Characterization of $\beta$pat-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 $\beta$ subunit from C. elegans which is shown to rescue the embryonic lethal mutation $\text{pat-3(rh54)}$ and is thus named $\beta$pat-3. Analysis of the deduced amino acid sequence revealed that $\beta$pat-3 is more similar to Drosophila integrin $\beta$PS and to vertebrate integrin $\beta$1 than to other integrin $\beta$ 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 $\beta$ subunits are conserved. A major transcript of $\sim 3$ kilo-base pairs was detected by RNA blot analysis. Immunoblot analysis using a polyclonal antiserum against the cytoplasmic domain showed that $\beta$pat-3 migrates in SDS-PAGE with apparent $M_\text{r}$ 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 $\alpha$ subunits coprecipitate with $\beta$pat-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 $\alpha$ subunits. Immunoprecipitations of $\beta$pat-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 $\beta$pat-3. Finally, immunocytochemical analysis revealed that $\beta$pat-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 $\beta$ 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, cell migration, and neurite outgrowth (for review see Hynes, 1992). Each receptor is a heterodimer composed of noncovalently associated $\alpha$ and $\beta$ subunits. To date, fourteen $\alpha$ subunits and eight $\beta$ 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-$\beta$1 antibody has been shown to interfere with neural crest cell migration (Bronner-Fraser, 1986), and anti-sense experiments have suggested that $\beta$1 integrins are required for neuroblast migration in the optic tectum (Galileo et al., 1992). In Pleurodeles embryos, injection of anti-$\beta$1 antibodies was found to inhibit gastrulation (Darribere et al., 1988). To date, the analysis of the Drosophila melanogaster integrin $\beta$PS subunit mutant lethal(l)myospheroid has been the most comprehensive study of integrin function during develop-
ment (e.g., Zusman et al., 1990, 1993). \textit{Lethal(L)mjospheroid} is an embryonic lethal mutation that results in defects in muscle attachment, dorsal closure, and constriction of the gut. Postembryonic phenotypes of \textit{lethal(L)mjospheroid} 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.

\textit{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, \textit{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 \(\beta\) 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 U197444”). We show that this gene rescues the \textit{C. elegans} embryonic lethal mutation \textit{pat-3(rh54)}; thus, it will be referred to as \(\beta\text{pat-3}\). A polyclonal antisera prepared against the cytoplasmic domain of \(\beta\text{pat-3}\) was used for immunoblot analysis, immunoprecipitations and immunocytochemistry. The immunoprecipitations showed that at least nine protein bands coprecipitate with \(\beta\text{pat-3}\); these bands have relative molecular weights in the same range as known integrin \(\alpha\) subunits. Immunocytochemical analysis showed that \(\beta\text{pat-3}\) is first expressed in the embryo and is subsequently widely expressed in larvae and adult animals. The complex pattern of the \(\beta\text{pat-3}\) expression and the potential for the association with multiple \(\alpha\) subunits suggests that receptors containing \(\beta\text{pat-3}\) have many important roles in \textit{C. elegans} development.

\section*{Materials and Methods}

\textbf{Polymerase Chain Reaction Amplification of an Integrin \(\beta\) Subunit from \textit{C. elegans}}

Degenerate oligonucleotides for use in PCR were designed from the deduced amino acid sequences of previously sequenced integrin \(\beta\) 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-C1, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 0.01% gelatin, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 1 \(\mu\)g of each primer, 1 \(\mu\)g of 
\textit{C. elegans} strain N2 genomic DNA, and 0.62 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). Oligonucleotide primers designed from the integrin \(\beta\) subunit ligand-binding region [(5GA/CT(T)N(TA (CT)ACT/CT(T)NATGGA); (5CT(N)AC/AGAA(N)GQA(N)CC/AGAA(N)NCC) where \(N = A,C,G,\) and \(T\)] were successfull 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 \(\beta\).

\section*{Isolation of \(\beta\text{pat-3}\) Genomic and cDNA Clones}

2 \(\times\) 10\(^6\) plaques from a \textit{C. elegans} strain N2 \(\lambda\text{EMBL3}\) 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 \(\times\) SSC with 0.1% SDS at 50°C. Seven hybridizing clones were purified.

\(5 \times 10^6\) plaques from a \textit{C. elegans} strain N2 2–3 kb size-selected \(\lambda\)SHLX2 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 Sall fragment derived from phage clone (2-4) isolated in the genomic library screen. Eleven hybridizing clones were isolated.

\section*{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 Sall fragment was subcloned from genomic phage clone (2-4) and partially sequenced using oligonucleotide primers \(\beta\text{35 5'}\) and \(\beta\text{35 3'}\) to verify that the clone contained sequence homologous to integrin \(\beta\) subunits. A 9-kb Sall fragment from genomic phage clone (1-1) that hybridized with oligonucleotides from the \(5'\) (\(\beta\text{35-PE}\) and 3' (\(\beta\text{35-7rev}\) 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, pBlI (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 Sall 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.

\section*{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 \(\beta\) subunits were performed using the Pattern-Induced Multisequence Alignment (PIAMA) algorithm (Smith and Smith, 1991). Sequence between cysteines not aligned using the PIAMA algorithm were aligned using the alignment program in PCGENE.

\section*{RNA Blot Analysis}

Total cellular RNA was prepared from mixed populations of worms using the method of Rosenquist and Kimble (1988). RNA was electrophoresed in formaldehyde-containing agarose gels according to Maniatis et al. (1982), transferred onto a Hybond-N membrane (Amer sham Corp., Arlington Heights, IL) using 20\(\times\) 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\(\times\) SSC with 0.05% SDS at 55°C. cDNA 1-1 was radiolabeled with \(^{32}\)PdCTP using random primers (Boehringer/Mannheim) to probe the RNA blot. The relative mol wt of the \(\beta\text{pat-3}\) transcripts was determined by comparison with RNA mol wt standards purchased from Gibco/BRL (Gaithersburg, MD).

\section*{Generation of Peptide Antiserum}

A peptide consisting of an NH₂-terminal cysteine plus the 27 COOH-terminal amino acids of the deduced sequence of the cytoplasmic domain of \(\beta\text{pat-3}\) (CKWDTNENPYIKQATTFTKPNYPYAGKAN) 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 on 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-3-cyto, 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 counted by Nomarski microscopy to determine the percentage of worms of each stage. To obtain dauer, *daf-2(e1370)* embryos were placed at the restrictive temperature of 25°C and the hatchlings monitored until dauer 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; 1 mM MgCl2; 1 mM CaCl2; 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 protease inhibitors (50 μg/ml final chymostatin, leupeptin, aprotinin, and pepstatin; 1 mM PMSF (Sigma Chemical Co., St. Louis, MO) or 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (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 BSA (50 mg/ml final) chymostatin, leupeptin, aprotinin, and pepstatin; 1 mM PMSF (Sigma Chemical Co., St. Louis, MO) or 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (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.

To label worm proteins with 35S, worms were fed radiolabeled bacteria as described in Gettner et al. (1983). Nematodes were grown to saturation in LB at 37°C to a density of 0.600 = 0.2. At this point, 500 μCi of [35S]methionine, 500 μCi of [35S]-cysteine, and 200 μCi of [35S]SO4 (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 in room temperature overnight to allow the radiolabeled bacteria to grow and the next day, bacitracin (1 μg/ml) and streptomycin (0.5 μg/ml) were added. The plates were then incubated at 37°C until the radiolabeled bacteria reached the high density. The radiolabeled bacteria were harvested by scraping the plates, sonicated, and centrifuged at 10,000 g at 4°C for 10 min. The supernatant was used for all of the experiments.

**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 protease 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 MgCl2; 1 mM CaCl2; 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 MgCl2; 1 mM CaCl2; 0.2% SDS, 0.1% Triton X-100 for radiolabeled samples; 100 mM Tris-Cl, pH 8.0, 500 mM NaCl, 1 mM CaCl2, 1% Triton X-100, 0.2% SDS for biotinylated samples) to reduce the background. 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'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 1:5000 or with HRP-streptavidin (Zymed, South San Francisco, CA) diluted 1:4000 in 1% BSA, fraction V plus 0.05% Tween-20. Blots incubated with alkaline-phosphatase conjugated streptavidin were washed 3× 15 min at room temperature with PBS, 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, 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 gel.
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-3 cyto diluted 1:200 or MH27 (a control for permeabilization) diluted 1:500 in PBS, pH 7.0, and mounted for viewing with 80% glycerol containing 2% n-propyl alcohol. 

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

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 Sal I 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 Sal I 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 Sal I 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 D149 to residue D206 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 Sal I 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

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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 \( \beta \text{pat}-3 \). 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.

sequences were examined for open reading frames. Although several open reading frames were detected, none were flanked by \( \text{C. elegans} \) 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 \( \beta \) subunit genes is evolutionarily conserved, the positions of introns in the \( \beta \text{pat}-3 \), human integrin \( \beta 2, \beta 3, \beta 7, \) and \( \text{Drosophila} \) 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 \( \text{C. elegans} \) 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 \( \beta \) subunit genes is evolutionarily conserved, the positions of introns in the \( \beta \text{pat}-3 \), human integrin \( \beta 2, \beta 3, \beta 7, \) and \( \text{Drosophila} \) 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, chicken β1, Xenopus β1, and Drosophila βPS (see Moyle et al., 1991; MacKrell et al., 1988).
Figure 3. Organization of the \( \beta \text{pat-3} \) subunit gene, RNA blot analysis of \( \beta \text{pat-3} \) transcripts, and alignment of \( \beta \text{pat-3} \) exons with the exons of human \( \beta_2 \), human \( \beta_3 \), human \( \beta_7 \), and Drosophila \( \beta \text{PS} \).

(A) The organization of exons and introns in the integrin \( \beta \text{pat-3} \) gene is shown relative to a schematic representation of the \( \beta \text{pat-3} \) 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 \( \beta \text{pat-3} \) transcripts. 50 \( \mu \)g of \( C. \text{elegans} \) total RNA were separated by agarose gel electrophoresis, blotted onto a nylon membrane, and probed with radiolabeled cDNA 1-1. A major transcript of \( \sim 3.0 \) kb was detected. The positions of the molecular weight markers (shown numerically in kilo-base pairs) are marked on the left.

(C) The exons of \( C. \text{elegans} \beta \text{pat-3} \) are aligned with the exons of human \( \beta_2 \) (Weitzman et al., 1991), human \( \beta_3 \) (Lanza et al., 1990; Zimrin et al., 1990), human \( \beta_7 \) (Jiang et al., 1992), and Drosophila \( \beta \text{PS} \) (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 \( \beta \text{pat-3} \) and the other \( \beta \) subunits are highlighted by an asterisk. The size of the \( \beta \) subunits was made uniform to demonstrate the alignment. Introns 1, 2, 3, and 7 of \( \beta \text{pat-3} \) correspond to introns 3, 4, 6, and 15 of human \( \beta_2 \); introns 2, 3, 5, and 14 of human \( \beta_3 \); introns 3, 4, 6, and 15 of \( \beta_7 \). Introns 2 and 7 of \( \beta \text{pat-3} \) correspond to introns 1 and 4 of \( \beta \text{PS} \).

The \( C. \text{elegans} \) \( \beta \text{pat-3} \) subunit 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 \( \beta_1 \) 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 \( \beta \text{pat-3} \) gene and the human integrin \( \beta_2 \), \( \beta_3 \), and \( \beta_7 \) genes, and the location of two of these four introns is conserved in the \( Drosophila \) integrin \( \beta \text{PS} \) gene. The first three introns conserved between the \( \beta \text{pat-3} \) gene and the human integrin \( \beta \) 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 \( \beta \) 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 \( \beta \text{pat-3} \) Protein**

To analyze the \( \beta \text{pat-3} \) 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-\( \beta \text{pat-3}-\text{cyto} \)) recognizes a single band at \( \sim 109 \) 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 \( \beta \) subunits and is presumably due to the high cysteine content. No \( C. \text{elegans} \) proteins were recognized by the preimmune serum (Fig. 4, lanes 1 and 4). In addition, the specificity of anti-\( \beta \text{pat-3}-\text{cyto} \) was demonstrated by competition with the peptide that was used to generate the antibody (Fig. 4, lanes 3 and 6).

**\( \beta \text{pat-3} \) Is Widely Expressed Postembryonically**

As a first step toward understanding the function of \( \beta \text{pat-3} \) during development, the cellular localization of \( \beta \text{pat-3} \) was determined using the \( \beta \text{pat-3}-\text{cyto} \) antibody. As a guide to data in later figures, a schematic representation of the tissues which express \( \beta \text{pat-3} \) 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, \( \beta \text{pat-3} \), 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). \( \beta \text{pat-3} \) was also detected in single sarcomere
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 $\times 10^3$.

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 $\times 10^3$.

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.

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original 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 Reichard, 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 35S. 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, 35S-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<sub>r</sub> 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<sub>r</sub> 180 kD band migrated at ~165 kD when reduced (not visible in this experiment); the M<sub>r</sub> 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 dauer larvae. 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 vml 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, midfocal 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, midfocal 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/F). (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 (vm1 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 MH27 staining can be seen in G. Bars, 20 µm.

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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 35S. 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, dauer. 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.
Discussion

As a first step toward understanding the function of integrin subunits 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 derived 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

Table 1. 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.
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 immuno-
precipitated by anti-βpat-3cyto. Fourth, the pat-3(rhl51)
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 attach-
ment. 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 att-
achment 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 organiza-
tion 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 ver-
tebrates differ, it is not possible to make a detailed compari-
on of the localization of βpat-3, βPS, or β1 in their respec-
tive 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 Dro-
sophila. 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 integ-
rin 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 Dro-
sophila, integrin βPS is present in ectodermal cells in em-
bryos (Leptin et al., 1989). In contrast, we did not detect
βpat-3 in hypodermal cells, either embryonically or postem-
bryonically. Finally, unlike vertebrate integrin β1, which is
highly expressed in the developing nervous system (for re-
view 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 ex-
est before the evolutionary divergence of protostomes from
deouterostomes. Several arguments favor the latter hypothe-
sis: (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 be-
tween 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 multi-
ple integrin α subunits. However, this property has been ob-
served with several integrin β subunit (β1, β2, β3, and β7)
(Hynes, 1992). (c) Comparison of the localization of βpat-3
with chicken integrin β1 in posthatch chick muscle (dis-
cussed above) has shown that their distributions are similar,
but not identical. Future studies will show whether the func-
tions of other integrin β subunits present in vertebrate mus-
cle are analogous to those performed by βpat-3; in particu-
lar, 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 ex-
pressed in the embryonic nervous system. (e) Human inte-
grins 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 in-
tron, it could be considered more closely related to the inte-
grin 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 increas-
ingly linked and more mutants are isolated, the identifica-
tion 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 pos-
sible to determine which proteins interact with integrin
receptors to produce cellular behaviors such as cell motility
and cell morphogenesis.

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References

Altruida, F., P. Cervella, G. Tarone, C. Botta, F. Balzac, G. Stefanuto, and L. Silengo. 1990. A human integrin β subunit with a unique cytoplasmic domain generated by alternative mRNA processing Gene (Amst.). 95:261-266.

Bao, Z., M. Lakonishok, S. Kaufman, and A. Horwitz. 1993. α7β1 integrin is a component of the myotendinous junction on skeletal muscle. J. Cell Sci. 106:579-590.

Bozyczko, D., C. Decker, J. Muschler, and A. Horwitz. 1989. Integrin on developing and adult skeletal muscle. Exp. Cell Res. 183:72-91.

Brenner, S. 1974. The genetics of Caenorhabditis elegans. Genetics. 77:71-94.

Bronner-Fraser, M. 1986. An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development in vivo. Dev. Biol. 117:528-536.

Brown, N., D. L. King, M. Wilcox, and F. C. Kafatos. 1989. Developmentally regulated alternative splicing of Drosophila integrin PS2α transcripts. Cell. 59:185-195.

Church, G. M., and W. Gilbert. 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA. 81:1991-1995.

Darrerre, R. K., M. Yamada, K. E. Johnson, and J. C. Boucaut. 1988. The 140-kDa fibronectin receptor complex is required for mesodermal cell adhesion during gastrulation in the amphibian Pleurodeles waltlii. Dev. Biol. 126:182-194.

D'Souza, S. E., M. H. Ginsberg, T. A. Burke, S. C.-T. Lam, and E. F. Plow. 1988. Location of an arg-gly-asp recognition site within an integrin adhesion receptor. Science (Wash. DC). 242:91-93.

Emmons, S. W., M. R. Klass, and D. Hirsh. 1979. Analysis of the constancy of DNA sequences during development and evolution of the nematode Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA. 76:1333-1337.

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.

Galileo, D. S., J. Majors, A. F. Horwitz, and J. R. Sanes. 1992. Retrovirally introduced antisense integrin RNA inhibits neuroblast migration in vivo. J. Cell Biol. 108:391-402.

Gettner, S. 1994. Isolation and functional analysis of C. elegans integrin βpat-3. Ph.D. thesis. University of California, San Francisco, CA. pp. 1-152.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY. pp. 1-726.

Hresko, M. C., B. D. Williams, and R. H. Waterston. 1994. Assembly of body wall muscle and muscle cell attachment structures in Caenorhabditis elegans. J. Cell Biol. 124:491-506.

Hynes, R. O. 1992. Integrins: versatility, modulation and signalling in cell adhesion. Cell. 69:11-25.

Jiang, W.-M., D. Jenkins, Q. Yuan, E. Leung, K. Choo, J. Watson, and G. Krissansen. 1992. The gene organization of the human β7 subunit, the common β subunit of the leukocyte integrins HML-1 and LPAM-1. Int. Immunol. 4:1031-1040.

Krause, M., and D. Hirsh. 1987. A trans-spliced leader sequence on actin mRNA in Caenorhabditis elegans. Cell. 49:753-761.

Lanza, F., N. Kieffer, D. Phillips, and L. A. Fitzgerald. 1990. Characterization of the human platelet glycoprotein IIIa gene: comparison with the fibronectin receptor β-subunit gene. J. Biol. Chem. 265:18098-18103.

Leptin, M., T. Bogaert, R. Lehmann, and M. Wilcox. 1989. The function of PS integrins during Drosophila embryogenesis. Cell. 56:401-408.

MacKrell, A. J., B. Blumberg, S. R. Haynes, and J. H. Fessler. 1988. The lethal myosinoid gene of Drosophila encodes a membrane protein homologous to vertebrate integrin β subunits. Proc. Natl. Acad. Sci. USA. 85:2633-2637.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, New York. pp. 1-545.

Moyle, M. A. Napier, and J. W. McLean. 1991. Cloning and expression of a divergent integrin subunit ββ. J. Biol. Chem. 266:19650-19658.

Neugebauer, K. M., and L. F. Reichardt. 1991. Cell-surface regulation of β1-integrin activity on developing retinal neurons. Nature (Lond.) 350:68-71.

Reichardt, L. F., and K. J. Tomasedi. 1991. Extracellular matrix molecules and their receptors: functions in neural development. Annu. Rev. Neurosci. 14:531-570.

Rosenquist, T. A., and J. Kimble. 1988. Molecular cloning and transcript analysis of fem-3, a sex-determination gene in Caenorhabditis elegans. Genes Dev. 2:606-616.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463-5467.

Smith, R. F., and T. F. Smith. 1991. Pattern-induced multi-sequence alignment (PIMA) algorithm employing secondary structure-dependent gap penalties for comparative protein modelling. Protein Engineering. 5:35-41.

Sulston, J. E., and R. H. Horvitz. 1977. Post-embryonic cell lineages of the nematode Caenorhabditis elegans. Dev. Biol. 56:110-156.

Sulston, J. E., D. G. Albertson, and J. N. Thomson. 1980. The Caenorhabditis elegans male: post-embryonic development of non-gonadal structures. Dev. Biol. 78:542-576.

Sulston, J. E., E. Schierenberg, J. G. White, and J. N. Thomson. 1983. The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev. Biol. 100:64-119.

van Kuppevelt, T. H. M. S. M., L. R. Languino, J. O. Galili, S. Suzuki, and E. Raoslabi. 1989. An alternative cytoplasmic domain of the integrin β3 subunit. Proc. Natl. Acad. Sci. USA. 86:5415-5418.

Volta, T., L. I. Fessler, and J. H. Fessler. 1990. A role for integrin in the formation of sarcomeric cytoarchitecture. Cell. 63:525-536.

Weitzman, F. B., C. E. Wells, A. H. Wright, P. A. Clark, and S. K. A. Law. 1991. The gene organization of the human β2 integrin subunit (CD18). FEBS Lett. (Fed. Eur. Biochem. Soc.) Lett. 294:97-103.

Williams, B. D., and R. H. Waterston. 1994. Genes critical for muscle development and function in Caenorhabditis elegans identified through lethal mutations. J. Cell Biol. 124:475-490.

Wilson, R., R. Ainscough, K. Anderson, C. Baynes, M. Berks, J. Bonfield, J. Burton, et al. 1994. 2.2 Mb of contiguous nucleotide sequence from chromosome III of C. elegans. Nature (Lond.). 368:32-38.

Wood, W. B. 1988. The Hematode Caenorhabditis elegans. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1-667.

Zembrin, A. B., S. Gidwitz, S. Lord, E. Schwartz, J. S. Bennett, G. C. White II, and M. Poncz. 1990. The genomic organization of platelet glycoprotein IIIa. J. Biol. Chem. 265:8590-8595.

Zusman, S., Y. Grinblat, G. Yee, F. C. Kafatos, and R. O. Hynes. 1993. Analyses of PS integrin functions during Drosophila development. Development. 118:737-750.

Zusman, S., R. S. Patel-King, C. French-Constant, and R. O. Hynes. 1990. Requirements for integrins during Drosophila development. Development. 108:391-402.