assembly of fibronectin into cell-associated matrices (37). In *l(1)mys* embryos, the accumulation of extracellular matrix is delayed as seen by periodic acid/Schiff reagent staining (10) and in electron micrographs (11). Therefore, a combination of effects may cause damaged basement membranes. It was recently reported (38) that at the cell culture level, treatment of vertebrate myoblasts with an antibody to an integrin receptor prevented a differentiation step that is essential prior to myoblast fusion (38). The same step might fail to occur in explanted cells of *Drosophila l(1)mys* embryos because these cells fail to fuse in culture (12). Therefore, the myospheroid protein may have important developmental functions that are distinct from its contribution to the mechanical requirements of the organism.

The widespread occurrence of the amino acid sequence Arg-Gly-Asp-$^{Ser}_{Thr}$ at adhesion sites greatly contributed to the identification of the integrin group of receptors (6), and a *Drosophila* integrin receptor may be expected to recognize such a sequence. Naidet *et al.* (39) found that injection of a peptide containing this sequence into *Drosophila* embryos caused arrest of development at gastrulation. If an integrin receptor is essential for gastrulation, then its β subunit will be encoded by a different gene than the one described here because *Drosophila l(1)mys* embryos proceed normally through gastrulation, even without a contribution from wild-type maternal copies of this gene. Embryos derived from maternal germ-line clones that lack the *l(1)mys* gene were deformed in later development but proceeded through gastrulation normally (40). Therefore, characterization of a potential repertoire of *Drosophila* integrin β subunits may help our understanding of several interactions of cells with extracellular matrix during development.

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