Characterization of βpat-3 Heterodimers, a Family of Essential Integrin Receptors in *C. elegans*

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Abstract. Members of the integrin family of cell surface receptors have been shown to mediate a diverse range of cellular functions that require cell–cell or cell–extracellular matrix interactions. We have initiated the characterization of integrin receptors from the nematode *Caenorhabditis elegans*, an organism in which genetics can be used to study integrin function with single cell resolution. Here we report the cloning of an integrin β subunit from *C. elegans* which is shown to rescue the embryonic lethal mutation *pat-3(rh54)* and is thus named βpat-3. Analysis of the deduced amino acid sequence revealed that βpat-3 is more similar to *Drosophila* integrin βPS and to vertebrate integrin β1 than to other integrin β subunits. Regions of highest homology are in the RGD-binding region and in the cytoplasmic domain. In addition, the 56 cysteines present in the majority of integrin β subunits are conserved. A major transcript of ~3 kilobase pairs was detected by RNA blot analysis. Immunoblot analysis using a polyclonal antiserum against the cytoplasmic domain showed that βpat-3 migrates in SDS-PAGE with apparent M_r of 109 k and 120 k under nonreducing and reducing conditions, respectively. At least nine protein bands with relative molecular weights in the range observed for known integrin α subunits coprecipitate with βpat-3, and at least three of these bands migrate in SDS-PAGE with increased mobility when reduced. This behavior has been observed for a majority of integrin α subunits. Immunoprecipitations of βpat-3 from developmentally staged populations of *C. elegans* showed that the expression of several of these bands changes during development. The monoclonal antibody MH25, which has been postulated to recognize the transmembrane component of the muscle dense body structure (Francis, G. R., and R. H. Waterston. 1985. Muscle organization in *Caenorhabditis elegans*: localization of proteins implicated in thin filament attachment and I-band organization. *J. Cell Biol.* 101:1532–1549), was shown to recognize βpat-3. Finally, immunocytochemical analysis revealed that βpat-3 is expressed in the embryo and in many cell types postembryonically, including muscle, somatic gonad, and coelomocytes, suggesting multiple roles for integrin heterodimers containing this β subunit in the developing animal.

THE interactions of cells with their environments are critical for the development and maintenance of tissues in multicellular organisms. Members of one family of cell surface receptors, the integrins, have been shown to mediate biological processes requiring cell–cell or cell–extracellular matrix adhesion such as cell attachment, cell migration, and neurite outgrowth (for review see Hynes, 1992). Each receptor is a heterodimer composed of noncovalently associated α and β subunits. To date, fourteen α subunits and eight β subunits have been identified in vertebrates. Ligands of integrin receptors include extracellular matrix proteins, such as laminin, fibronectin, and several collagens, as well as cell surface proteins, such as VCAM-1 and the three ICAMs.

In vitro studies and immunocytochemical analyses have suggested several roles for integrins in developmental processes. However, few experiments have addressed integrin function during development in vivo. In chick, injection of an anti-β1 antibody has been shown to interfere with neural crest cell migration (Bronner-Fraser, 1986), and anti-sense experiments have suggested that β1 integrins are required for neuroblast migration in the optic tectum (Galileo et al., 1992). In *Pleurodeles* embryos, injection of anti-β1 antibodies was found to inhibit gastrulation (Darribere et al., 1988). To date, the analysis of the *Drosophila melanogaster* integrin βPS subunit mutant *lethal(l)myospheroid* has been the most comprehensive study of integrin function during develop-

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ment (e.g., Zusman et al., 1990, 1993). *Lethal(l)myospheroid* is an embryonic lethal mutation that results in defects in muscle attachment, dorsal closure, and constriction of the gut. Postembryonic phenotypes of *lethal(l)myospheroid* include defects in wing morphogenesis and organization of photoreceptors in the retina. Surprisingly, no phenotypes due to defects in cell migration or axon outgrowth were observed, although subtle phenotypes may not have been detected.

C. elegans is a particularly attractive experimental animal for the study of integrin function during development because each cell in the animal can be visualized, and the identity and lineage of each somatic cell is known from the embryo to the adult (Sulston and Horvitz, 1977; Sulston et al., 1980, 1983). In addition, *C. elegans* is amenable to genetic analysis; thus, the localization and function of integrins in single cells can be determined in exquisite detail. In this report, we describe the cloning of an integrin β subunit, the primary sequence of its cDNA and the organization of its gene (Sequence information has been deposited in Gene Bank. The accession name and number are "integrin beta pat-3 U19744"). We show that this gene rescues the *C. elegans* embryonic lethal mutation *pat-3(rh54)*; thus, it will be referred to as βpat-3. A polyclonal antiserum prepared against the cytoplasmic domain of βpat-3 was used for immunoblot analysis, immunoprecipitations and immunocytochemistry. The immunoprecipitations showed that at least nine protein bands coprecipitate with βpat-3; these bands have relative molecular weights in the same range as known integrin α subunits. Immunocytochemical analysis showed that βpat-3 is first expressed in the embryo and is subsequently widely expressed in larvae and adult animals. The complex pattern of the βpat-3 expression and the potential for the association with multiple α subunits suggest that receptors containing βpat-3 have many important roles in *C. elegans* development.

Materials and Methods

Polymerase Chain Reaction Amplification of an Integrin β Subunit from C. elegans

Degenerate oligonucleotides for use in PCR were designed from the deduced amino acid sequences of previously sequenced integrin β subunits. The conditions for PCR were denaturing temperature, 94°C for 1 min; annealing temperature, 45°C for 1 min; extension temperature, 55°C for 3 min; forty cycles were run in a thermocycler. The reaction mixes for PCR contained 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 1 μg of each primer, 1 μg of *C. elegans* strain N2 genomic DNA, and 0.62 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). Oligonucleotide primers designed from the integrin β subunit ligand-binding region [β135#7 (5'GA(CT)CT(N)TA (CT)TA(CT)(CT)T(N)ATGGA); β135#8 (5'TC(N)AC(AG)AA(N)GA(N) CC(AG)AA(N)CC) where N = A,C,G, and T], were successful in amplifying a DNA fragment of the predicted size. The amplified DNA was subcloned and sequenced; 16 of the 32 predicted amino acids were identical to the corresponding region from human integrin β1.

Isolation of βpat-3 Genomic and cDNA Clones

2×10^4 plaques from a *C. elegans* strain N2 λEMBL3 genomic library were transferred to nitrocellulose filters (Schleicher and Schuell, Inc., Keene, NH) which were prehybridized and hybridized in a modified Church buffer (0.5 M sodium phosphate, pH 7.2; 7% SDS; 1 mM EDTA; 1% BSA; 10% formamide) at 55°C (Church and Gilbert, 1984) and washed with 2 × SSC with 0.1% SDS at 50°C. Seven hybridizing clones were purified.

5×10^5 plaques from a *C. elegans* strain N2 2–3 kb size-selected λSHLX2 cDNA library provided by Dr. Chris Martin were screened as described above, except the prehybridization and hybridization temperatures were 50°C. The radiolabeled DNA used to screen the cDNA library was prepared from a gel-purified 5-kb SalI fragment derived from phage clone (2-4) isolated in the genomic library screen. Eleven hybridizing clones were isolated.

Subcloning and Sequencing

Restriction enzyme mapping by single and double restriction enzyme digests and Southern blot analysis of the seven genomic phage clones using the radiolabeled probe described for the genomic library screen were carried out according to Maniatis et al. (1982). A 5-kb SalI fragment was subcloned from genomic phage clone (2-4) and partially sequenced using oligonucleotide primers β135 5' and β135 3' to verify that the clone contained sequence homologous to integrin β subunits. A 9-kb SalI fragment from genomic phage clone (1-1) that hybridized with oligonucleotides from the 5' (β135-PE) and 3' (β135-7rev) ends of the cDNA sequence was subcloned and sequenced to determine intron and noncoding 5' and 3' sequence. cDNA phage clone 1-1 contained the longest insert and was therefore sequenced completely. The 5' ends of the other clones were sequenced and found to contain sequence identical to that found in cDNA 1-1.

PCR-amplified DNA, cDNA inserts, and DNA fragments from the genomic phage clones were subcloned into M13, pIBI (IBI, New Haven, CT), or Bluescript (Stratagene, La Jolla, CA). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA) or Boehringer/Mannheim Corp. (Indianapolis, IN). The nucleotide sequence of cDNA clone 1-1 was determined from a single-stranded template derived from M13; both DNA strands were sequenced. The nucleotide sequences of the PCR products and the genomic clones were determined from double-stranded templates. The PCR-amplified DNA, cDNA 1-1, and the exon/intron borders in the 9-kb SalI fragment from genomic phage clone 1-1 were sequenced using the dideoxy chain termination method of Sanger et al. (1977), and reagents from the Sequenase kit (U. S. Biochemicals, Cleveland, OH). Intron sequence was determined by the Biomolecular Resource Center DNA sequencing facility (UCSF) using a PCR System 9600 (Perkin Elmer Cetus) for DNA amplification and a 370A DNA sequencer (Appl. Biosystems, Inc., Foster City, CA). Gene-specific oligonucleotide primers for sequencing were synthesized at the Biomolecular Resource Center and the oligonucleotide synthesis facility in the Howard Hughes Medical Institute at UCSF.

DNA and Protein Sequence Analysis

DNA and amino acid sequence analysis was performed using PCGene (IntelliGenetics, Mountain View, CA). Alignments of the amino acid sequences of integrin β subunits were performed using the Pattern-Induced Multisequence Alignment (PIMA) algorithm (Smith and Smith, 1991). Sequences between cysteines not aligned using the PIMA algorithm were aligned using the alignment program in PCGENE.

RNA Blot Analysis

Total cellular RNA was prepared from mixed populations of worms using the method of Rosenquith and Kimble (1988). RNA was electrophoresed in formaldehyde-containing agarose gels according to Maniatis et al. (1982), transferred onto a Hybond-N membrane (Amersham Corp., Arlington Heights, IL) using 20× SSC, and cross-linked to the membrane with ultraviolet light. The membrane was prehybridized and hybridized in a modified Church buffer (0.5 M sodium phosphate, pH 7.2; 7% SDS; 1 mM EDTA; 1% BSA; 30% formamide) and washed with 1× SSC with 0.05% SDS at 55°C. cDNA 1-1 was radiolabeled with [^{32}P]dCTP using random primers (Boehringer/Mannheim) to probe the RNA blot. The relative mol wt of the βpat-3 transcripts was determined by comparison with RNA mol wt standards purchased from GIBCO/BRL (Gaithersburg, MD).

Generation of Peptide Antiserum

A peptide consisting of an NH_2-terminal cysteine plus the 27 COOH-terminal amino acids of the deduced sequence of the cytoplasmic domain of βpat-3 (CKWDTNENPIYKQATTTFKNPVYAGKAN) was coupled to keyhole limpet hemocyanin (KLH) (Calbiochem-Behring Corp., San Diego, CA) and sent to Caltag Laboratories (South San Francisco, CA) for immunizations into pathogen-free rabbits. For affinity purification, an IgG fraction was prepared by precipitation with ammonium sulfate and purification

on DEAE-cellulose (DE-52, Whatman, Clifton, NJ) as described in Harlow and Lane (1988). IgG-containing-fractions were pooled and run over a column containing the βpat-3 cytoplasmic peptide coupled to thiopropyl-Sepharose 6B (Pharmacia LKB Biotechnology, Piscataway, NJ). The affinity-purified antibody, which will be referred to as anti-βpat-3cyto, was used for all of the experiments.

Nematodes

C. elegans strain Bristol (N2) worms were used for all experiments except that the temperature-sensitive dauer-constitutive mutant *daf-2(e1370)* was used to obtain a pure population of dauer larvae. N2 and *daf-2(e1370)* worms were propagated at 20°C on agar in petri dishes seeded with *Escherichia coli* strain OP50 as a food source (Brenner, 1974). Animals were staged by dissolving gravid hermaphrodites in bleaching solution to release embryos (Emmons et al., 1979). For each staged sample collected, 100 worms were checked by Nomarski microscopy to determine the percentage of worms of each stage. To obtain dauers, *daf-2(e1370)* embryos were placed at the restrictive temperature of 25°C and the hatchlings monitored until dauers appeared on the plates. Subsequently, the *daf-2(e1370)* worms were kept at 25°C for an additional 24 h to ensure that the entire population had entered the dauer state. For biochemical analysis, worms were collected in M9 buffer and pelleted in a clinical centrifuge setting at medium speed; pellets were frozen in a dry ice/ethanol bath and stored at −80°C if not used immediately.

Extraction of Proteins from Worms

To extract protein, 1 ml of ice cold Western lysis buffer (20 mM Tris-Cl, pH 7.5; 0.32 M sucrose) or immunoprecipitation lysis buffer (50 mM Tris-Cl, pH 8.0; 150 mM NaCl; mM $MgCl_2$; 1 mM $CaCl_2$; 1% Triton X-100 for radiolabeled proteins or PBS/0.1 M Hepes, pH 7.0 with 0.32 M sucrose for proteins to be labeled with biotin) supplemented with freshly added proteinase inhibitors (50 μg/ml final chymostatin, leupeptin, aprotinin, and pepstatin; 1 mM PMSF (Sigma Chem. Co., St. Louis, MO) or 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochlorine) (AEBSF) (ICN Biochemicals, Cleveland, OH) and 0.5 ml white quartz sand (Sigma) was added per 0.2–0.5 ml pellet of worms. The worms were vortexed at maximum speed for 45 s alternating with 30 s on ice until most of the worms were lysed. The extract was transferred to an Eppendorf tube and spun at low speed (1,000 *g*) at 4°C for 2 min to remove debris. The supernatant was transferred to a fresh Eppendorf tube and spun at 2,500 *g* at 4°C for 5 min to remove nuclei and cytoskeleton.

Immunoblot Analysis

For immunoblot analysis of βpat-3, protein extracts were spun at high speed (15,000 *g*) at 4°C for 30 min to pellet membranes. The membranes were resuspended in 6× Laemmli buffer and the concentration of protein determined by the Amido Schwartz method. The samples were electrophoresed through a 6% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane (Schleicher and Schuell). The nitrocellulose was blocked with Blotto (50 mM Tris-Cl, pH 7.5; 150 mM NaCl; 5% Carnation dry milk); incubations with antibodies were also done in Blotto for 1 h at room temperature. Anti-βpat-3cyto, MH25, and the βpat-3 cytoplasmic peptide used for competition experiments were diluted to a final concentration of 10 μg/ml. Alkaline phosphatase-conjugated anti-rabbit secondary antibodies (Promega, Madison, WI) were diluted 1:7,500. The blots were washed 3× for 5 min with Blotto after each antibody incubation and 1× with alkaline phosphatase buffer (0.1 M Tris-Cl, pH 9.0; 0.1 M NaCl; 5 mM $MgCl_2$) before incubation with nitroblue tetrazolium (NBT) (50 mg/ml) diluted 1:150 and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (50 mg/ml) (Sigma) diluted 1:300 in alkaline phosphatase buffer.

Radiolabeling of Worms

To label worm proteins with ^{35}S, worms were fed radiolabeled bacteria prepared as follows: OP50 bacteria were grown to saturation in LB at 37°C and diluted 1:100 in low-sulfate M9 (M9 containing 0.5 mM magnesium sulfate) supplemented with 0.1% glucose, 1 mM uracil, and 0.01 mg/ml thiamine. The culture was grown again to saturation, diluted 1:100 in low sulfate medium a second time and grown until a density of OD600 = 0.2 was reached. At this point, 500 μCi of [^{35}S]methionine, 500 μCi of [^{35}S]-cysteine, and 200 μCi of $^{35}SO_4$ (New England Nuclear, Boston, MA) were added to the culture; the culture was returned to 37°C until saturation was reached. The radiolabeled bacteria were pelleted, resuspended in 2 ml of low-sulfate M9 and plated onto 2 10-cm NG agar plates. The plates were left at room temperature overnight to allow the bacteria to grow to confluency. 200 μl of a mixed population of worms were placed on each plate until no trace of bacteria remained. The worms were shaken gently in M9 for 15 min and washed 3× in M9 before protein was extracted.

Nonradioactive Labeling of Worm Protein Extract

Before labeling, the protein extract was spun at high speed (15,000 *g*) to pellet membranes. The supernatant was discarded and the pellet resuspended in 1 ml of PBS/0.1 M Na-Hepes, pH 7.0, supplemented with proteinase inhibitors as described above. 20 μl of 10 μg/ml freshly prepared sulfo-NHS-biotin (Pierce, Rockford, IL) were added to the protein extract; the extract was incubated at 4°C for 2 h with rocking. To stop the labeling reaction and remove unreacted biotin, 100 μl of 1 M Tris-Cl, pH 8.0, was added to the extract and the membranes were pelleted at 27,000 *g* at 4°C for 25 min and washed 3× with PBS/0.1 M Tris-Cl, pH 8.0, supplemented with PMSF or AEBSF. After the final wash, the membranes were solubilized in 1 ml ice cold immunoprecipitation buffer (100 mM Tris-Cl, pH 8.0; 150 mM NaCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 1% Triton X-100) plus PMSF or AEBSF. The extracts were precleared with 100 μl of protein A–Sepharose (Pharmacia) for 1 h at 4°C with rocking followed by a preclear step with 100 μl of protein A–Sepharose plus 30 μl of preimmune serum or nonimmune serum for 1 h at 4°C.

Immunoprecipitations

To immunoprecipitate βpat-3, a protein extract derived from radiolabeled worms or the precleared supernatant derived from a biotinylated protein extract was divided into the same number of Eppendorf tubes as samples (200–500 μl/sample) and 20 μl of serum or 20 μg of purified antibodies were added to each sample. The samples were rocked at 4°C overnight. The samples were precleared 2× for 1 min with 75 μl of Sepharose Cl-4B beads (Pharmacia LKB Biotechnology) before incubation with 75 μl of protein A–Sepharose Cl-4B beads (Pharmacia LKB Biotechnology) at 4°C for 45 min. After the incubation, the beads were washed 5× with an excess volume of wash buffer (50 mM Tris-Cl, pH 8.0; 150 mM NaCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 0.2% SDS, 0.1% Triton X-100 for radiolabeled samples; 100 mM Tris-Cl, pH 8.0, 500 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100, 0.2% SDS for biotinylated samples).

To visualize the immunoprecipitated proteins, the precipitates were resuspended in 6× Laemmli buffer, boiled for 3 min, and electrophoresed through a 6% SDS-polyacrylamide gel. Gels containing radiolabeled samples were stained with Coomassie blue, destained, dried, sprayed with En^{3}Hance (New England Nuclear), and exposed to Kodak X Omat AR film (Rochester, NY). Nitrocellulose blots of biotinylated proteins were blocked for 1 h at room temperature with 10% BSA fraction V (Sigma) and 0.05% Tween-20 before incubation for 1 h at room temperature with alkaline-phosphatase conjugated streptavidin (Accurate, San Diego, CA) diluted 1:50,000 or with HRP-streptavidin (Zymed, South San Francisco, CA) diluted 1:4,000 in 1% BSA, fraction V plus 0.05% Tween-20. Blots incubated with alkaline-phosphatase conjugated streptavidin were washed 3 × 5 min at room temperature with PBS, pH 7.0, and developed as described above. Blots incubated with HRP-streptavidin were washed 1 × 5 min at room temperature with 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 2 × 5 min at room temperature with 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 2 × 5 min at room temperature with 50 mM Tris-Cl, pH 7.5, 150 mM NaCl and developed with ECL reagents (Amersham Corp.) according to the manufacturer's instructions. Relative molecular weights of the proteins were determined by comparison with molecular weight standards purchased from BioRad (Richmond, CA).

Two-dimensional SDS-PAGE of immunoprecipitated biotinylated proteins was carried out as follows: In the first dimension, samples were electrophoresed through a 6% SDS-polyacrylamide gel under nonreducing conditions. Nonreduced prestained markers (Sigma) as well as biotinylated molecular weight standards (BioRad) preincubated with iodoacetamide were added to the sample to visualize the location of the sample in the gel, and iodoacetamide was added to a final concentration of 2.5%. After electrophoresis, a strip of gel containing the sample was incubated in 2× Laemmli buffer with 10% β-mercaptoethanol for 1 h at room temperature followed by 5 min on a hot plate at 100°C. For electrophoresis in the second dimension, the gel strip was rotated 90° and placed at the top of the gel plates, the gel plates were sealed, and a 4% SDS-polyacrylamide stacking gel followed by a 7.5% SDS-polyacrylamide gel was poured on top of the

strip. The sample was electrophoresed through the gel, electroblotted and developed for the alkaline phosphatase reaction as described above.

Immunocytochemistry

Worms were prepared for immunocytochemistry using the freeze/crack method according to Wood (1988). Briefly, 10–12 μl of worms in distilled water were placed on microscope slides freshly coated with 0.1% poly-L-lysine and allowed to settle. After a coverslip was placed on top of the worms, the slide was placed under a dissecting microscope for viewing and liquid withdrawn with bibulous paper until the worms were immobile. The slides were then frozen on dry ice for a minimum of 5 min, the coverslip removed with a razor blade, and the slides stored at −80°C until used for staining. The fixation of the animals consisted of an incubation for 5 min in either −20°C methanol or −20°C ethanol, followed by 1 min in −20°C acetone and three washes in PBS, pH 7.0, for 10 min. The slides were blocked for 1 h at 37°C with 5% BSA, fraction V, 5% normal goat serum (NGS)[1] in PBS, pH 7.0, before incubation for 2 h at 37°C with anti-βpat-3cyto diluted 1:200 or MH27 (a control for permeabilization) diluted 1:500 in 5% BSA/5% NGS in PBS, pH 7.0. The slides were washed 3× in an excess volume of PBS, pH 7.0, before incubation for 1 h at 37°C with TRITC-conjugated goat anti-rabbit or anti-mouse antibodies (Accurate Chemical & Scientific Corp., Westbury, NY), diluted 1:500 in 5% BSA/5% NGS in PBS, pH 7.0. The slides were washed three times for 10 min in PBS, pH 7.0, and mounted for viewing with 80% glycerol containing 2% *n*-propyl gallate, and 1 μg/ml diamidinophenolindole (DAPI) using a Zeiss Axioscope microscope equipped with fluorescence optics. All images were photographed onto Kodak Techpan film and printed on Agfa paper (Leverkusen, Germany) at high contrast.

Results

Cloning of an Integrin β Subunit from C. elegans

Using degenerate oligonucleotide primers designed from the deduced amino acid sequence of the ligand-binding region of human integrin β1, human integrin β2, human integrin β3, chicken integrin β1, *Xenopus* integrin β1, and *Drosophila* integrin βPS, a DNA fragment of the expected size was amplified from *C. elegans* genomic DNA by PCR. The amino acid sequence predicted from the *C. elegans* DNA amplified between the primers contained an open reading frame with sixteen of the 32 amino acids (50%) identical to the corresponding residues in the human integrin β1 sequence. To clone more of the gene, the PCR-amplified DNA was used to screen a *C. elegans* genomic library. A 5-kb SalI fragment derived from one of the genomic phage clones isolated in the screen was partially sequenced to confirm that an integrin β subunit gene had been cloned. The sequence analysis revealed an open reading frame containing 114 codons with thirty-one of seventy-four predicted residues (42%) identical with the human integrin β1 sequence. The gene encoding this subunit was mapped to the left arm of chromosome III by Dr. A. Coulson (Cambridge University). Of the mutants that map to this region, pat-3 (paralyzed, arrested elongation at twofold) has phenotypes consistent with integrin functions observed in vertebrates and *Drosophila*. We therefore tested the ability of a 9-kb SalI fragment containing the entire coding region of the integrin β subunit to rescue the *C. elegans* embryonic lethal mutation *pat-3(rh54)* and observed that this DNA fragment does indeed rescue this mutation. Sequence analysis of three different alleles of *pat-3* (Gettner, 1994; also to be published elsewhere) has shown that each contains a nonsense or missense mutation in the coding sequence of the integrin β subunit gene; therefore, the integrin β subunit cloned in this study has been named βpat-3.

Coding Sequence Analysis

To obtain the full-length coding sequence of βpat-3, a cDNA library was screened using a probe prepared from the 5-kb SalI fragment. Sequence analysis revealed that the longest cDNA isolated, cDNA 1-1, encodes a transcript of 2908 nucleotides in length. The 5′ end of the cDNA contains 11 bp of the 22-bp *C. elegans* SL1 sequence, which is a leader sequence *trans*-spliced from an unlinked exon onto ~10% of *C. elegans* transcripts (Krause and Hirsh, 1987). After the SL1 sequence, 20 bp preceded the proposed initiator methionine and a large open reading frame of 2,427 bp. The 3′ untranslated sequence preceding the poly A sequence is 451 bp in length. On the basis of computer analysis and comparisons with other integrin β subunits, the predicted protein is composed of 809 residues and has a signal peptide of 19 amino acids, an extracellular domain of 719 amino acids, a transmembrane domain of 23 amino acids, and a cytoplasmic domain of 49 amino acids. The predicted molecular weight of the mature protein is 88,120; with an additional 22,500 D from nine potential N-linked glycosylations (average 2,500 D/N-linked glycosylation), the predicted mol wt is 110,620. The complete cDNA and deduced amino acid sequences are shown in Fig. 1.

Comparison of βpat-3 with Previously Cloned Integrin β Subunits

Comparison of the βpat-3 amino acid sequence with human integrins β1-β8 and *Drosophila* integrin βPS revealed that βpat-3 is most similar to *Drosophila* integrin βPS (44.7% identity), although βpat-3 does not contain the 40-amino acid insert 5′ to the ligand-binding region unique to *Drosophila* (Fig. 2). Of the integrin β subunits cloned from human, βpat-3 is most similar to β1 (41.2% identity). The 56 cysteines characteristic of the extracellular domains of integrin β1, β2, β3, β5, and β6 are present in βpat-3 and their spacing is conserved. The inferred ligand-binding region, which was originally identified by cross-linking the RGD peptide to the integrin β3 subunit (D'Souza et al., 1988) and is highly conserved in all integrin β subunits sequenced to date, is highly conserved in βpat-3. This region extends from D^{149} to residue D^{206} in βpat-3, and has 60.3% identity with the human β1 sequence and 69% identity with the *Drosophila* βPS sequence. The cytoplasmic domain of βpat-3 is also highly conserved, with 59.6% identity with human integrin β1, 52.5% with human integrin β3, and 72.3% identity with *Drosophila* integrin βPS, and identities ranging between 23–45% for human integrins β2, β5, β6, and β7. A consensus sequence derived from the alignment of human integrins β1–β8, chicken, mouse, and *Xenopus* integrin β1, and *Drosophila* integrin βPS shows that 96 residues in the extracellular domain are invariant (Fig. 2); 41 of these residues are cysteines.

Genomic Organization

To determine the genomic organization of the βpat-3 gene, a 9-kb SalI genomic fragment that hybridized on a Southern blot with oligonucleotide primers designed from the 5′ and 3′ ends of the cDNA sequence was subcloned and partially

1. *Abbreviations used in this paper*: NGS, normal goat serum; pat-3, paralyzed arrested elongation at twofold.

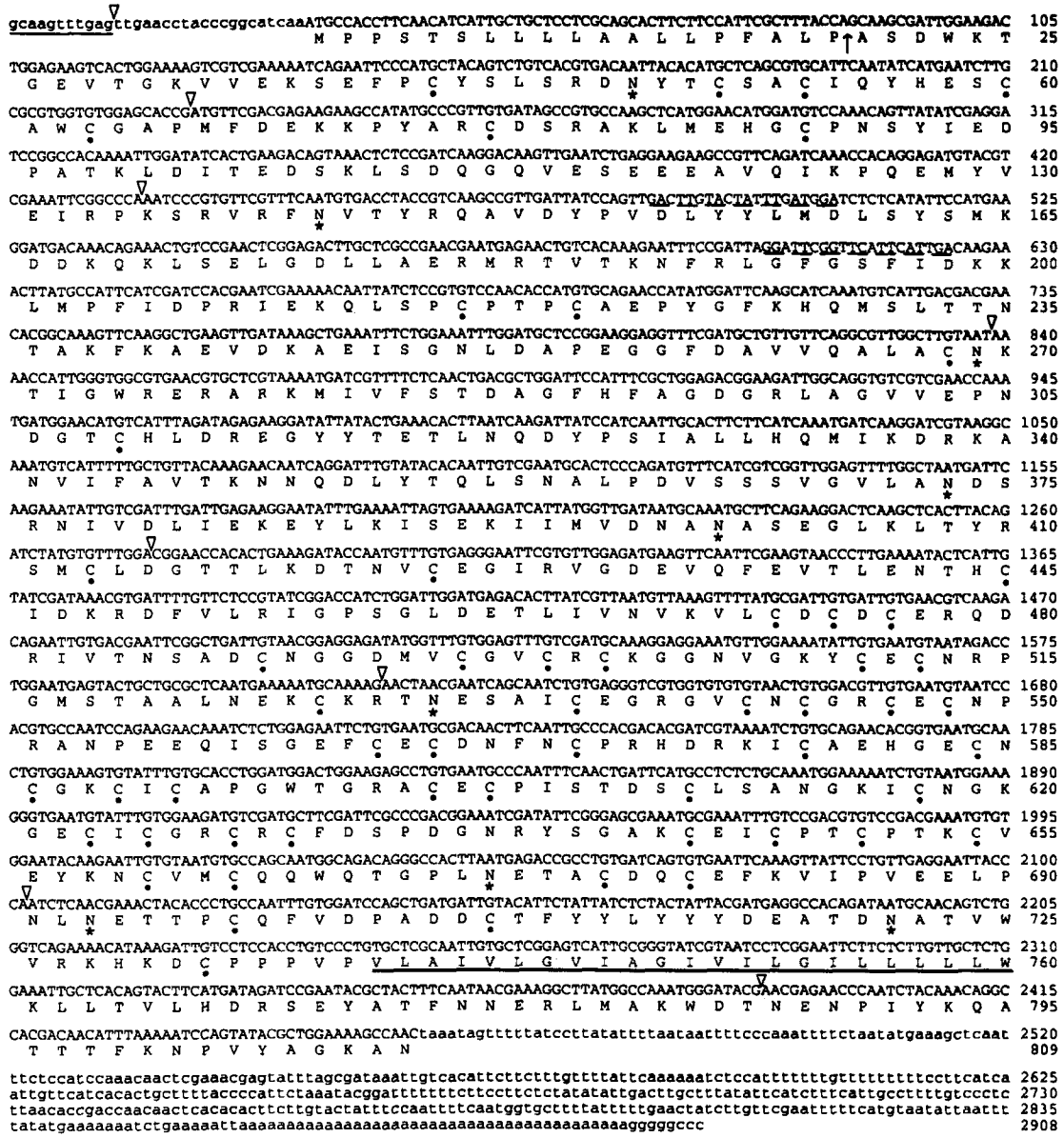

Figure 1. Nucleotide sequence and deduced amino acid sequence of βpat-3. Coding DNA sequence from cDNA 1-1 is shown in upper case letters; noncoding sequence is shown in lower case letters. The partial SL1 *trans*-splice leader sequence (nucleotides 1–11) is highlighted by a fine underline. The regions corresponding to the oligonucleotides used for PCR are marked by a dashed underline. Downward triangles indicate the positions of introns. The amino acid sequence is shown in the single-letter code below the second nucleotide of each codon. The putative signal peptide cleavage site (between residues 19 and 20) is marked by an arrow and the predicted transmembrane sequence (residues 738–760) is highlighted by a heavy underline. Cysteines are indicated by dots under the amino acid sequence, and the nine potential N-linked glycosylation sites are denoted by asterisks.

sequenced (gene bank accession #U19744). The length of the gene from the first nucleotide of exon 1 to the nucleotide in the poly A sequence where the genomic sequence diverges from the cDNA sequence is 5,224 bp. The coding sequence is divided into eight exons ranging from 69 bp to 488 bp in length; the seven introns range between 53 bp and 617 bp. The location of the introns with respect to the cDNA and amino acid sequences is shown in Figs. 1 and 3 *A*. Since alternative splicing has been found in vertebrate integrins (van Kuppevelt et al., 1989; Altruda et al., 1990) and *Drosophila* integrins (Brown et al., 1989; Zusman et al., 1993), intron sequences were examined for open reading frames. Although several open reading frames were detected, none were flanked by *C. elegans* consensus splice donor or splice acceptor sequences. In addition, a single major transcript of 3 kb was detected by RNA blot analysis (Fig. 3 *B*), which is equivalent in length to cDNA 1-1. This result suggests that if any alternatively spliced transcripts exist, they must be either rare or equivalent in length to cDNA 1-1.

To learn whether the organization of integrin β subunit genes is evolutionarily conserved, the positions of introns in the βpat-3, human integrin β2, β3, β7, and *Drosophila* inte-

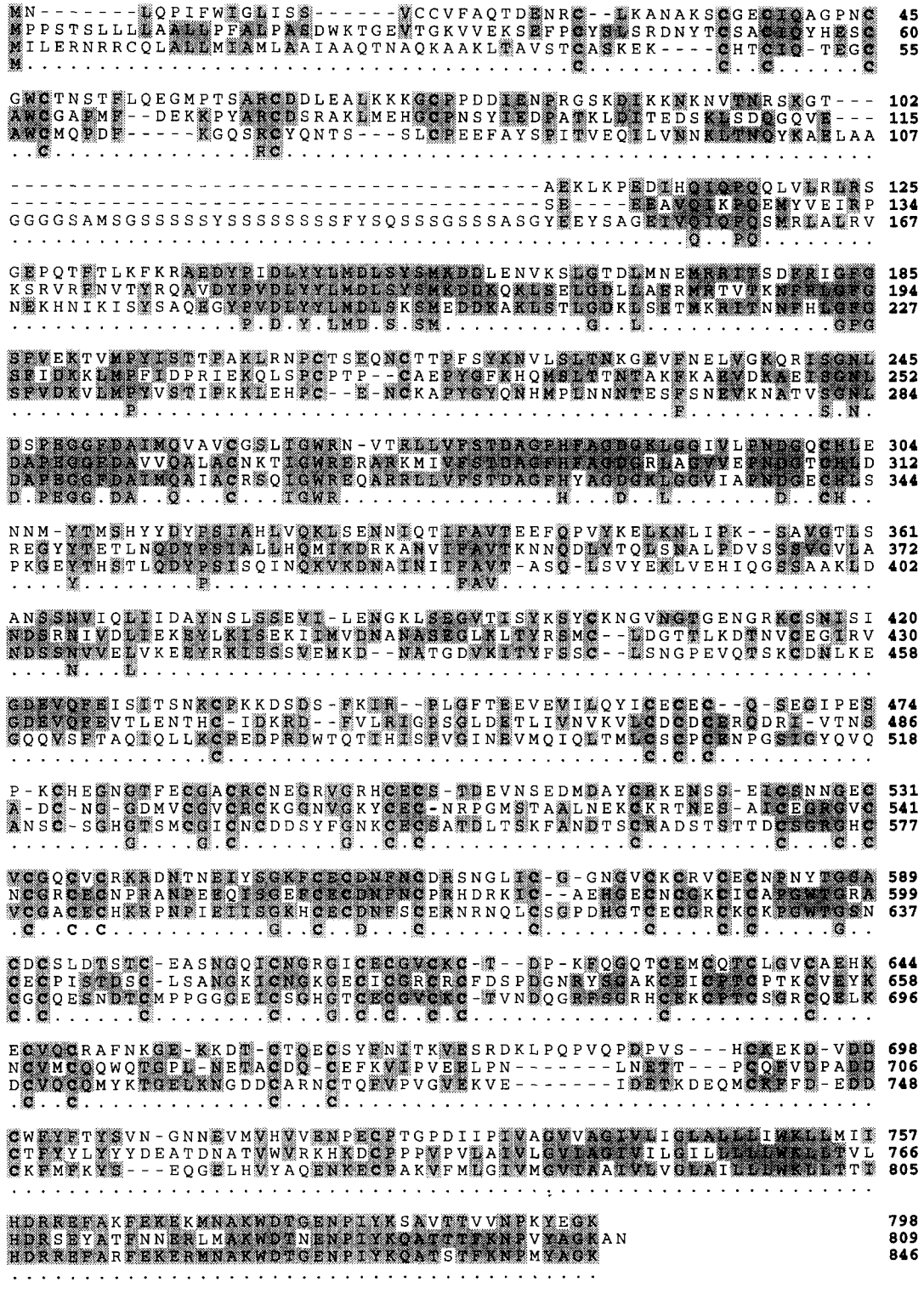

Figure 2. Alignment of the βpat-3 deduced amino acid sequence with the sequences of human integrin β1, *Drosophila* integrin βPS, and a consensus sequence of integrin β sequences. The deduced amino acid sequences are shown in the one letter code and are numbered starting from the initial methionine. Dashes denote gaps introduced into the sequences to maximize the alignment. Residues that are identical between the sequences are shaded. The consensus sequence was derived from the sequences of *C. elegans* βpat-3, human β1-β8, chicken β1, *Xenopus* β1, and *Drosophila* βPS (see Moyle et al., 1991; MacKrell et al., 1988).

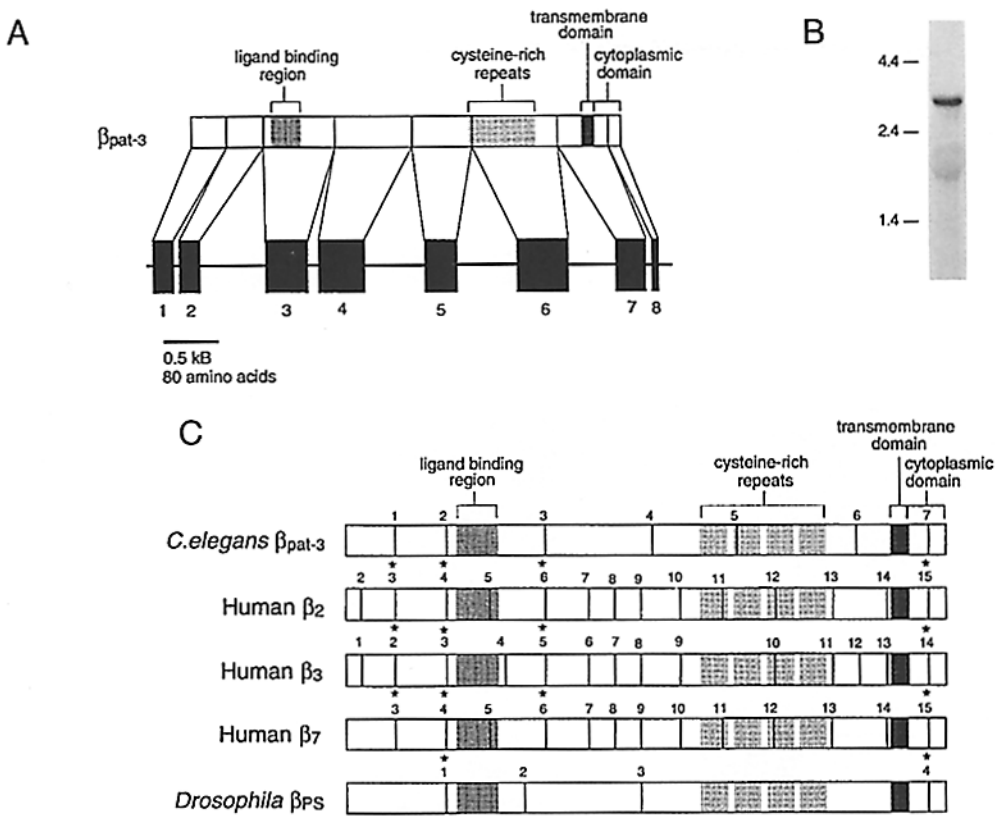

Figure 3. Organization of the βpat-3 subunit gene, RNA blot analysis of βpat-3 transcripts, and alignment of βpat-3 exons with the exons of human β2, human β3, human β7, and *Drosophila* βPS. (*A*) The organization of exons and introns in the integrin βpat-3 gene is shown relative to a schematic representation of the βpat-3 protein. Exons are represented by numbered black boxes and introns by a solid black line. The length of the exons and introns in the gene and the position of the exons relative to the protein sequence are drawn to scale. (*B*) RNA blot analysis of βpat-3 transcripts. 50 μg of *C. elegans* total RNA were separated by agarose gel electrophoresis, blotted onto a nylon membrane, and probed with radiolabeled cDNA 1-1. A major transcript of ~3.0 kb was detected. The positions of the molecular weight markers (shown numerically in kilobase pairs) are marked on the left. (*C*) The exons of *C. elegans* βpat-3 are aligned with the exons of human β2 (Weitzman et al., 1991), human β3 (Lanza et al., 1990; Zimrin et al., 1990), human β7 (Jiang et al., 1992), and *Drosophila* βPS (MacKrell, A., personal communication). The positions of the introns are indicated by vertical lines and numbered in order along the top of the protein. Introns conserved between βpat-3 and the other β subunits are highlighted by an asterisk. The size of the β subunits was made uniform to demonstrate the alignment. Introns 1, 2, 3, and 7 of βpat-3 correspond to introns 3, 4, 6, and 15 of human β2; introns 2, 3, 5, and 14 of human β3; introns 3, 4, 6, and 15 of β7. Introns 2 and 7 of βpat-3 correspond to introns 1 and 4 of βPS.

grin βPS genes (Lanza et al., 1990; Zimrin et al., 1990; Weitzman et al., 1991; Jiang et al., 1992; MacKrell, Albert, personal communication) were compared. The human integrin β1 gene was not included, as information regarding its genomic organization is incomplete. The comparison revealed that the location of four introns is conserved between the βpat-3 gene and the human integrin β2, β3, and β7 genes, and the location of two of these four introns is conserved in the *Drosophila* integrin βPS gene. The first three introns conserved between the βpat-3 gene and the human integrin β subunit genes are located near the ligand-binding region, and the fourth intron conserved in all of the genes divides the coding sequence of the cytoplasmic domain in two (Fig. 3 *C*). In each of the integrin β subunit genes examined, introns interrupt the one or more of the cysteine-rich repeats; thus, the cysteine-rich repeats do not appear to be encoded by discrete exons.

Characterization of the βpat-3 Protein

To analyze the βpat-3 protein, we generated a polyclonal antiserum to a peptide corresponding to the 27 carboxyl-terminal residues of the cytoplasmic domain. In immunoblot analysis, the affinity-purified antibody (hereafter referred to as anti-βpat-3cyto) recognizes a single band at ~109 kD nonreduced (Fig. 4, lane *2*) and 120 kD reduced (Fig. 4, lane *5*). This decrease in electrophoretic mobility is noteworthy as it has been observed for other integrin β subunits and is presumably due to the high cysteine content. No *C. elegans* proteins were recognized by the preimmune serum (Fig. 4, lanes *1* and *4*). In addition, the specificity of anti-βpat-3cyto was demonstrated by competition with the peptide that was used to generate the antibody (Fig. 4, lanes *3* and *6*).

βpat-3 Is Widely Expressed Postembryonically

As a first step toward understanding the function of βpat-3 during development, the cellular localization of βpat-3 was determined using the βpat-3cyto antibody. As a guide to data in later figures, a schematic representation of the tissues which express βpat-3 shown in Fig. 5. The most striking staining throughout development was detected in muscle cells. In the four quadrants of body wall muscle used for locomotion, each of which is composed of two rows of obliquely oriented uninucleate cells, βpat-3, is localized to the dense bodies, the M-lines, and the borders between cells (Fig. 6, *A* and *B*). The dense bodies, which are Z-disc analogs, and the M-lines are structures thought to anchor the thin and thick filaments, respectively, of the sarcomere (Wood, 1988). βpat-3 was also detected in single sarcomere

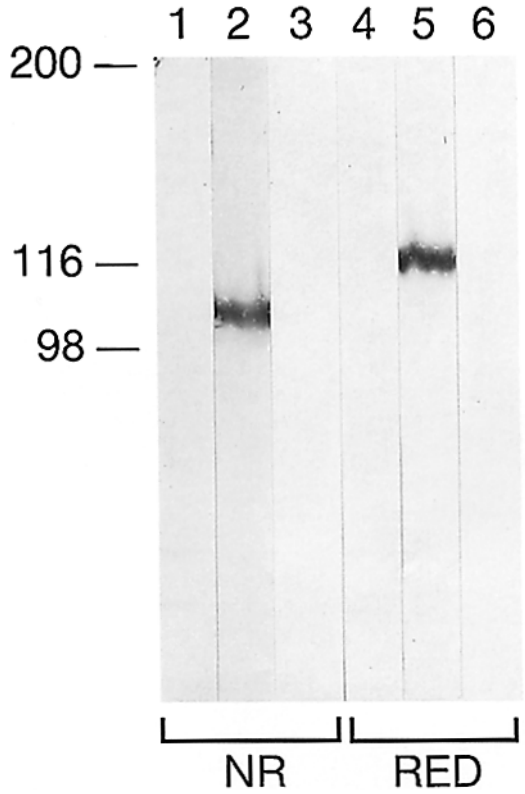

Figure 4. Immunoblot analysis of the βpat-3 protein. A polyclonal antibody was generated against a peptide corresponding to 27 amino acids of the predicted cytoplasmic domain of βpat-3; the affinity-purified antibody is referred to as the anti-βpat-3cyto. A protein extract was prepared from *C. elegans* N2 worms, separated on a 6% SDS-polyacrylamide gel under nonreducing conditions (lanes *1–3*) and under reducing conditions (lanes *4–6*), and electroblotted into a nitrocellulose membrane. The nitrocellulose was incubated with preimmune serum (lanes *1* and *4*), anti-βpat-3cyto (lanes *2* and *5*), and anti-βpat-3cyto plus the cytoplasmic peptide (lanes *3* and *6*). Anti-βpat-3cyto recognizes a band of 109 kD under nonreducing conditions (lane *2*) and a band of 120 kD band under reducing conditions (lane *5*). The numbers at the left indicate the positions and sizes of the molecular mass markers in Daltons × 10^3.

muscles. In the anal depressor muscle, which is an H-shaped cell that opens the anus during defecation, βpat-3 is localized at the ventral attachment site of the muscle, apposing the anus; in addition, staining was detected along the middle strut that straddles the anus (Fig. 6 *C*). More anteriorly along the intestinal-rectal tract, βpat-3 was detected on the ventral surface of the sphincter muscle and the basal surface of the intestinal muscles (Fig. 6 *C*). The intestinal staining is at least partially nonspecific (see below). In the vulval muscles, which open the vulva during egg laying, βpat-3 was located ventrally, in apposition to the opening of the vulva, and laterally, along the uterus (Fig. 6, *D* and *E*). βpat-3 was also detected in the uterine muscles localized to the lateral ridge (Fig. 6 *E*). No staining was detected in the muscles comprising the pharynx (data not shown).

In addition to muscle cells, βpat-3 was detected in several other tissues. The uterus and the spermatheca were stained with anti-βpat-3cyto (Fig. 6, *D* and *E*). All six coelomocytes, which have gland-like morphologies and may act as scavenger cells, have βpat-3 localized on the surface of the cell in a punctate pattern (Fig. 6, *C* and *D*). Staining was not detected in muscles or in coelomocytes in peptide competition experiments (Fig. 6 *G*) or in *pat-3(rh54)* embryos (Fig. 6 *H*). As *pat-3(rh54)* contains a non-sense mutation within the first quarter of the predicted coding sequence (Gettner, 1994), staining in these tissues must depend on βpat-3 expression. Lastly, the apical surface of the intestine was frequently strongly stained in wild-type animals. However, it was also stained in *pat-3(rh54)* embryos (Fig. 6 *H*), suggesting that at least some of the intestinal staining detected is not dependent on the presence of the βpat-3 protein. At this time, we cannot determine whether βpat-3 is expressed in this tissue.

βpat-3 Is Expressed during Embryogenesis

Embryos were also examined with the βpat-3cyto antibody. At the comma stage of embryogenesis (~400 min), weak staining in body wall muscle quadrants was detected (Fig. 7 *A*). At this stage, the twitching movements that indicate contractile muscles are first observed. Staining was also detected in the pharynx and tentatively assigned to the mar-

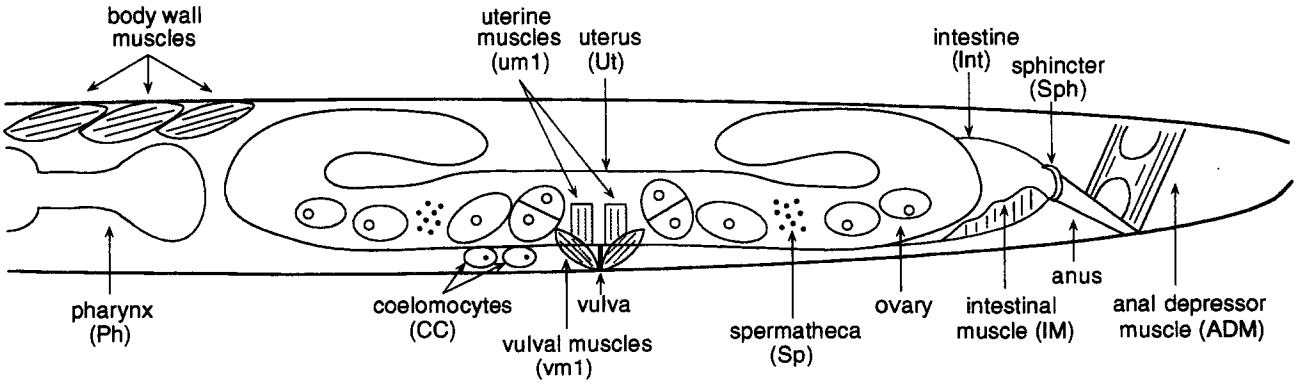

Figure 5. Representation of anatomical structures expressing βpat-3. Structures and their abbreviations are depicted schematically in this figure.

ginal cells, although further work will be necessary to verify this assignment (Fig. 7 *B*). By the 1.5-fold stage of embryogenesis ~430 min strong staining was detected in the body wall muscles (Fig. 7 *C*) and in the pharynx (Fig. 7 *D*). No staining in the muscle or pharynx was observed in *pat-3(rh54)* embryos (Fig. 5 *H*), or in peptide competition experiments (data not shown). After the threefold stage of embryogenesis (~520 min), staining was no longer detected in the pharynx (Fig. 7 *E*). However, staining of the anal depressor muscle (Fig. 7 *E*) and the coelomocytes (Fig. 7 *F*), which have migrated to their adult position by this stage, was present. In addition, transient labeling of several processes, which appeared to be neuronal, was observed. The neuron shown in Fig. 7 *G* has been tentatively identified as the touch neuron ALM due to the shape and the position of the cell body, the direction of the projection, and the timing of the outgrowth of the process. However, further work is needed to unambiguously identify the processes stained by anti-βpat-3cyto. Finally, although many stages of development were examined, the analysis was not complete; therefore, expression of βpat-3 of a more transient nature, such as in migrating cells, may have been missed.

Putative Integrin α Subunits Can be Coprecipitated with βpat-3

The observations that βpat-3 is present in several cell types and can be discretely localized to multiple structures within a single cell suggest that βpat-3 may associate with multiple integrin α subunits in order to carry out distinct functions within each cell. To determine whether proteins with molecular mass in the range observed for integrin α subunits (125–180 kD) associate with βpat-3, immunoprecipitations were performed in conditions identical to those used for vertebrate integrins (e.g., Neugebauer and Reichardt, 1991). For these experiments, two different methods for labeling protein were used. In the first, worm protein extracts were biotinylated, immunoprecipitated, fractionated by SDS-PAGE, and visualized by chemiluminescence, as illustrated in Fig. 8 *A*. Under nonreducing conditions, βpat-3 is represented by a relatively broad band at ~106 kD (Fig. 8 *A*, lane *2*). Seven additional protein bands of ~125, 130, 135, 140, 145, 150, and 180 kD can be distinguished in this experiment. No bands in this molecular mass range were immunoprecipitated by the preimmune serum (Fig. 8 *A*, lane *1*). In the second method, worms were metabolically radiolabeled with ^{35}S. After extraction, proteins were immunoprecipitated, fractionated by SDS-PAGE, and visualized by autoradiography (Fig. 8 *B*). Due to lack of sensitivity in this method, only a subset of the bands observed in the biotinylation method can be observed. Under nonreducing conditions (Fig. 8 *B*, lane *1*), βpat-3 is seen at ~109 kD, and three major bands of 135, 150, and 180 kD are present. In addition, a minor band of 140 kD can be detected.

A majority of integrin α subunits are proteolytically cleaved into two disulfide-linked chains, and, as a result, their mobility in SDS-PAGE increases in reducing conditions. To learn whether any of the proteins coprecipitated with βpat-3 migrate with increased mobility when reduced, ^{35}S-labeled immunoprecipitates were electrophoresed under reducing conditions (Fig. 8 *B*, lane *2*). βpat-3, as shown by immunoblot analysis (Fig. 4), migrated at ~120 kD when reduced. Interestingly, the M_r of the 135, 150, and 180 kD bands observed under nonreducing conditions (Fig. 8 *B*, lane *1*) have also shifted; instead, bands at 112, 138, and 165 kD were observed (Fig. 8 *B*, lane *2*). To determine more precisely how the individual bands have shifted, immunoprecipitates were electrophoresed in two dimensional SDS-PAGE in which proteins were electrophoresed under nonreducing conditions in the first dimension and reducing conditions in the second (Fig. 8 *C*). βpat-3, as expected, migrated above the diagonal demarcated by the molecular weight markers. In addition, several bands were detected below the diagonal: the M_r 180 kD band migrated at ~165 kD when reduced (not visible in this experiment); the M_r 150 kD band migrated at ~135 kD; and a third band at 130–135 kD migrated at ~112 kD. The resolution of the two-dimensional gel was not sufficient to determine whether this third band represents one or both of the 130-kD and 135-kD bands shown in Fig. 8 *A*, lane *2*. The relative molecular weight of each of these coprecipitated proteins decreased by ~15–25 kD when reduced; this decrease is similar to that observed when integrin α subunits known to be composed of two disulfide-linked chains are electrophoresed under reducing conditions instead of nonreducing conditions (for review see Hynes, 1992). Thus, at least three of the proteins that coprecipitate with βpat-3 share an unusual biochemical property characteristic of many known integrin α subunits.

To learn whether the levels of expression of the proteins that coprecipitate with βpat-3 changes during development, immunoprecipitations from developmentally staged populations of worms were performed (Fig. 9). Six developmental stages were examined: embryos (lane *1*); young larvae (L1/L2, lane *2*); larvae beginning to undergo extension of the sexual structures (L3/L4, lane *3*); larvae in which the uterus is developing combined with young adults without oocytes (L4/young adults, lane *4*); gravid adult hermaphrodites (lane *5*); and dauer larvae (lane *6*). The dauer stage is an alternative third larval stage induced by overcrowding and food limitation. For this experiment, the dauer-constitutive mutant *daf-2(e1370)* was used to ensure a pure population of dauers. A total of nine bands with distinct mobilities coprecipitating with βpat-3 could be distinguished. Seven of these bands had molecular weights equivalent to those described above (Fig. 8 *A*). Interestingly, the bands at 160 kD and 165 kD, which were not detected in Fig. 8 *A*, appear to be specific to embryos (Fig. 9, lane *1*). Also of note, the 125-kD band appears most clearly as a doublet with the 130-kD band in gravid adults (Fig. 9, lane *5*), and the 135-kD and 140-kD bands are more highly expressed in larvae (Fig. 9, lanes *2*, *3*, and *4*) than in embryos, gravid adults, or dauer larvae (Fig. 9, lanes *1*, *5*, and *6*). The level of expression of the 150-kD band relative to βpat-3 appears to remain constant postembryonically. The 145-kD band is difficult to distinguish due to the strong labeling of the 150-kD band, and is not marked in Fig. 9. Finally, the 180-kD band is present in all stages except for dauer larvae. The properties of these bands are summarized in Table I.

The Monoclonal Antibody MH25 Recognizes βpat-3

The observation that anti-βpat-3cyto stains muscle cells in a pattern identical to the mAb MH25 and the proposal of Francis and Waterston (1985) that MH25 recognizes a trans-

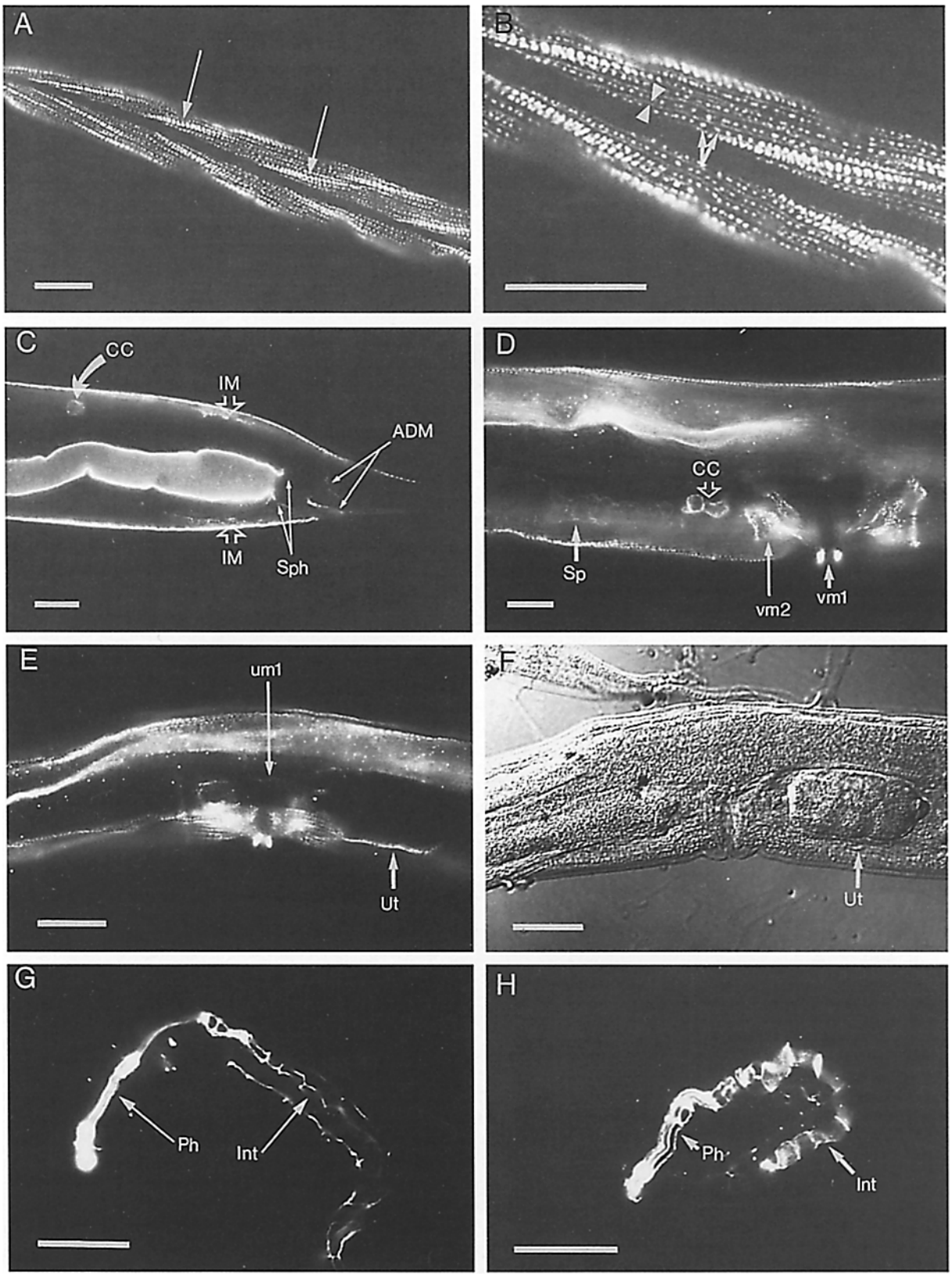

Figure 6. Immunocytochemical analysis of whole-mount worms using anti-βpat-3cyto. Animals are oriented with the head to the left, dorsal up. (*A*) βpat-3 staining in two quadrants of body wall muscles. Arrows indicate staining localized to the longitudinal cell boundaries. (*B*) Enlarged view of body wall muscle cells showing βpat-3 staining localized to the dense bodies (*arrows*) and M lines (*arrowheads*). (*C*) Lateral view, showing βpat-3 staining of the ventral attachment sites (apposed to the anus) and the middle strut of the anal depressor muscle (*ADM*), the ventral attachment sites of the sphincter muscle (*Sph*), the basal surface of the intestinal muscles (*IM*), and the cell surface of the left postembryonically derived coelomocyte (*CC*). βpat-3 staining in the dorsal and ventral body wall muscle quadrants is out of the plane of focus. (*D*) Lateral view, left side, showing βpat-3 staining at the ventral and lateral attachment sites of the vm1 and

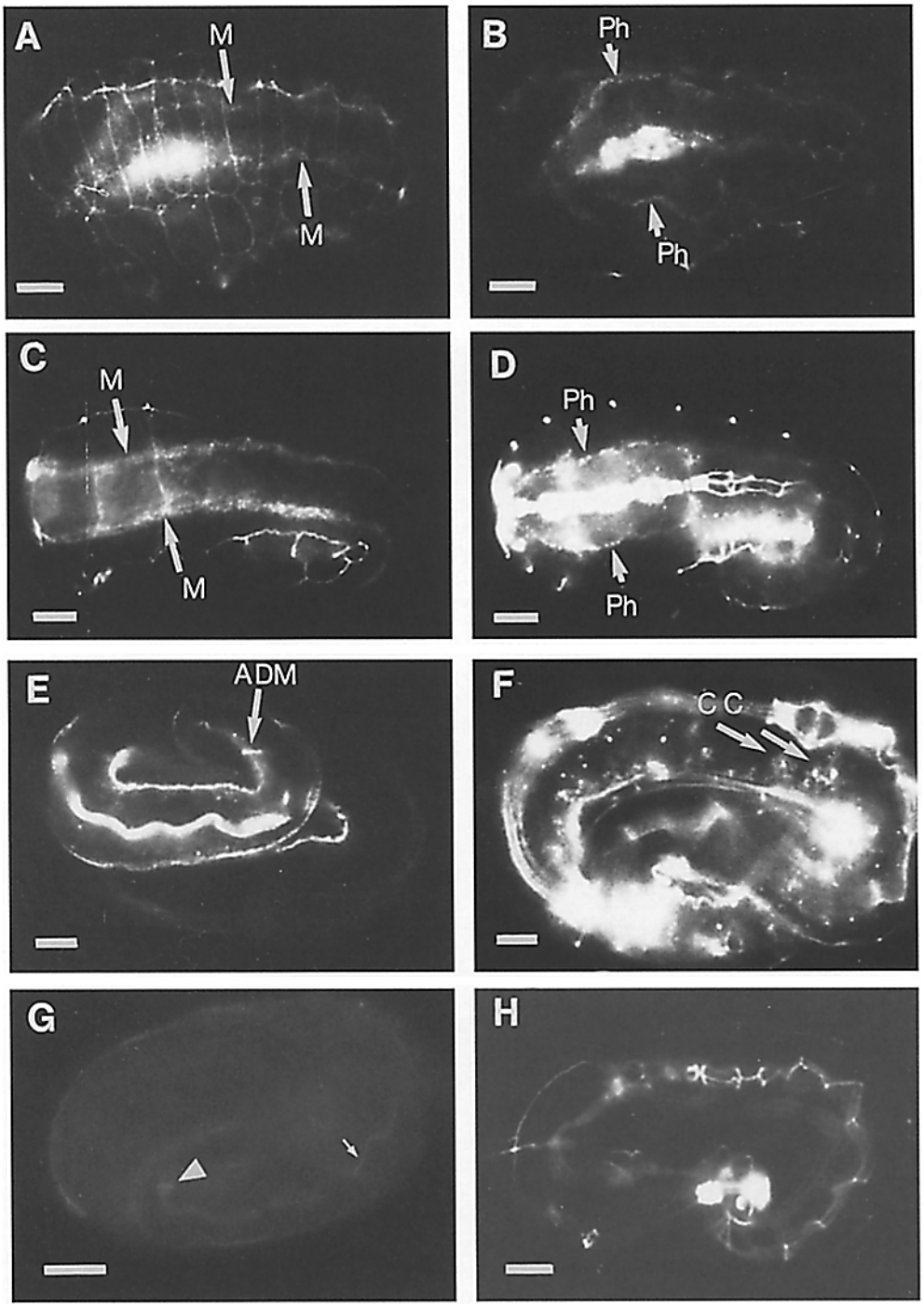

Figure 7. Immunocytochemical analysis of whole-mount embryos using anti-βpat-3cyto. Embryos are oriented with the head to the left. All embryos except those shown in *E* and *G* were colabeled with the monoclonal antibody MH27, which recognizes a desmosomal component at the apical surface of hypodermal and a subset of pharyngeal cells. The pattern of MH27 staining, which forms a grid-like pattern, can be seen in *H*. (*A*) Dorsal view of staining in a comma stage embryo, showing βpat-3 staining in developing muscle quadrants. (*B*) Same embryo as in *A*, mid-focal plane of embryo, showing βpat-3 staining in a subset of cells comprising the pharynx, tentatively identified as the marginal cells. (*C*) Dorsal view of a 1.5-fold embryo, showing βpat-3 staining in the dorsal muscle quadrants. (*D*) Same embryo as in *C*, mid-focal plane of embryo, showing staining in the pharynx, again tentatively identified as the marginal cells. (*E*) Threefold embryo, showing βpat-3 staining in the anal depressor muscle. βpat-3 staining in body wall muscles is out of focus. Staining in the intestine is most likely independent of βpat-3 expression as it was also detected in *pat-3(rh54)* embryos (see Fig. 5 *H*) (*F*) Threefold embryo, right side, showing βpat-3 staining in the right side anterior coelomocytes. (*G*) Threefold embryo, showing βpat-3 staining in a neuronal cell body and process located laterally in the anterior portion of the embryo, with the process extending anteriorly. This neuron has been tentatively identified as the touch neuron ALM (see text). (*H*) Embryos stained with anti-βpat-3cyto and preincubated with 10 μg/ml of the cytoplasmic peptide show no detectable staining. Embryos were colabeled with MH27 to ensure that the animals had been permeabilized; the pattern of MH27 staining is shown for comparison with the other figures. Bars: (*A–F* and *H*) 5 μm; (*G*) 10 μm.

vm2 vulval muscles (*vml* and *vm2*), the anterior spermatheca (*Sp*), and the embryonically derived coelomocytes (*CC*). (*E*) Lateral view, right side, showing βpat-3 staining in the vulval muscles as described in *D*, and staining at the lateral ridge attachment sites of the uml uterine muscles (*uml*), in the anterior spermatheca and in the posterior uterus (*Ut*) of a young adult hermaphrodite. Intestinal and body wall muscle staining are out of focus. (*F*) Nomarski image of the same animal as in *E*. (*G*) Competition of anti-βpat-3cyto with 10 μg/ml of the cytoplasmic peptide. To ensure that the animals had been permeabilized, the monoclonal antibody MH27, which recognizes a desmosomal component at the apical surface of hypodermal, pharyngeal, and intestinal tract cells, was included in the sample. No βpat-3 staining was observed. The pattern of MH27 staining is shown for comparison with *H*; the pharynx (*ph*) and the intestine (*int*) are indicated. (*H*) Staining of *pat-3(rh54)* embryos with anti-βpat-3cyto show that staining in the intestine (*int*) is not dependent on the expression of βpat-3. Animals were colabeled with MH27 to provide an outline of the intestine; the pattern of MH 27 staining can be seen in *G*. Bars, 20 μm.

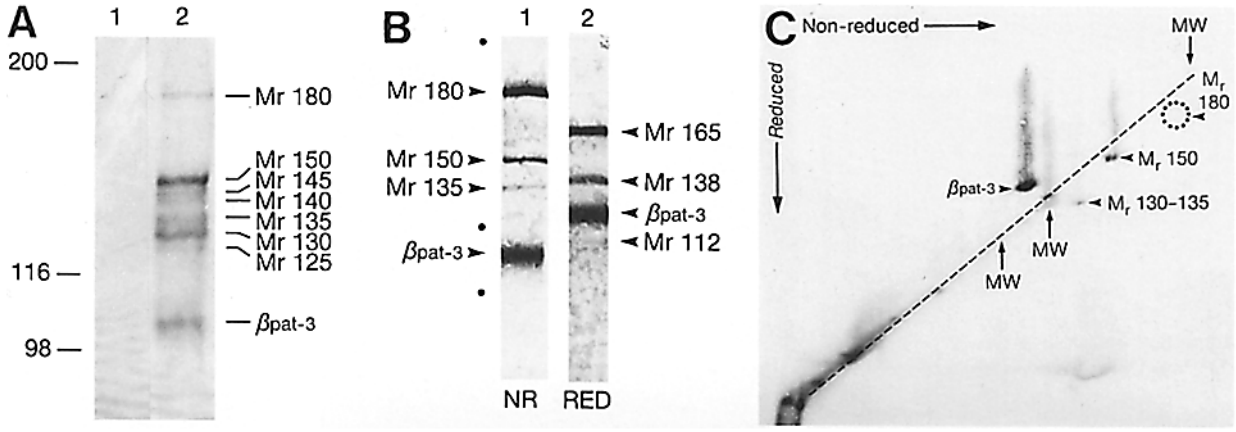

Figure 8. Immunoprecipitation of βpat-3 complexes using anti-βpat-3cyto. (*A*) Immunoprecipitations of biotinylated proteins. *C. elegans* N2 membrane proteins were labeled with sulfo-NHS-biotin and immunoprecipitated by the preimmune serum (lane *1*) or anti-βpat-3cyto (lane *2*). The immunoprecipitated proteins were electrophoresed under nonreducing conditions through a 6% SDS-polyacrylamide gel and electroblotted onto nitrocellulose. The blot was incubated with HRP-conjugated avidin and developed with reagents for chemiluminescence. The immunoprecipitated proteins were visualized by autoradiography. βpat-3 is represented by a band at 109 kD (lane *2*). Seven other bands with relative molecular masses between 125 kD and 180 kD can be detected. The numbers at the left indicate the positions and sizes of the molecular mass markers in Daltons × 10^3. (*B*) Immunoprecipitations of radiolabeled proteins. Radiolabeled proteins were immunoprecipitated by anti-βpat-3cyto from *C. elegans* N2 worms metabolically labeled with ^{35}S. The immunoprecipitated proteins were separated on a 6% SDS-polyacrylamide gel and visualized by autoradiography. Under nonreducing conditions (lane *1*), βpat-3 and three major bands at 130, 150, and 180 kD can be detected. Under reducing conditions (lane *2*), βpat-3 migrates at ~120 kD and bands at 112, 138, and 165 kD can be detected. The molecular mass markers are the same as in *A*; their positions are indicated on the left by dots. (*C*) Two-dimensional SDS-PAGE of immunoprecipitated biotinylated proteins. *C. elegans* N2 membrane proteins were labeled with sulfo-NHS-biotin and immunoprecipitated with anti-βpat-3cyto. The immunoprecipitated proteins were electrophoresed through a 6% SDS-polyacrylamide gel under nonreducing conditions in the first dimension and a 7.5% SDS-polyacrylamide gel under reducing conditions in the second dimension before transfer onto nitrocellulose. The nitrocellulose blot was incubated with alkaline phosphatase-conjugated streptavidin and developed with reagents for the alkaline phosphatase reaction. Prestained nonreduced molecular weight markers and biotinylated molecular mass markers pretreated with iodoacetamide were included in the sample. βpat-3 migrates above the diagonal demarcated by the molecular mass markers (*dashed line*), and proteins at approximately 135, 158, and 180 kD in the first dimension migrate below the diagonal. The position of the 180-kD protein is indicated by a dotted circle as it is faint. The molecular mass markers are the same as in *A*; their positions along the diagonal are marked by arrows labeled MW.

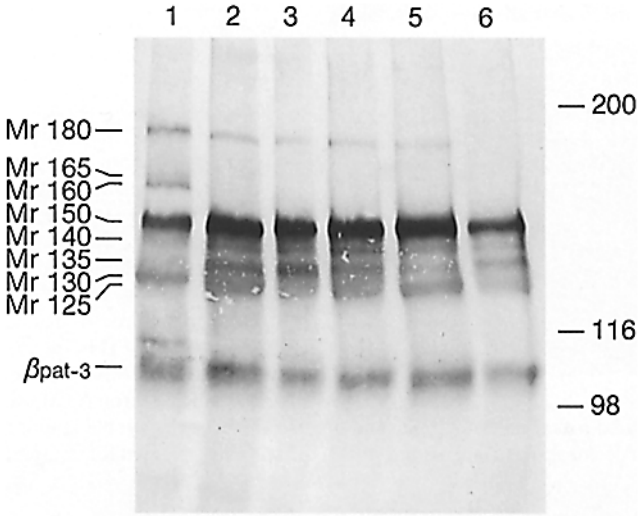

Figure 9. Immunoprecipitations from developmentally staged populations of *C. elegans* using anti-βpat-3cyto. Proteins labeled with sulfo-NHS-biotin from six stages of development were immunoprecipitated using anti-βpat-3cyto, electrophoresed through a 6% SDS-polyacrylamide gel under nonreducing conditions and transferred onto nitrocellulose. The nitrocellulose blot was incubated with alkaline phosphatase-conjugated streptavidin and the immunoprecipitated proteins visualized by the alkaline phosphatase reaction product. The stages of development that were examined are as follows: lane *1*, embryos; lane *2*, young larvae (L1/L2); lane *3*, larvae beginning to undergo development of the sexual structures (L3/L4); lane *4*, larvae in which the uterus is developing combined with young adults without oocytes (L4/young adults); lane *5*, gravid adult hermaphrodites; lane *6*, dauers. Dauers were obtained for this experiment using the dauer-constitutive mutant strain *daf-2(e1370)*. Two embryonic-specific bands of 160 kD and 165 kD can be seen (lane *1*); in addition, the 180-kD band cannot be detected in dauers (lane *6*). The band migrating above βpat-3 in lane *1* (not marked) has not been observed reproducibly. The numbers at the left indicate the positions and sizes of the molecular mass markers in Daltons × 10^3.

membrane component of the dense body prompted us to examine whether MH25 recognizes βpat-3. Nonlabeled proteins were immunoprecipitated by MH25 or anti-βpat-3cyto. Then, after SDS-PAGE in reducing conditions of the immunoprecipitates, blots were incubated with anti-βpat-3cyto (Fig. 10, lanes *1* and *2*). An antigen of 120 kD was detected in each lane, demonstrating that MH25 recognizes βpat-3. Interestingly, MH25 recognizes βpat-3 on immunoblots only after electrophoresis of samples under nonreducing conditions (Gettner, 1994). This result suggests that the epitope recognized by MH25 is dependent on the presence of intact disulfide linkages and therefore is likely to be extracellular and thus distinct from the epitope recognized by anti-βpat-3cyto.

Table I. Summary of Proteins Coprecipitated with C. elegans Integrin βpat-3

M_r (nonreduced)	M_r (reduced)	Comments
180 k	155 k	Not expressed in dauers or recognized by MH25.
165 k	ND	Embryo specific.
160 k	ND	Embryo specific.
150 k	138 k	Expressed at all stages.
145 k	ND	Difficult to detect.
140 k	ND	Expressed at all stages.
135 k	112 k*	Expressed at all stages; most strongly expressed in L3/L4 larvae.
130 k	112 k*	Expressed at all stages.
125 k	ND	Not present at all stages.

* The resolution of the gel was not sufficient to determine which of these bands, or whether both of these bands, have an increased mobility when reduced.

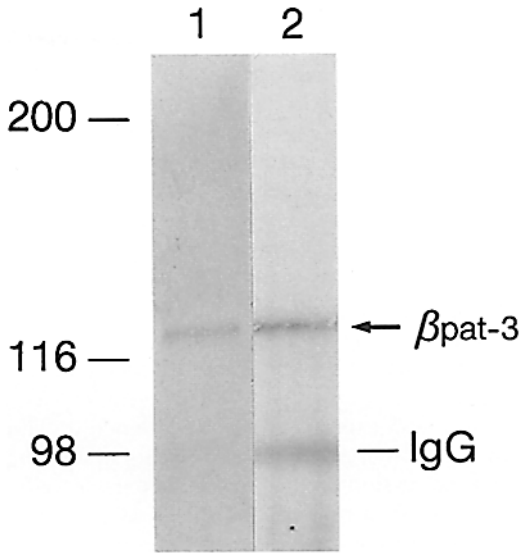

Figure 10. The monoclonal antibody MH25 recognizes βpat-3. Unlabeled proteins immunoprecipitated by anti-βpat-3cyto (lane *1*) and MH25 (lane *2*) were separated on a 6% SDS-polyacrylamide gel under reducing conditions and electroblotted onto nitrocellulose. The blot was incubated with anti-βpat-3cyto followed by an alkaline phosphatase–conjugated anti-rabbit antibody. Bands of 120 kD can be detected in both lanes.

Discussion

As a first step toward understanding the function of integrin receptors during *C. elegans* development, we have cloned an integrin β subunit from *C. elegans* using the PCR and degenerate oligonucleotides designed from the highly conserved RGD-binding region of integrin β subunits. The gene encoding this subunit maps to the left arm of chromosome III in the vicinity of *pat-3*. A genomic fragment containing the entire coding region of the *C. elegans* integrin β subunit has been shown to rescue the embryonic lethality of the *C. elegans* mutation *pat-3(rh54)*. This allele has been shown to contain a nonsense mutation in the coding region of βpat-3 (Gettner, 1994; also to be published elsewhere). Thus, the integrin β subunit described in this paper has been named βpat-3. The pattern of expression of βpat-3 was determined by staining whole mount preparations of worms with an affinity-purified polyclonal antibody generated against a peptide designed from the predicted cytoplasmic domain of βpat-3. The immunocytochemical analysis revealed that βpat-3 was expressed in several tissues in the embryo and postembryonically, with a particularly striking pattern of subcellular localization in body wall muscles. Immunoprecipitations using anti-βpat-3cyto showed that at least nine distinct protein bands with molecular weights in the range observed for integrin α subunits coprecipitate with βpat-3. A subset of these bands can also be immunoprecipitated by the monoclonal antibody MH25 (Gettner, 1994), which was shown in this study to recognize βpat-3.

Three lines of evidence suggest that we have determined the complete sequence of the βpat-3 transcript. First, the longest cDNA isolated (cDNA 1-1) was equivalent in length to the major transcript observed by RNA blot analysis. Second, 11 nt of an SL1 variant *trans*-splice leader sequence was present at the 5′ end of the longest cDNA isolated, and a poly A stretch was present at the 3′ end. Third, since a single-length transcript was detected using RNA blot analysis and no potential exons were found in intron sequences, it is unlikely that alternatively spliced transcripts exist. However, since the genomic DNA was only sequenced 500 bases 3′ to the last exon containing the carboxy-terminal end of the cytoplasmic domain, the possibility that this exon is alternatively spliced has not been ruled out. This exon has been observed to be alternatively spliced in the human integrin β1 and β3 subunits (van Kuppevelt et al., 1989; Altruda et al., 1990).

Biochemical analyses using anti-βpat-3cyto demonstrated that βpat-3 has features observed for previously isolated integrin β subunits. For example, under nonreducing conditions, βpat-3 migrates with decreased mobility in SDS-PAGE as compared with reducing conditions; this behavior is presumably due to the disulfide-linked cysteines in the extracellular domains of integrin β subunits producing a more compact structure when nonreduced. Furthermore, at least nine protein bands with molecular weights in the range observed for integrin α subunits coprecipitate with βpat-3. We believe that at least some of these bands may represent integrin α subunits for the following reasons. First, the conditions used to immunoprecipitate βpat-3 in this study were identical to those used for vertebrate integrins, and under these conditions, protein bands in the molecular weight range 125–180 kD coprecipitating with integrin β subunits have been shown to be integrin α subunits. Second, when the immunoprecipitates were electrophoresed in SDS-polyacrylamide gels under reducing conditions, at least three of the coprecipitated bands migrate with increased mobility. This result is significant because ~60% of identified integrin α subunits are proteolytically cleaved into a disulfide-linked heavy chain and light chain and, when reduced, migrate with increased mobility in SDS-PAGE (for review see Hynes, 1992). Third, the level of expression of several of the coprecipitated bands appeared to change with development; most notably, two bands were detected only in embryos, and a third band was absent in dauer larvae. These bands may represent integrin α subunits that mediate processes specific to distinct developmental events. Fourth, since bands with molecular

weights immunoprecipitated by anti-βpat-3cyto were also immunoprecipitated by the mAb (data not shown) MH25, it is not likely that these proteins are nonspecifically immunoprecipitated by anti-βpat-3cyto. Fourth, the *pat-3(rh151)* mutation has been shown to affect the association of these coprecipitated proteins with βpat-3 (Gettner et al., 1994). Finally, at least two integrin α subunits have been identified in sequencing of the *C. elegans* genome (Wilson et al., 1994), providing direct evidence that *C. elegans* has at least two integrin α subunits. Future experiments will be able to determine whether each of the bands that coprecipitate with βpat-3 is an integrin α subunit and whether the integrin α subunits that have been identified by genomic sequencing associate with βpat-3.

The pattern of βpat-3 expression was determined to gain insight into the function of βpat-3 during development. The most prominent expression of βpat-3 was in muscle cells, where βpat-3 appeared to be localized to points of attachment. In body wall muscle, βpat-3 was concentrated in the dense bodies, the M lines, and the obliquely oriented borders between muscle cells. The borders between cells contain attachment plaques; further work is necessary to determine whether βpat-3 is a component of these plaques. The pattern of staining in body wall muscles with anti-βpat-3cyto is identical to that seen with MH25 (Francis and Waterston, 1985). Our demonstration that MH25 recognizes βpat-3 validates the proposal of Francis and Waterston (1985) that MH25 binds to a membrane or cell surface component of the dense body. These authors also showed that vinculin and α-actinin are present in the dense bodies. As in vertebrates (for review see Hynes, 1992), βpat-3 may function to attach muscles by acting as a transmembrane link to the actin cytoskeleton via vinculin and α-actinin (see Williams and Watterson, 1994). In addition, a recent study showed that the organization of the sarcomere is initiated at membrane-proximal components such as the antigen recognized by MH25 (Hresko et al., 1994). Since MH25 recognizes βpat-3, this result suggests that βpat-3 participates in the organization of the structural components of the sarcomeres in body wall muscles. The staining of βpat-3 in the process and cell body of the neuron tentatively identified as ALM appears to be transient and occurs coincidently with the migration and process extension of this neuron, suggesting that βpat-3 may have a role in these processes. It is less apparent what role βpat-3 plays in tissues such as the coelomocyte, the uterus or the spermatheca; however, the analysis of *pat-3* mutants should reveal βpat-3 function in these and other tissues.

Because the anatomy of *C. elegans*, *Drosophila* and vertebrates differ, it is not possible to make a detailed comparison of the localization of βpat-3, βPS, or β1 in their respective organisms of origin. However, several observations can be noted. The localization of βpat-3 in striated muscle is similar, but not identical to that seen in vertebrates and *Drosophila*. The vertebrate integrin β1 and *Drosophila* integrin βPS, like βpat-3, localize in the Z bands (Bozyczko et al., 1989; Volk et al., 1990); however, unlike βpat-3, these integrin β subunits are absent from M lines. Moreover, while the vertebrate integrin β1 and *Drosophila* integrin βPS are strongly localized at myotendinous junctions (Bao et al., 1993; Leptin et al., 1989), no increased density of staining can be detected at the analogous site in *C. elegans*. In *Drosophila*, integrin βPS is present in ectodermal cells in embryos (Leptin et al., 1989). In contrast, we did not detect βpat-3 in hypodermal cells, either embryonically or postembryonically. Finally, unlike vertebrate integrin β1, which is highly expressed in the developing nervous system (for review see Reichardt and Tomaselli, 1991), no staining of βpat-3 was detected in the dorsal or ventral nerve cords, the nerve ring or the pre-anal ganglion. However, transient staining was observed in a neuronal cell body and process tentatively identified as ALM.

One question raised by this study is whether βpat-3 is a homolog of the vertebrate integrin β1 subunit, or whether it is representative of a prototypic integrin β subunit that existed before the evolutionary divergence of protostomes from deuterostomes. Several arguments favor the latter hypothesis: (*a*) Although computer alignments revealed that βpat-3 was slightly more homologous with human integrin β1 (41%) than with other integrin β subunits, the level of identity between integrin β subunits found in the same species ranges from 28–55%. Thus, βpat-3 cannot be called a homolog of integrin β1 on the basis of sequence homology alone. (*b*) βpat-3 appears to have the potential to associate with multiple integrin α subunits. However, this property has been observed with several integrin β subunits (β1, β2, β3, and β7) (Hynes, 1992). (*c*) Comparison of the localization of βpat-3 with chicken integrin β1 in posthatch chick muscle (discussed above) has shown that their distributions are similar, but not identical. Future studies will show whether the functions of other integrin β subunits present in vertebrate muscle are analogous to those performed by βpat-3; in particular, an integrin β subunit distinct from βpat-3 may function to attach the distal ends of muscle cells to the basement membrane. (*d*) Unlike integrin β1, βpat-3 is not strongly expressed in the embryonic nervous system. (*e*) Human integrins β1, β2, and β7 and integrins β3, β5, and β6 have been placed into two subgroups based on sequence homology (Moyle et al., 1991). As discussed above, the integrin β1, β2, and β7 genes also contain an intron in the highly conserved ligand-binding region, while the integrin β3, β5, and β6 genes do not. Since the βpat-3 gene does not contain this intron, it could be considered more closely related to the integrin β3, β5, and β6 subgroup. However, considering that βpat-3 has properties of integrin β subunits in general, it is most likely that this intron appeared after the divergence of nematodes from vertebrates but before the duplication and divergence of the distinct vertebrate integrin β subunit genes.

With the completion of the *C. elegans* genome sequencing project, it will soon be clear exactly how many integrin α and β subunits genes are present in *C. elegans*. Furthermore, as the genetic map and physical map are becoming increasingly linked and more mutants are isolated, the identification of mutations in other integrin genes will be facilitated. In particular, the identification of mutations in integrin α subunit genes whose protein products associate with βpat-3 should be informative, as their phenotypes are likely to be discrete. Using *C. elegans* genetics, it may ultimately be possible to determine which proteins interact with integrin receptors to produce cellular behaviors such as cell motility and cell morphogenesis.

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