

Molecular Characterization of Human Zyxin*

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Zyxin is a component of adhesion plaques that has been suggested to perform regulatory functions at these specialized regions of the plasma membrane. Here we describe the isolation and characterization of cDNAs encoding human and mouse zyxin. Both the human and mouse zyxin proteins display a collection of proline-rich sequences as well as three copies of the LIM domain, a zinc finger domain found in many signaling molecules. The human zyxin protein is closely related in sequence to proteins implicated in benign tumorigenesis and steroid receptor binding. Antibodies raised against human zyxin recognize an 84-kDa protein by Western immunoblot analysis. The protein is localized at focal contacts in adherent erythroleukemia cells. By Northern analysis, we show that zyxin is widely expressed in human tissues. The zyxin gene maps to human chromosome 7q32–q36.

Focal contacts represent specialized regions of the plasma membrane where the cell engages in integrin-dependent cell adhesion to the extracellular matrix. At these sites, the cell establishes a transmembrane connection between elements of the extracellular matrix and the actin cytoskeleton. The linkage of the cytoskeleton to the integrin receptors appears to be critical for the ability of integrins to function as true receptors that relay information about the extracellular environment to the cell interior to affect many aspects of cell behavior including cell locomotion and cell proliferation (Zigmond, 1996; Shattil *et al.*, 1994; Clark and Brugge, 1995; Miyamoto *et al.*, 1995). The integrin receptors contribute to the regulation of cell function by affecting the activation states of intracellular signal transduction cascades (Hynes, 1992; Miyamoto *et al.*, 1995). For example, integrin engagement regulates the tyrosine phosphorylation of proteins that are co-localized with the receptors at the adhesion plaques (Ferrell and Martin, 1989; Golden *et al.*, 1990; Shattil and Brugge, 1991; Guan *et al.*, 1991; Kornberg *et al.*, 1991; Schaller *et al.*, 1992). Tyrosine phosphorylation

events couple components of the focal contact to downstream effector molecules that are likely to influence behaviors such as cell proliferation (Clark and Brugge, 1995; Miyamoto *et al.*, 1995). Cytoskeletal elements that are co-localized with integrin receptors may be essential for the docking of signaling proteins in association with the adhesive membrane or may participate directly in certain responses to integrin occupancy, such as membrane protrusion and cell spreading.

One protein that may function in both a signaling and an architectural capacity in conjunction with the actin cytoskeleton is zyxin (Crawford and Beckerle, 1991; Sadler *et al.*, 1992). Zyxin is a low abundance phosphoprotein that is concentrated at adhesion plaques and along the actin filament bundles near where they insert at the adhesion plaques (Crawford and Beckerle, 1991). Zyxin has the molecular features of an intracellular signal transducer (Sadler *et al.*, 1992). The protein exhibits a striking proline-rich sequence that has been shown to associate with SH3 domains that are found in a number of protein components of signal transduction pathways. For example, zyxin interacts with the human proto-oncogene product Vav, an SH3 protein that is required for antigen-dependent signaling in T- and B-cells (Hobert *et al.*, 1996). The proline-rich sequences in zyxin are also related to sequences in the ActA protein of the intracellular bacterial pathogen *Listeria monocytogenes* that play a role in coordinated actin assembly at the surface of the bacterium (Lasa *et al.*, 1995; Pistor *et al.*, 1995.)

In addition to the proline-rich region, zyxin displays three copies of a cysteine- and histidine-rich motif called the LIM motif, which is found in a number of proteins involved in the regulation of cell proliferation and differentiation (Sadler *et al.*, 1992; Schmeichel and Beckerle, 1994). The LIM motif has been shown to mediate specific protein-protein interactions (Schmeichel and Beckerle, 1994; Wu and Gill, 1994; Feuerstein *et al.*, 1994). One of the LIM domains in zyxin interacts with members of the cysteine-rich protein family that have been implicated in myogenic potentiation (Sadler *et al.*, 1992; Schmeichel and Beckerle, 1994; Arber *et al.*, 1994). The demonstration that zyxin displays a tandem array of domains that mediate specific protein-protein interactions has led to the suggestion that zyxin may function as a scaffold for the assembly of multimeric complexes (Schmeichel and Beckerle, 1994). These protein assemblages could mediate integrin-dependent signaling events that lead to cell differentiation or modulation of cytoarchitecture.

Thus far, zyxin has only been characterized in avian cells (Crawford and Beckerle, 1991; Crawford *et al.*, 1992; Sadler *et al.*, 1992). To identify sequences in zyxin that are central to its function and to develop materials to facilitate the study of zyxin in mammals, we have initiated an effort to identify and characterize human zyxin. Here we report the sequence, expression pattern, subcellular distribution, and chromosomal map position corresponding to human zyxin.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y07711 and X99063 (murine zyxin) and X94991 (human zyxin).

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MATERIALS AND METHODS

Isolation of Human and Mouse Zyxin cDNAs—A human umbilical vein endothelial cell cDNA (VII-91-5) library cloned into the *EcoRI* site of λ gt11 was obtained from E. Sadler (Washington University, St. Louis, MO). The library was screened with a chicken zyxin cDNA probe (Sadler *et al.*, 1992) by standard procedures. Briefly, a chicken zyxin cDNA fragment corresponding to nucleotides 1133–1718 was labeled with ^{32}P using the Prime It II labeling kit (Stratagene, La Jolla, CA). Hybridization was performed overnight at 50 °C in 5 × SSPE, 5 × Denhardt's solution, 0.5% (w/v) SDS, and 250 $\mu\text{g/ml}$ yeast tRNA (Boehringer Mannheim). Following hybridization, the filters were washed twice at room temperature for 10 min each with 2 × SSPE and 0.1% (w/v) SDS and once at 60 °C for 15 min with 1 × SSPE and 0.1% (w/v) SDS. Plaques giving specific signals with the probe were isolated by four rounds of purification. The sizes of the inserts were determined by PCR¹ using λ gt11 phage arm primers and purified plaques from the fourth-round screen as templates. The phage isolates carrying large inserts were amplified, and the insert DNA fragments were isolated and subcloned into the *EcoRI* site of the pBluescript KS⁻ vector.

Two methods were used to isolate cDNAs that included sequence corresponding to the complete 5'-untranslated region of zyxin mRNA. In the first method, poly (A)⁺ RNAs prepared from HEL cells and purified on oligo(dT) columns (QIAGEN Inc., Chatsworth, CA) were reverse-transcribed and amplified using the Marathon cDNA amplification kit (CLONTECH, Palo Alto, CA). cDNA fragments were generated by 5'-rapid amplification of cDNA ends, subcloned into pBluescript KS⁻, and sequenced. In the second method, a ^{32}P -labeled 5'-probe prepared from the hZyx-20 (where "h" is human) clone was used to rescreen the original human umbilical vein endothelial cell cDNA library as well as two other human endothelial cell cDNA libraries that were kindly provided by E. Sadler. A total of 12 clones that were expected to correspond to the 5'-untranslated region of human zyxin mRNA were analyzed, but no additional meaningful sequence beyond what was available from analysis of hZyx-20 was obtained. The complete sequence of hZyx-20 cDNA can be found in the GenBank™/EMBL Data Bank under accession number X94991.

Two approaches were utilized to obtain full-length mouse cDNAs. The independently isolated mouse cDNA clones were identical in the coding regions, but exhibited different amounts of 5'- and 3'-untranslated regions. First, a mouse postnatal brain capillary cDNA library cloned in vector λ gt10 (Schntrich and Risau, 1993) was screened with a human zyxin cDNA probe. Several cross-hybridizing clones were isolated from this library. One clone with a 2-kb insert was characterized by restriction analysis and DNA sequencing. This sequence was complete with respect to coding capacity. The sequence of this cDNA has been deposited in the GenBank™/EMBL Data Bank under accession number X99063. Mouse cDNAs were also isolated from an 11.5-day mouse embryo cDNA library cloned into the expression vector λ gt11 (CLONTECH). To screen this library, the sequences encoding the LIM domains were amplified from first strand mouse fibroblast cDNA by the polymerase chain reaction using degenerate oligonucleotide primers that corresponded to conserved regions of the human and chicken zyxin sequences. A clone with a cDNA insert of ~2 kb was characterized by sequence analysis. This cDNA contained the majority of the coding sequence as well as an extensive 3'-untranslated region with an polyadenylation signal. The remaining coding sequence was determined by analysis of mouse genomic DNA. The resulting composite mouse zyxin cDNA sequence has been deposited in the GenBank™/EMBL Data Bank under accession number Y07711.

DNA Sequencing and Analysis—The sequences of human and mouse zyxin cDNAs were determined from both strands by the method of dideoxynucleotide chain termination sequencing of polymerase chain reaction products (Life Technologies, Inc.) or by a modification of the dideoxy chain termination method (Schuurman and Keulen, 1991). Some areas were also analyzed using automated sequencing technology on a Model 373A DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Sequencing was performed with standard primers and custom-made oligonucleotide primers. Oligonucleotides were synthesized using an Applied Biosystems Model 380B DNA synthesizer. Sequence analysis was performed with software from the Wisconsin Genetics Computer Group (Devereux *et al.*, 1984) using the program manual for the GCG package (Genetics Computer Group, 1994). The program Bestfit was used to compare the derived amino acid sequences of chicken and human zyxin. In this analysis, optimal alignment is achieved when nine

gaps are introduced; no gaps are required for alignment of the LIM regions. The Bestfit and Gap programs were used to compare the human and mouse zyxin sequences.

Southern Blot Analysis—Total human genomic DNA was purchased from Promega (Madison, WI). DNA samples (10 μg each) were digested with *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Dra*I, or *Hind*III. The resulting DNA fragments were resolved by electrophoresis on a 0.8% agarose gel and transferred to Hybond-N⁺ nylon membrane as described by the manufacturer (Amersham Corp.). A ^{32}P -labeled cDNA corresponding to nucleotides 1153–1730, the region encoding the LIM domains, was used as a probe. Hybridization was carried out overnight at 65 °C in 5 × SSPE, 5 × Denhardt's solution, 0.5% (w/v) SDS, and 250 $\mu\text{g/ml}$ yeast tRNA. The blot was washed twice at room temperature for 10 min each in a solution containing 2 × SSPE and 0.1% (w/v) SDS and once at 65 °C for 15 min in 1 × SSPE and 0.1% (w/v) SDS and subjected to a final high stringency wash at 65 °C for 10 min in 0.1 × SSPE and 0.1% (w/v) SDS.

Northern Blot Analysis—Human multiple tissue Northern blots (containing poly (A)⁺ RNA derived from heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and blood leukocytes) were purchased from CLONTECH. Hybridization was carried out overnight at 37 °C using ^{32}P -labeled human zyxin cDNA coding region in 5 × SSPE, 10 × Denhardt's solution, 100 $\mu\text{g/ml}$ denatured sheared salmon sperm DNA (Stratagene), 50% formamide (Life Technologies, Inc.), and 2% (w/v) SDS. The blots were washed four times at room temperature for 10 min each in a solution containing 2 × SSC and 0.05% (w/v) SDS and twice at 50 °C for 20 min each in 0.1 × SSC and 0.1% (w/v) SDS. Hybridization with a ^{32}P -labeled probe encoding glyceraldehyde-3-phosphate dehydrogenase was also carried out as a control. Blot signals were quantified by PhosphorImager analysis using ImageQuant software (Version 3.2, Molecular Dynamics, Inc.).

Indirect Immunofluorescence—HEL cells were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone Laboratories). Coverslips for indirect immunofluorescence with HEL cells were coated with 1 mg/ml fibronectin (Life Technologies, Inc.) in 1 × phosphate-buffered saline, incubated at 4 °C overnight, rinsed in 1 × phosphate-buffered saline, and placed in a Corning 60-mm Petri dish. Approximately 10⁶ cells in 2 ml of medium were added to each dish and were incubated for 4 h at 37 °C on fibronectin-coated coverslips with 1 μM 12-*O*-tetradecanoylphorbol-13-acetate in dimethyl sulfoxide (or dimethyl sulfoxide alone). Indirect immunofluorescence was performed as described previously (Beckerle, 1984) using the B38 rabbit polyclonal anti-platelet zyxin antibody as the primary antibody and rhodamine-conjugated goat anti-rabbit IgG as the secondary antibody. Visualization was performed on a Zeiss Axiophot microscope. The B38 anti-zyxin antibody used in this study is a rabbit polyclonal anti-peptide antibody directed against the human zyxin amino acid sequence KKFGPVPVAPKPK; an N-terminal cysteine residue was added to the zyxin peptide sequence to enable coupling of the peptide to carrier proteins prior to immunization. The human zyxin protein sequence used for antibody generation was obtained by direct microsequence analysis of zyxin isolated from human platelets.² The specificity of this antibody was demonstrated by Western immunoblot analysis using purified zyxin and whole cell lysates.

Western Immunoblot Analysis—A 10% SDS-polyacrylamide gel was run according to the method of Laemmli (1970) except with 0.13% bisacrylamide. Western immunoblot analysis was performed using the B38 antibody and a procedure that has been described previously (Beckerle, 1986). ¹²⁵I-Protein A followed by autoradiography was used as the detection method. HEL cell lysates were prepared as follows. A Corning 100-mm Petri dish was coated with 1 mg/ml fibronectin, incubated at 4 °C overnight, and rinsed once with phosphate-buffered saline. Cells were collected from a confluent 100-mm dish and spun in a table-top centrifuge at 1500 rpm for 2 min. The cell pellet was resuspended in 5 ml of medium, and the cells were added to the fibronectin-coated dish. Tumor-promoting phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate) was added to a final concentration of 1 μM , and the cells were incubated at 37 °C for 4 h. The cells were rinsed four times with 1 × phosphate-buffered saline and were harvested in Laemmli sample buffer (Laemmli, 1970). The resulting lysate was passed through a 26-gauge tuberculin syringe, and the sample was boiled for 5 min immediately prior to loading onto a 10% SDS-polyacrylamide gel.

Chromosomal Mapping—The somatic cell hybrid clones employed for chromosomal localization have been described previously (Boyle *et al.*, 1992; Croce and Koprowski, 1974; Luerssen *et al.*, 1990; Rommens *et*

¹ The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s).

² M. E. Hensler and M. C. Beckerle, unpublished observations.

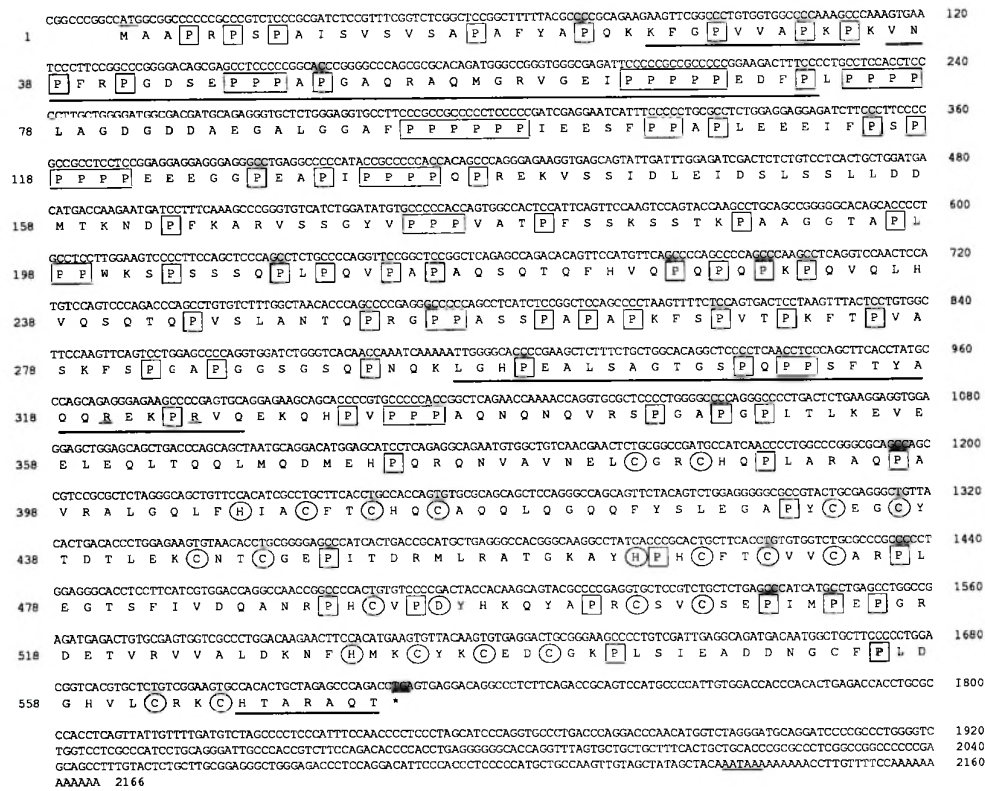


FIG. 1. Nucleotide and deduced amino acid sequences of human zyxin cDNA. Nucleotide numbers are indicated on the right, and amino acid numbers are indicated on the left. The regions of the deduced amino acid sequence that correspond to the four endoproteolytic peptides microsequenced from purified human platelet zyxin protein are underlined in bold; two underlined arginine residues were not unambiguously assigned in the peptide sequence analysis. The ATG start codon, stop codon, and polyadenylation signal are underlined. Proline residues are boxed; cysteine, histidine, and aspartic acid residues that comprise the conserved metal-binding residues of the LIM domain are circled. The nucleotide and deduced amino acid sequences have been deposited in the GenBank™/EMBL Data Bank under accession number X94991.

al., 1988; Vortkamp *et al.*, 1991). *EcoRI*-restricted DNA (12 μ g each) from the somatic cell hybrid lines and the control cell lines used in the chromosomal mapping panel was fractionated on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized to a ³²P-labeled fragment corresponding to nucleotides 1153–1730 of the zyxin cDNA. The hybridization protocol was essentially equivalent to that described above for Southern analysis. The probe detects human-specific hybridization of a 6.8-kb *EcoRI* fragment.

For higher resolution analysis of the position of the zyxin gene, PCR amplification with zyxin-specific primers was used to assay the presence of zyxin sequences in genomic DNA from several human-rodent somatic cell lines that display parts of human chromosome 7. The PCR primers (Pl, 5'-ACTCCATTCAGTTCCAAAGT-3'; and Pr, 5'-ACTG-GAGAAAACCTTAGG-3') were derived from the known cDNA sequence and give rise to a 270-bp amplification product from the zyxin gene. Reactions were performed in a total volume of 50 μ l containing 200 ng of genomic DNA template, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.02% gelatin, 200 μ M each dNTP, 100 ng of each primer, and 1 unit of *Pfu* DNA polymerase. The template DNA was subjected to 32 cycles of amplification (1 min at 94 °C, 30 s at 50 °C, and 2 min at 72 °C).

RESULTS

Isolation and Characterization of Human and Mouse Zyxin cDNAs—In an effort to identify human zyxin cDNA, a partial chicken cDNA that encoded the LIM region of zyxin was used to screen a human umbilical vein endothelial cell cDNA library. From 750,000 recombinants screened, seven positive plaques were isolated and purified. Five of the seven isolates were subcloned into the *EcoRI* site of pBluescript and sequenced. Comparison of the nucleotide sequences with the reported chicken zyxin sequence suggested that all five isolated cDNAs carried inserts that encoded human zyxin. The largest clone (hZyx-20) was sequenced in entirety on both strands. The nucleotide and deduced amino acid sequences of human zyxin

derived from cDNA clone hZyx-20 are shown in Fig. 1. The human zyxin cDNA is 2166 bp long. This size is very close to the transcript size observed by Northern analysis. The nucleotide sequence presented in Fig. 1 represents the extent of unambiguous zyxin cDNA sequence that we obtained by a variety of methods. Additional 5'-cDNA sequences defined either by employing a 5'-rapid amplification of cDNA ends strategy or by exhaustive rescreening of multiple cDNA libraries were divergent both in terms of absolute sequence and length and are therefore not included in this report. The cDNA sequence exhibits a polyadenylation signal (AATAAA) at the 3'-end and a potential initiation codon (ATG) beginning at nucleotide 11. The nucleotide sequence around this codon (GC-CATGG) fits the Kozak consensus for initiation sequences (Kozak, 1986). The open reading frame of 1716 bp encodes a protein of 572 amino acid residues with a predicted unmodified M_r of 61,273. Comparison of the deduced amino acid sequence with peptide sequences derived by endoproteolytic cleavage of purified human zyxin² strongly suggested that the cDNA we had isolated corresponds to the human zyxin transcript. In particular, with the exception of two amino acid residues that were ambiguous in the protein sequence analysis, the sequences of all four endoproteolytic peptides microsequenced were equivalent to regions of the deduced amino acid sequence. Since the peptide sequences analyzed (85 residues) arose from different parts of the purified zyxin protein, the results strongly suggest that the hZyx-20 clone is a *bona fide* human zyxin cDNA.

The overall molecular architecture and amino acid sequence of human zyxin are closely related to those of its avian homologue (Fig. 2). Human zyxin displays 58% identity and 70%

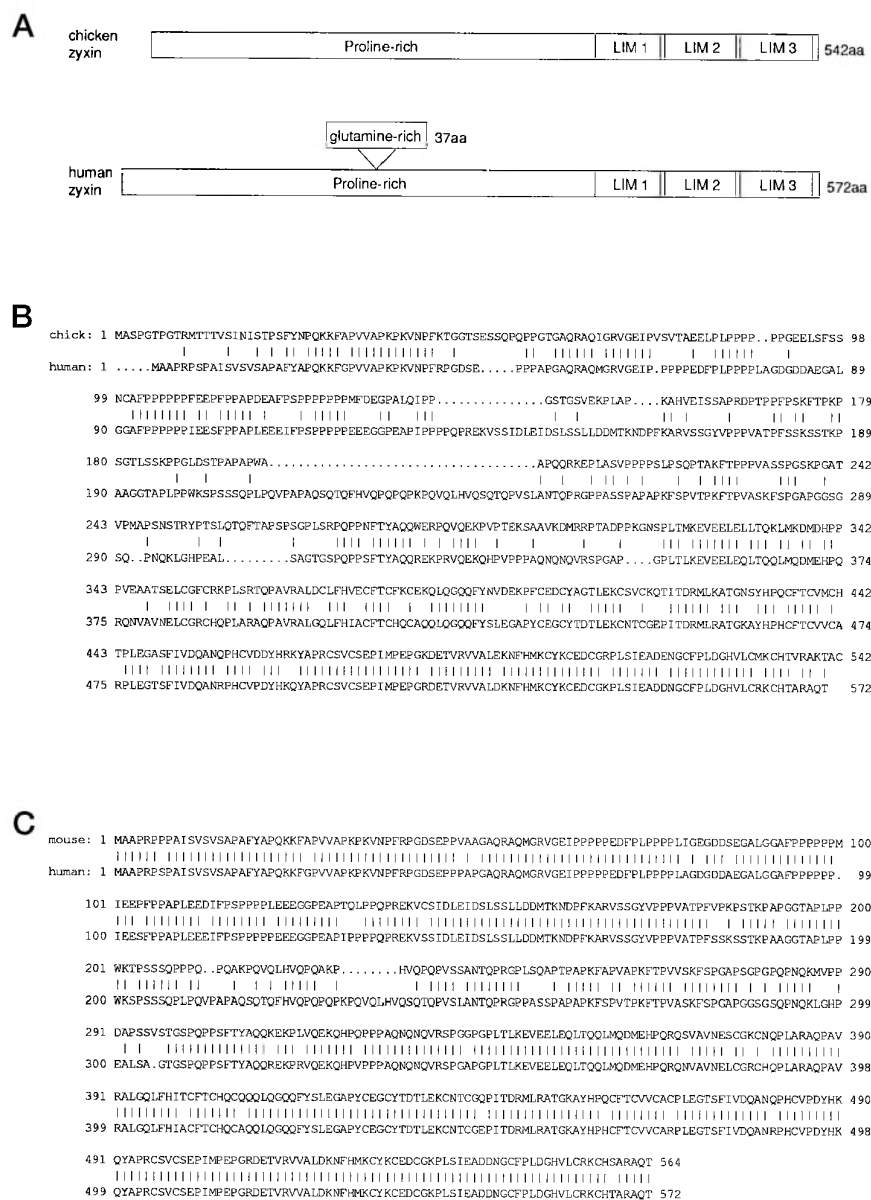


FIG. 2. Comparison of the amino acid sequences of chicken and human zyxin. *A*, a schematic representation of chicken and human zyxin proteins. The global architecture of the protein is conserved in the human and avian proteins; however, the human protein exhibits a few short sequences that are not found in the avian protein. *aa*, amino acids. *B*, a comparison of the amino acid sequences of chicken and human zyxin. The proteins display substantial similarity, particularly in the C-terminal LIM region. Identical residues are indicated by vertical lines. *C*, a comparison of the amino acid sequences of human and mouse zyxin. Identical residues are indicated by vertical lines.

similarity to the chicken protein. The predicted unmodified pI of human zyxin is 6.6, a value that is similar to the neutral pI predicted from the chicken zyxin sequence. Interestingly, as is the case for chicken zyxin (Sadler *et al.*, 1992), specific regions of the human zyxin protein display substantial position-dependent fluctuations in their predicted isoelectric points. When analyzed in intervals of 100 amino acids, we observe acidic domains (amino acids 1–99, pI 5.0; amino acids 100–199, pI = 4.1) and a basic region (amino acids 200–299, pI 12.1) that is completely devoid of aspartate and glutamate residues. The remaining protein sequence (amino acids 300–572) displays a near-neutral predicted pI of 6.9.

Like chicken zyxin, the human zyxin protein features a proline-rich sequence in the N-terminal region of the protein and three tandemly arranged LIM domains in the C-terminal region of the protein. The proline residues of human zyxin are boxed in Fig. 1. Both human zyxin and chicken zyxin exhibit a high proline content (18%) compared with the 5% found in typical eukaryotic proteins (Sadler *et al.*, 1992). The majority (86%) of the prolines are found in the N-terminal 350 amino acids of human zyxin; the proline content in this region exceeds 25%. Between amino acids 64 and 137, the proline content is

43%, and numerous arrays of up to six contiguous proline residues are observed. The proline-rich region of human zyxin is punctuated by the insertion of certain short sequences including a 37-amino acid glutamine-rich sequence (amino acids 211–247) that is not found in its avian counterpart. The overall glutamine content of human zyxin is also high, 8% compared with 4% in a typical eukaryotic protein. In addition, a serine-rich region is observed from amino acids 169 to 291; in this area, the serine content is 16% compared with 7% in a typical protein. The region of the protein that displays the LIM domains (amino acids 384–565) is the most highly conserved. Cysteine and histidine residues that are involved in zinc coordination by the LIM domains are circled in Fig. 1. In this area (amino acids 384–565), human zyxin displays 76% identity to chicken zyxin. Comparison of the sequences of the three individual LIM domains in human and chicken zyxin reveals 62% identity for LIM1, 71% identity for LIM2, and 92% identity for LIM3. In LIM3, there are only five amino acid differences between the human and chicken sequences, and four of these changes are very conservative substitutions (Fig. 2*B*). Regions of striking similarity between the human and chicken zyxin sequences are likely to correspond to binding sites for zyxin's

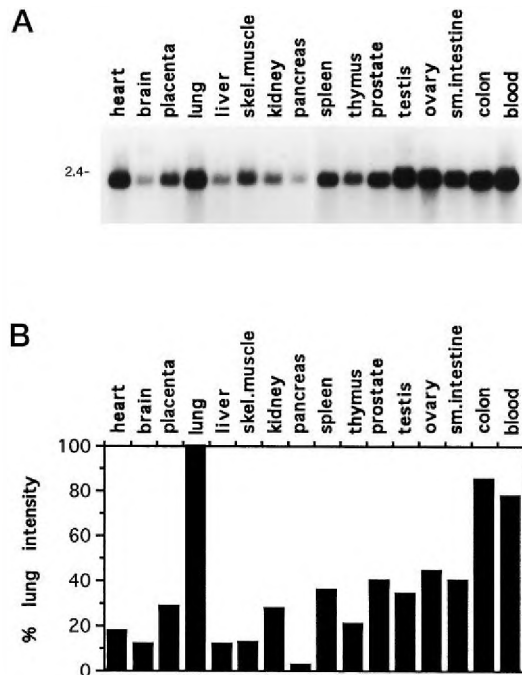


FIG. 3. Northern blot analysis of the distribution and expression of human zyxin mRNA. *A*, Northern blot analysis of mRNA derived from various human tissues. Each lane contains from 1 to 6 μ g of poly (A)⁺ RNA: heart, 5 μ g; brain, 1.5 μ g; placenta, 1.5 μ g; lung, 1 μ g; liver, 2 μ g; skeletal muscle, 4 μ g; kidney, 1 μ g; pancreas, 6 μ g; spleen, 2 μ g; thymus, 2 μ g; prostate, 2 μ g; testis, 4 μ g; ovary, 3 μ g; small intestine, 2.25 μ g; colon, 2 μ g; and blood leukocytes, 2.75 μ g. The blots were hybridized with a ³²P-labeled human zyxin cDNA probe that corresponded to the protein coding region. The relative position of an RNA size marker is indicated on the left. *B*, quantitative analysis of the results shown in *A*. The signals from the Northern blots were quantified by PhosphorImager analysis, and values were normalized with respect to the specific amounts (micrograms) of poly (A)⁺ RNA used for each tissue. mRNA levels are expressed as percent of the maximum expression detected in RNA samples obtained from lung tissue.

protein partners, including α -actinin, cysteine-rich protein, and SH3 proteins (Crawford *et al.*, 1992; Sadler *et al.*, 1992; Hobert *et al.*, 1996).

We have also isolated and characterized full-length mouse cDNAs. The open reading frame codes for a protein of 564 amino acids with an unmodified pI of 6.4 (Fig. 2C). Mouse zyxin displays the same overall molecular architecture as human and chicken zyxin, exhibiting an extensive proline-rich region and three tandemly arrayed LIM domains. The mouse protein exhibits 88% identity to human zyxin (Fig. 2C) and 57% identity to chicken zyxin at the amino acid level. The complete nucleotide sequences for the mouse cDNAs characterized in this study are available from the GenBankTM/EMBL Data Bank under accession numbers X99063 and Y07711.

Distribution and Expression of Human Zyxin mRNA—A Northern blot analysis of mRNA from 16 human tissues using the human zyxin cDNA coding region as probe revealed a single transcript of ~2.2 kb in all tissues (Fig. 3A). Northern analysis revealed that human zyxin is ubiquitously expressed. A glyceraldehyde-3-phosphate dehydrogenase probe was used to assess the quality of the RNA in each lane (data not shown). Because transcripts encoding glyceraldehyde-3-phosphate dehydrogenase are not expressed uniformly in different tissues (Spanakis, 1993), we quantitated the relative levels of zyxin expression by normalizing the PhosphorImager values to amounts (micrograms) of poly(A)⁺ RNA loaded in each lane. By this approach, we determined that the level of human zyxin mRNA is highest in the lung. Colon and blood were also relatively rich in this mRNA, while expression was relatively low in

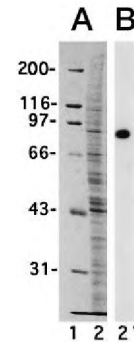


FIG. 4. Characterization of the anti-zyxin antibody. *A*, shown is a Coomassie Blue-stained SDS-polyacrylamide gel showing molecular mass markers (lane 1; in kilodaltons) and proteins of a HEL cell lysate (lane 2). *B*, proteins from a parallel gel were transferred to nitrocellulose and probed with the anti-zyxin antibody (lane 2'). A single protein with an apparent molecular mass of 84 kDa is detected by the anti-zyxin antibody.

the pancreas (Fig. 3B).

Association of Human Zyxin with Sites of Cell-Substratum Adhesion—To characterize further the relationship between the product of our cDNA clone and zyxin, we generated an anti-peptide antibody against the human protein and used this to examine the subcellular distribution of the protein by indirect immunofluorescence. Because zyxin expression appears to be relatively high in blood cells (Fig. 3), we examined the expression and subcellular distribution of zyxin in human erythroleukemia (HEL) cells. By Western immunoblot analysis, the antibody specifically recognizes a polypeptide that migrates at 84 kDa in HEL cells (Fig. 4). The immunoreactive protein in human cells behaves as if it is slightly larger than avian zyxin, which migrates at an apparent molecular mass of 82 kDa. This is consistent with the slightly greater size of the protein product in humans; chicken zyxin is 542 amino acids in length compared with 572 for the human protein. The high proline content of zyxin contributes to the reduced mobility observed on SDS-polyacrylamide gels (Sadler *et al.*, 1992; Schmeichel and Beckerle, 1994).

The subcellular distribution of zyxin in HEL cells was evaluated by indirect immunofluorescence (Fig. 5). HEL cells grow in suspension, but can be induced to spread and form focal adhesions when plated on a fibronectin substratum and stimulated with a tumor-promoting phorbol ester. Zyxin is diffusely distributed in suspension cells (Fig. 5A). When adhesion and spreading is stimulated, zyxin localizes to patches at the cell borders that are reminiscent of focal contacts (Fig. 5B). Some nuclear staining is occasionally observed, but the extent of this staining varies in different preparations. No staining is detected with preimmune serum (Fig. 5C).

To confirm that the staining observed in adherent cells coincides with the distribution of focal contacts, we examined the same cells by indirect immunofluorescence to localize zyxin and by interference reflection contrast microscopy to identify unequivocally the regions of close membrane-substratum contact. As can be seen in Fig. 5 (D and E), zyxin accumulates precisely at regions of cell-substratum adhesion. The localization of the human protein to these specialized regions of the plasma membrane provides further support for the suggestion that we have isolated the human homologue of zyxin.

Zyxin-related Sequences in the Human Genome—When total human genomic DNA was digested with *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, or *Hind*III and probed at high stringency with a human zyxin cDNA probe, a simple hybridization pattern was obtained (Fig. 6A). The hybridization pattern is characteristic of what would be expected for a single copy gene.

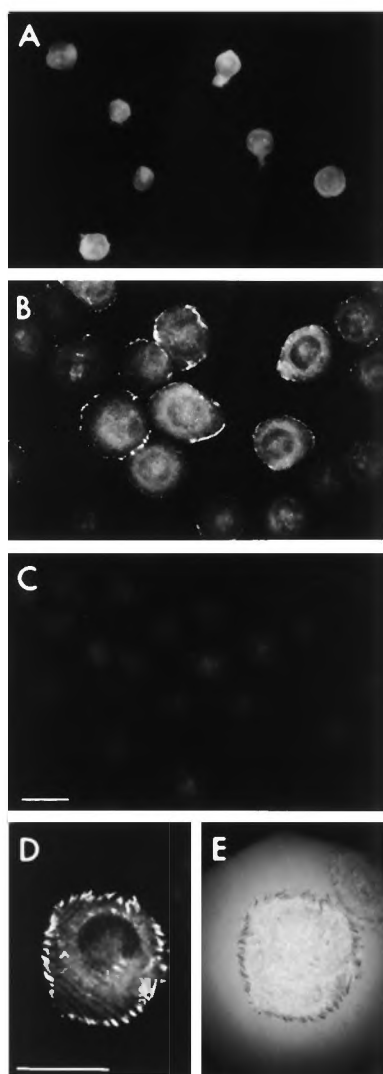


FIG. 5. Antibody directed against the zyxin peptide stains focal contacts of adherent HEL cells. The localization of zyxin was examined by indirect immunofluorescence. *A*, shown are nonadherent HEL cells stained with anti-zyxin antibody. *B*, HEL cells were stimulated to adhere by exposure to a fibronectin-coated coverslip in the presence of tumor-promoting phorbol ester and were stained with the anti-zyxin antibody. The cells are brightly labeled at the cell periphery, where newly formed adhesion plaques are prominent. Occasional nuclear staining is also observed, but the significance of this labeling is not clear. *C*, no staining is observed with preimmune serum. *D*, shown is a higher magnification view of an adherent HEL cell stained with the anti-zyxin antibody. Note the prominent staining of structures at the cell periphery. *E*, the same cell as in *D* is observed by interference reflection contrast microscopy to visualize the areas of closest membrane-substratum contact; these regions appear *black* and correspond precisely to the areas of prominent labeling with the anti-zyxin antibody observed in *D*. Bars = 30 μ m.

Searches of the GenBankTM/EMBL Data Bank revealed two other human proteins that bear a striking resemblance to zyxin (Fig. 6*B*). The human zyxin protein sequence is very similar to the sequence of a protein that is frequently fused to the HMGI-C transcription factor in human lipomas (Schoenmakers *et al.*, 1995; Ashar *et al.*, 1995). The LPP (lipoma-preferred partner) protein is closely related to zyxin in the LIM domain region and also exhibits a proline-rich N-terminal domain with proline-rich repeats similar to those found in human zyxin (Petit *et al.*, 1996). In addition to LPP, zyxin is related to a protein referred to as Trip6 (thyroid receptor-interacting protein), which was identified as a ligand-dependent binding partner for the thyroid hormone receptor (Lee *et al.*, 1995). The

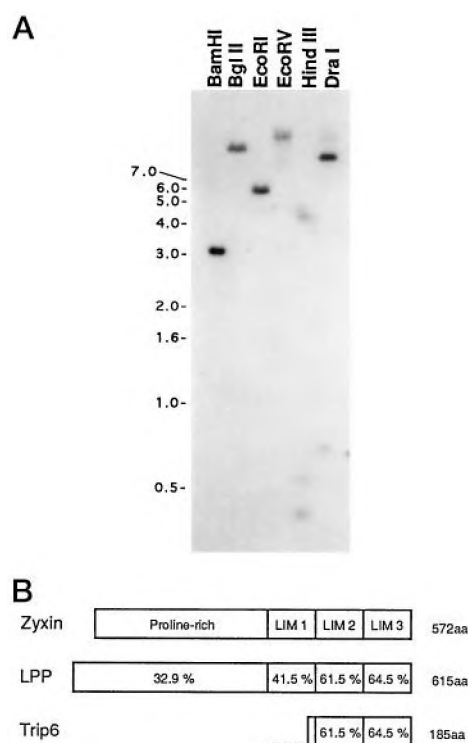


FIG. 6. Zyxin-related sequences in humans. *A*, a Southern blot analysis of the zyxin gene. Each lane contained 10 μ g of total human genomic DNA digested with *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, or *Dra*I. The blot was hybridized with ³²P-labeled cDNA corresponding to the region encoding the LIM domain sequences of human zyxin. The positions of the DNA size markers in kb are indicated on the left. *B*, a schematic representation of the zyxin protein and two other human sequences that show significant similarity to zyxin. Trip6 is a thyroid receptor-interacting protein, and LPP is a lipoma-preferred partner that is fused to the DNA-binding domain of HMGI-C in common benign mesenchymal tumors. The amino acid sequences corresponding to the boxed regions were compared individually with the sequence of human zyxin, and the relationship to human zyxin (percent amino acid identity) is shown for each area analyzed. aa, amino acids.

available Trip6 sequence information corresponds to the C terminus of the protein and specifies two complete and one partial LIM domain. Interestingly, the second and third LIM domains of both LPP and Trip6 independently exhibit 62 and 65% amino acid sequence identities to the corresponding LIM domains of zyxin. LPP and Trip6 are more similar to each other than either is to zyxin, displaying 77% amino acid identity in LIM2 and 74% amino acid identity in LIM3.

Chromosomal Mapping—Southern analysis and a PCR-based screen were employed to map the chromosomal location of the human zyxin gene. In our initial mapping studies, the human zyxin gene was assigned to chromosome 7 by discordancy analysis using Southern blot hybridization of a zyxin cDNA probe to a human-rodent somatic cell hybrid panel (Table I). The panel represents the complete population of human chromosomes with individual hybrids containing different chromosome complements (Boyle *et al.*, 1992; Luerssen *et al.*, 1990). *Eco*RI-restricted DNA corresponding to each hybrid cell type in the panel was hybridized with a human zyxin cDNA probe (0.6 kb). Analysis of the correlation between the presence (cell lines 1–5, 7, 8, 14, and 18) and absence (cell lines 6, 9–13, and 15–17) of the specific human signal (6.8 kb) and the human chromosome content shows that zyxin is encoded by a single locus in the human genome that is present on chromosome 7 (Table I). Hybridization to DNA isolated from the monochromosomal chromosome 7 human-mouse cell hybrid 5387-3 (cl.10) corroborates the assignment to this chromosome.

TABLE I
Segregation of human zyxin in human-rodent somatic cell hybrids

+, chromosome present; -, chromosome absent; o, chromosome fragment; ±, chromosome or chromosome fragment(s) present only in a minority of cells (<30%); (o and ± data were not included in the correlation analysis). Cell lines were as follows: 1, human DNA; 2-10 and 14-16, human-mouse cell hybrid DNA; 11 and 12, human-hamster cell hybrid DNA; 13, Chinese hamster DNA; 17, mouse DNA; and 18, monochromosomal chromosome 7 human-mouse somatic cell hybrid DNA.

Cell line	Human chromosomes																									
	Zyxin	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	
1. GM 3104	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2. B82/MS2, cl.1a-14-1	+	-	±	+	+	o	+	+	-	-	-	±	-	+	+	+	+	+	+	+	+	+	+	+	+	+
3. Rag/ANLY, cl.1	+	±	-	+	+	+	±	+	-	o	-	o	+	-	o	o	o	o	+	-	-	+	-	+	o	-
4. Rag/PI, cl.7-2	+	+	-	+	-	+	+	+	±	+	-	o	±	-	+	o	-	-	+	-	-	-	±	o	-	-
5. Rag/PI, cl.5-15	+	±	o	+	+	+	+	±	-	+	+	±	-	+	+	+	+	+	+	+	+	+	+	+	o	-
6. Rag/GM 194, cl.7	-	+	-	o	+	+	+	-	+	-	+	o	±	-	+	o	-	+	+	+	+	+	+	o	-	-
7. Rag/GM 194, cl.5-5	+	-	-	o	+	+	+	+	-	+	-	+	+	+	+	-	+	-	-	o	+	+	-	o	-	-
8. Rag/GO, cl.4	+	-	-	±	+	-	-	+	+	-	o	-	-	+	+	+	-	-	±	-	-	-	+	o	-	-
9. Rag/SU, cl.3-1-2-3	-	-	-	o	-	+	o	o	-	-	-	-	o	-	-	-	-	-	-	-	-	-	+	-	+	-
10. A9/SU, cl.1-2	-	-	+	o	o	-	+	o	-	-	-	o	-	+	+	-	-	o	+	-	-	+	-	-	-	-
11. V79/Ly, cl.3-2	-	±	-	±	+	-	+	-	+	-	+	o	+	-	+	-	-	+	-	-	-	+	+	+	+	+
12. V79/Ly, cl.3-3	-	+	-	+	+	-	-	-	±	-	+	o	+	-	+	-	-	+	-	-	-	+	+	+	+	+
13. P3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14. A9/GM 89, cl.9c-7	+	-	-	+	+	-	-	+	-	-	-	+	-	-	-	+	o	-	+	o	o	+	+	o	-	-
15. A9/MS 58, cl.2b	-	-	o	-	-	-	-	-	-	-	+	-	+	o	+	-	-	-	-	-	-	-	-	o	-	-
16. Rag/GM79, cl.8-13-3	-	o	-	-	+	-	-	-	-	+	-	+	-	+	-	-	-	+	-	-	-	-	o	o	-	-
17. Rag	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18. 5387-3, cl.10	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
No. of concordant hybrids	(+/+)	1	0	5	6	4	3	8	2	1	2	3	2	2	4	4	3	2	5	2	2	5	4	1	1	
No. of discordant hybrids	(-/-)	3	5	2	2	5	4	6	4	6	4	2	4	3	2	6	6	3	6	6	7	2	4	1	5	
	(+/-)	5	6	1	2	4	3	0	4	6	5	3	4	5	3	3	3	5	2	4	5	3	3	1	7	
	(-/+)	2	1	1	4	2	2	0	2	1	3	2	2	2	4	1	0	3	1	1	0	5	2	3	2	
% discordancy		64	58	22	43	40	42	0	50	50	57	50	50	58	54	29	25	61	21	38	36	53	38	67	60	

We have further defined the location of the gene within human chromosome 7 using PCR amplification of DNA from well characterized human-rodent somatic cell hybrids that contain fragments of this chromosome (Fig. 7). All the hybrid cell lines employed have been described previously (Croce and Koprowski, 1974; Rommens *et al.*, 1988; Vortkamp *et al.*, 1991). PCR amplification of zyxin sequences using zyxin-specific primers results in a 270-bp product. As can be seen in Fig. 7, sequences present on the long arm of human chromosome 7 are required for amplification driven by zyxin-specific primers. The lack of amplification using 194 Rag 6-13 genomic DNA as template localizes the gene encoding zyxin to chromosomal region 7q32-q36. No amplification occurs when murine DNA is used as template (Fig. 7, Rag DNA).

DISCUSSION

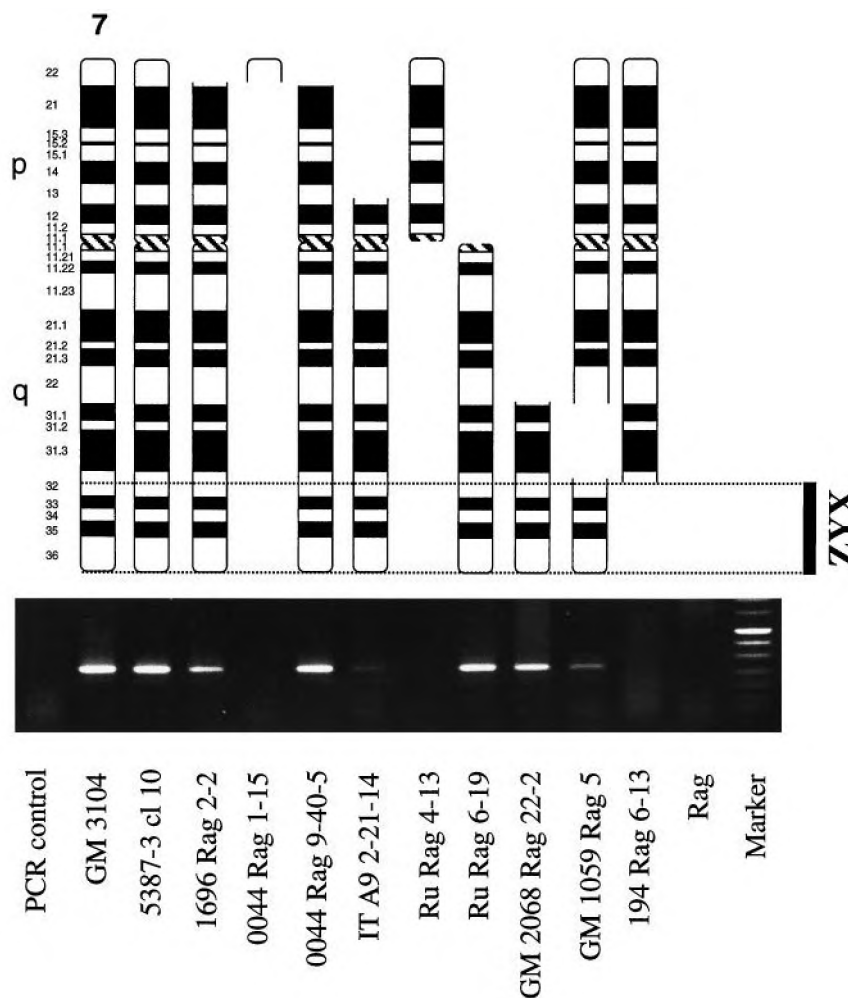
In this paper, we have reported the molecular characterization of human zyxin. Evidence that we have isolated cDNAs that encode the human homologue of zyxin includes the substantial similarity in domain organization and sequence as well as the common subcellular distributions of the protein when compared with avian zyxin. Moreover, Southern analysis has revealed that the human sequences we have characterized are the sequences that are most closely related to the coding region of avian zyxin. Zyxin is widely expressed in human tissues, but is most prominent in blood cells and in tissues that are rich in smooth muscle and endothelial cells, such as lung and colon.

The amino acid sequence of zyxin suggests certain functions for the protein. Chicken zyxin, as well as the human and mouse forms of zyxin characterized here, displays substantial proline-rich repeats as well as three copies of the LIM domain. Proline-rich regions of proteins can serve as molecular spacers (Stryer, 1978; Stryer and Haugland, 1967) or as surfaces for interaction with specific ligands such as proteins that exhibit SH3 domains (Ren *et al.*, 1993). SH3 domains were first defined in the human proto-oncogene product Src and have now been identified in a large number of proteins, many of which participate in signal

transduction. In aqueous solution, proline arrays typically adopt an extended conformation called a polyproline II helix, which is well suited for high affinity binding since it displays very limited mobility in solution (Williamson, 1994). Interestingly, proteins that are rich in proline residues are often rich in glutamines as well. Like prolines, glutamines are predicted to prefer a polyproline II helical secondary structure (Adzhubei and Sternberg, 1993). In this regard, it is intriguing that human zyxin displays a 37-amino acid sequence (not found in avian zyxin) that is 50% proline and glutamine.

The proline-rich regions of zyxin derived from human, murine, and avian sources also exhibit a number of specific sequences that are very similar to proline-rich repeats ((D/E)FP-PPP) found in the ActA protein of the intracellular pathogen *L. monocytogenes* (reviewed by Lasa and Cossart (1996)). The ActA protein is a bacterial surface component that is critical for transmission of the pathogen (Kocks *et al.*, 1992; Domann *et al.*, 1992). In a eukaryotic cell that is infected with *Listeria*, the cytoplasmic bacteria harness host cell machinery to assemble actin-rich comet tails that allow the bacteria to move within the host cytoplasm and to infect adjacent cells (reviewed by Tilney and Tilney (1993)). The *Listeria* ActA protein is required for the ability of a bacterium to nucleate and polymerize the surface-bound actin that is necessary for its effective transmission to uninfected cells (Kocks *et al.*, 1992; Domann *et al.*, 1992). Analysis of the ActA protein has revealed that proline-rich repeats found in the central region of the protein, although not required for the initial polymerization of actin, stimulate the assembly of filamentous actin in association with the bacterial surface (Southwick and Purich, 1994; Pistor *et al.*, 1994, 1995; Lasa *et al.*, 1995). The similarity of zyxin sequences to the bioactive proline-rich repeats in ActA raises the possibility that zyxin may be a cellular analogue of ActA. If this were the case, zyxin, like ActA, would be expected to play a role in the spatial control of actin assembly within cells. This suggestion gains some support from the observation that zyxin interacts with a pro-

FIG. 7. **Regional mapping of the human zyxin gene.** *Top*, schematic representation of the human chromosome 7 content of each somatic cell hybrid and of the control template. Hybrid 5387-3, cl.10, contains only chromosome 7 as human material. The bar indicates the chromosomal region (7q32–q36) containing the human zyxin gene (*ZYX*). *Bottom*, the ethidium bromide-stained agarose gel of the PCR products shows the presence or absence of the 270-bp human zyxin-specific fragment in the genomic template DNAs indicated below the corresponding lanes. For size determination, the DNA molecular weight marker system VIII from Boehringer Mannheim was used.



tein called VASP (Reinhard *et al.*, 1995b). VASP, a vasodilator-stimulated phosphoprotein that was originally characterized in human platelets, is a profilin-binding partner that is itself implicated in actin assembly (Halbrugge and Walter, 1989; Haffner *et al.*, 1995; Reinhard *et al.*, 1995a, 1995b).

The three LIM domains present in human zyxin are closely related to those found in avian zyxin (Sadler *et al.*, 1992). Interestingly, it was recently discovered that sequences related to the LIM region of zyxin are encoded by chimeric transcripts associated with lipomas, a common mesenchymal neoplasm in humans (Ashar *et al.*, 1995; Schoenmakers *et al.*, 1995; Petit *et al.*, 1996). A major subclass of lipomas are characterized by chromosomal translocations involving chromosome 12 that result in rearrangements in the gene encoding HMGI-C, a high mobility group protein that binds to DNA and influences transcription by modulating the local conformation of the nucleic acid (Ashar *et al.*, 1995; Schoenmakers *et al.*, 1995; Thanos and Maniatis, 1992; Du *et al.*, 1993; Wolffe, 1994). The *LPP* gene on chromosome 3 is a preferred translocation partner in these lipomas (Ashar *et al.*, 1995; Schoenmakers *et al.*, 1995). The *LPP* protein is similar to zyxin in both sequence and overall molecular architecture (Fig. 6B) and may represent another zyxin family member. In the HMGI-C/*LPP* chimeric proteins that characterize the tumor cells, LIM domains derived from *LPP* are linked to the DNA-binding domain of HMGI-C. Since the LIM domain mediates specific protein-protein interactions (Schmeichel and Beckerle, 1994; Wu and Gill, 1994; Feuerstein *et al.*, 1994), it seems likely that LIM-dependent recruitment of protein factors adversely affects transcriptional regulation by HMGI-C and thus contributes to tumorigenesis.

The LIM region of zyxin is also very similar to a sequence in the Trip6 protein that was identified based on its ability to interact with the thyroid hormone receptor in a ligand-dependent fashion (Lee *et al.*, 1995). The physiological relevance of this relationship is not understood at present. Although Trip6, *LPP*, and zyxin are clearly encoded by distinct human genes, the LIM regions contained in these proteins are closely related in sequence. Since LIM domains display specific protein binding preferences (Schmeichel and Beckerle, 1994), it is possible that these closely related LIM domains may exhibit common binding partners and thus may have related functions.

Zyxin has been implicated in a number of important signaling pathways that regulate cell differentiation, proliferation, and morphology. Because of the domain structure of zyxin, we have postulated that zyxin may function by mediating the assembly of multimeric protein complexes (Schmeichel and Beckerle, 1994). Each LIM domain of zyxin may be able to associate with specific protein partners, and the proline region clearly has the capacity to interact with a number of proteins (Sadler *et al.*, 1992; Reinhard *et al.*, 1995b; Crawford *et al.*, 1992; Hobert *et al.*, 1996). Based on the recent identification of zyxin-binding partners, it appears likely that zyxin may participate in a variety of processes within cells. For example, zyxin has been shown to interact with members of the cysteine-rich protein family that are required for muscle differentiation (Arber *et al.*, 1994). In addition, zyxin's ability to associate with Vav, a human proto-oncogene product that is required for signaling and proliferation of T- and B-cells, suggests a role for zyxin in Vav-dependent processes (Hobert *et al.*, 1996). As discussed above, recent work has also raised the possibility

that zyxin and its binding partners may participate in the regulated, spatially localized assembly of filamentous actin by virtue of its similarity to the *Listeria* protein ActA and its ability to interact with the profilin-binding proteins (VASP).

Most work on zyxin has focused on functions related to its protein binding activity. However, structural features of the LIM domain (Pérez-Alvarado *et al.*, 1994) suggest that these zinc fingers may also have the capacity to associate with nucleic acid. Of particular interest in this regard are recent experiments in which zyxin sequences were identified as a binding partner for the thrombomodulin gene promoter.³ Thus, zyxin may be able to function in both the nucleus and the cytoplasm. Over the past few years, zyxin has emerged as an interesting constituent of the focal contact that may play a role in signal transduction at those specialized regions of the plasma membrane. The availability of molecular probes for zyxin should enable higher resolution analysis of zyxin's physiological function.

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