Mutually Exclusive Splicing of Calcium-binding Domain Exons in Chick α-Actinin*

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We have determined the complete sequence of chick brain α-actinin (892 amino acids; 107,644 Da). The sequence differs from that of smooth muscle α-actinin only in the region of the first EF-hand calcium-binding motif, where 27 residues in brain α-actinin are replaced by just 22 residues in the smooth muscle isoform. This probably accounts for the different calcium sensitivities of the two isoforms with respect to actin binding. Analysis of the gene structure showed that this region of sequence divergence is encoded by two separate exons whose incorporation is mutually exclusive. We have determined the proportion of the two transcripts in various tissues and cell lines using poly(A)* RNA and a quantitative assay based on the polymerase chain reaction. MRC-5 fibroblasts and HeLa cells express mRNAs encoding both isoforms, whereas Namalwa lymphoblastoid cells, which lack actin stress fibers, express only the non-muscle mRNA. Both isoforms of α-actinin became incorporated into stress fibers and cell-matrix junctions when full-length chick α-actinin cDNAs were expressed in monkey COS cells. The levels of chick α-actinin mRNAs were found to be serum-inducible, suggesting that α-actinin may be an early response gene.

α-Actinin is a rod-shaped F-actin cross-linking protein with a subunit molecular mass of 94–103 kDa. It is a homodimer in which the subunits are antiparallel in orientation (reviewed in Blanchard et al., 1989). Analysis of the sequence of a variety of α-actinin cDNAs (Baron et al., 1987a, 1987b; Noegel et al., 1987; Arimura et al., 1988; Millake et al., 1988; Fryberg et al., 1990) has shown the molecule to contain three distinct domains. The N-terminal actin-binding domain is followed by four spectrin-like repeats thought to be important in dimer formation and two C-terminal EF-hand calcium-binding motifs. A number of distinct isoforms of α-actinin have been characterized, including skeletal, smooth, and non-muscle α-actinins. The only recorded functional difference between these α-actinins is that binding of the non-muscle isoform to F-actin is inhibited by calcium, whereas binding of the muscle isoforms is calcium insensitive (Burridge and Feramisco 1981; Bennett et al., 1984; Duhtaiman and Bamburg 1984; Landon et al., 1985).

Comparison of the complete sequence of chick skeletal (Arimura et al., 1988) with smooth muscle α-actinins (Baron et al., 1987b) show that they are the products of separate genes. However, comparison of the smooth muscle sequence with that deduced from two partial chick fibroblast cDNAs (Baron et al., 1987a; Arimura et al., 1988) shows that these isoforms are identical in the region of overlap except for a short region of sequence divergence covering the second part of the first EF-hand. Such sequence divergence might account for the different calcium sensitivities of the two isoforms. The result suggests that the smooth muscle and fibroblast isoforms of α-actinin arise by alternative splicing of the primary transcript of a single gene. Whether other splice variants of the non-muscle isoform of α-actinin exist has not been established.

One of the best characterized non-muscle α-actinins is that derived from chick brain (Duhtaiman and Bamburg, 1984). We have now determined the complete sequence of chick brain α-actinin and report that it is identical to that expressed in fibroblasts. We have analyzed the structure of the gene encoding the EF-hand region of the smooth and non-muscle isoforms and have shown that the region of divergence between the two isoforms is encoded by two separate exons which are alternatively spliced. In an attempt to gain some insight into the functions of the two isoforms, we have investigated the pattern of splicing in different tissues and in various cell types grown in culture.

MATERIALS AND METHODS

Chicken smooth and non-muscle α-actinins were purified from gizzard and brain by the methods of Feramisco and Burridge (1980) and Duhtaiman and Bamburg (1984), respectively. The purified proteins were incubated with thermolysin (50:1 protein to enzyme ratio) in 100 mM ammonium bicarbonate, 5 mM CaCl2, pH 7.6, for varying times. Digestion was stopped by addition of 10 mM EDTA, and the cleavage products were analyzed by SDS-polyacrylamide gel electrophoresis. Polypeptides were sequenced after transfer to polyvinylidene difluoride membranes (Matsudaira, 1987) using an Applied Biosystems 470A automated gas phase sequencer.

Isolation of Chick Brain α-Actinin cDNAs and Genomic Clones—A Δgt10 chick embryo brain cDNA library (kindly provided by Dr. M. Darlison, MRC Molecular Neurobiology Unit, Cambridge, United Kingdom) was screened with a 2.1-kb cDNA (C17) encoding part of chick fibroblast α-actinin (Baron et al., 1987a) and a 1-kb SmaI/EcoRI restriction enzyme fragment derived from the 3'-end of a full-length chick smooth muscle α-actinin cDNA (C17) (Baron et al., 1987b). The probes were labeled with [α-32P]dCTP by the random

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† The abbreviations used are: SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair; bp, base pair; DMEM, Dulbecco's minimal essential medium; RT-PCR, reverse transcription-polymerase chain reaction.
priming method (Feinberg and Vogelstein, 1984) and plaque screening by DNA-DNA hybridization was by standard methods (Sambrook et al., 1989) with a final wash in 15 mM NaCl, 1.5 mM sodium citrate, pH 7, 0.1% (w/v) SDS at 65 °C. Positive plaques were purified to homogeneity and recombiant λ phage isolated by CsCl gradient centrifugation (Sambrook et al., 1989). The cDNA inserts were liberated with EcoRI, subcloned into the Bluescript SK+ vector (Stratagene), and sequenced by the dideoxy chain termination method (Sanger et al., 1977).

A chick genomic library in the λEMBL3 replacement vector (kindly provided by Professor D. Engel, Northwestern University, Evanston, IL) was screened with the chick smooth muscle a-actinin cDNA probe and a 546-bp HaeII restriction enzyme fragment of C17 encoding the EF-hand domain of the protein. The size of the inserts in the EMBL3 clones was determined by gel electrophoresis of Southern-digested DNA and the relationships between clones established by comparison of the SaI/EcoRI/BamHI triple digest restriction enzyme maps.

Identification of Intron/Exon Boundaries in Chick a-Actinin Genomic Clones—Recombinant phage DNA cleaved with combinations of SaI, EcoRI, and BamHI restriction endonucleases was separated in a 0.5% agarose gel and transferred to Hybond N (Amersham Corp.). In order to identify the exons encoding the EF-hand calcium-binding motifs, blots were hybridized at 65 °C (as described for library screening) with four different [α-32P]dCTP-labeled cDNA probes in succession. The probes used were the full-length a-actinin cDNA (C17), a 546-bp HaeII restriction enzyme fragment derived from C17 encoding both EF-hand calcium-binding motifs, an XmnI/NcoI fragment of C17 encoding EF-hand 1, and an NcoI/SmaI fragment of C17 encoding EF-hand 2. Filters were washed between each exon hybridization and the efficiency of probe removal checked by autoradiography. Selected hybridized fragments were gel-purified and subcloned into the Bluescript SK+ vector (Stratagene) for further analysis. Intron/exon boundaries were mapped using a combination of exonuclease III deletion, Southern blotting, and DNA sequencing. Blots were hybridized with 17-mer oligonucleotides designed to the 5'-end of each exon, as predicted from the cDNA sequences of smooth and non-muscle a-actinin. Hybridization was carried out as for library screening at temperatures calculated for each oligonucleotide. The same oligonucleotides were also used to sequence across the 3' intron/exon boundaries. Oligonucleotides to determine intron sequence were then used to sequence across the 5' intron/exon boundaries. To sequence across the boundaries of exons flanking the EF-hand region of a-actinin, oligonucleotides were made to the 3'-end of the fourth spectrin-like repeat and to a region downstream of EF-hand 2.

Isolation of RNA from Chick Tissues—The following fresh tissues were obtained from young adult chickens, snap-frozen, and stored at −70°C: gizzard, stomach, heart, breast muscle, leg muscle, brain, liver, kidney, lung, spleen. Total RNA was extracted from tissues using the guanidine isothiocyanate/hot phenol procedure (Maniatis et al., 1982) and poly(A)+ RNA purified by chromatography on oligo(dT)-cellulose (Collaborative Research, Lexington, MA). In order to identify the exons encoding the EF-hand calcium-binding motifs, filters were hybridized at 65 °C with [α-32P]dCTP-labeled cDNA probes in succession. The probes used were the full-length a-actinin cDNA (C17), a 546-bp HaeII restriction enzyme fragment derived from C17 encoding both EF-hand calcium-binding motifs, an XmnI/NcoI fragment of C17 encoding EF-hand 1, and an NcoI/SmaI fragment of C17 encoding EF-hand 2. Filters were washed between each exon hybridization and the efficiency of probe removal checked by autoradiography. Selected hybridized fragments were gel-purified and subcloned into the Bluescript SK+ vector (Stratagene) for further analysis. Intron/exon boundaries were mapped using a combination of exonuclease III deletion, Southern blotting, and DNA sequencing. Blots were hybridized with 17-mer oligonucleotides designed to the 5'-end of each exon, as predicted from the cDNA sequences of smooth and non-muscle a-actinin. Hybridization was carried out as for library screening at temperatures calculated for each oligonucleotide. The same oligonucleotides were also used to sequence across the 3' intron/exon boundaries. Oligonucleotides to determine intron sequence were then used to sequence across the 5' intron/exon boundaries. To sequence across the boundaries of exons flanking the EF-hand region of a-actinin, oligonucleotides were made to the 3'-end of the fourth spectrin-like repeat and to a region downstream of EF-hand 2.

RESULTS

In order to determine the relationship between chick brain and smooth muscle α-actinin, we carried out peptide mapping of the two proteins. Thermolysin liberates a well characterized series of polypeptides from chick smooth muscle α-actinin (Mimura and Asano, 1986; Davison et al., 1989). These include a 53-kDa fragment which starts at Leu-266 and is large enough to contain all four spectrin-like repeats, and 32-, 29-, and 27-kDa fragments which are derived from the N-terminal actin-binding domain of the protein (Fig. 1A). Interestingly, generation of the 53-kDa fragment from brain α-actinin was much slower than was the case with the smooth muscle isoform, and the 64-kDa polypeptide was prominent (Fig. 1B). The N-terminal sequences of the above polypeptides derived from brain and smooth muscle α-actinin were identical (data not shown). The 64-, 53-, and 51-kDa brain polypeptides all have the same N terminus (Leu-266) (Fig. 1A). As the 64-

FIG. 1. Comparison of the thermolysin digestion pattern of chick brain and smooth muscle α-actinin. A, the domain structure of α-actinin is shown along with the relative positions of the major thermolysin cleavage products derived from the smooth muscle isoform, as determined by N-terminal sequencing. B, time course of digestion (1–150 min) of chick brain and smooth muscle α-actinin with thermolysin as analyzed by SDS-PAGE. The position and molecular mass of the major proteolytic fragments is shown. Th indicates the position of thermolysin.
kDa polypeptide is large enough to extend to the EF-hand domain, it must be cleaved within this domain to yield the lower molecular mass fragments. The relative resistance of the brain 64-kDa polypeptide to further proteolysis, compared with that in smooth muscle, suggests that brain and smooth muscle α-actinin must differ in the EF-hand region of the protein.

To investigate this difference further, chick smooth muscle and fibroblast α-actinin cDNA probes (Baron et al., 1987a, 1987b) were used to isolate several partial cDNA clones from a λgt10 brain cDNA library. Analysis of the aligned cDNAs (3469 bp) revealed 18 bp of 5′-untranslated sequence followed by a putative initiation codon contained within the sequence CC GCG CATTG, which is common to many translation start sites (Kozak, 1984). Unfortunately, the N terminus of brain α-actinin is blocked so we were unable to confirm the authenticity of this translation start site directly. The putative initiation codon is followed by a single open reading frame of 2682 bp terminating in a stop codon (TAA) at position 2688. This sequence encodes a protein of 892 amino acids with a deduced molecular mass of 107,644 Da (excluding the initiating methionine). This is in agreement with the molecular mass of brain α-actinin estimated from SDS-PAGE. The stop codon is followed by 726 bp of 3′-untranslated sequence, a polyadenylation sequence ATTAAA, and a poly(A) tail of at least 43 residues. The complete deduced amino acid sequence of brain α-actinin was identical to the published partial fibroblast α-actinin sequence (Baron et al., 1987a; Arimura et al., 1988) in the region of overlap, indicating that there may be just one non-muscle isoform of the protein. The deduced sequence of brain α-actinin differed from that of smooth muscle α-actinin in the region spanning the first EF-hand (Fig. 2). The first 11 residues of EF-hand 1 are identical between the two isoforms of α-actinin, but the second part of EF-hand 1 contains 27 residues in non-muscle α-actinin which are distinct from the 22 residues found in the smooth muscle isoform. The second EF-hand is identical in both isoforms, as is the rest of the molecule.

These results are consistent with the existence of two separate exons encoding the second part of EF-hand 1, which are alternatively spliced to produce mRNAs encoding the smooth and non-muscle isoforms of the protein. To examine this hypothesis, we isolated a number of chick α-actinin genomic clones using cDNA probes encoding the EF-hand region of the protein. The restriction enzyme maps of two of these genomic clones are shown in Fig. 3A. Restriction enzyme fragments containing exons were subcloned into Bluescript SK+ for further analysis using a combination of EcoRI deletion, Southern blotting, and DNA sequencing. The organization of this region of the chick smooth/non-muscle α-actinin gene is shown in Fig. 3B. The exon referred to as EF1a encodes from residue 712 (the end of repeat 4) to residue 760 in EF-hand 1, the exact position of sequence divergence between the isoforms. The adjacent exon (EF1b NM) encodes the 27 amino acids unique to EF-hand 1 of the non-muscle isoform and is followed by exon EF1b SM which encodes the 22 residues unique to EF-hand 1 of the smooth muscle isoform. The second EF-hand, which is common to both isoforms, is contained entirely within its own exon.

To investigate the tissue-specific pattern of alternative splicing of the exons encoding the EF-hand region of α-actinin, we analyzed poly(A)+ RNA from various tissues using an assay based on reverse transcription to generate a cDNA and the polymerase chain reaction to amplify sequences encoding the EF-hand region (RT-PCR). The nucleotide sequence of non-muscle α-actinin contains a 15-bp insert between EF-hands 1 and 2 which has no counterpart in smooth muscle α-actinin. Thus, amplification from primers (one of which is 32P-labeled) which anneal to the common flanking sequences (shown in Fig. 3B), followed by gel electrophoresis and autoradiography, would allow the ratios of the mRNA isoforms to be determined. To establish that the approach was quantitative, we showed first that the ratios of the products of RT-PCR were the same as those of RNA substrates transcribed in vitro from appropriate α-actinin cDNA clones and mixed in various ratios. The method resolved the products from the two isoforms (Fig. 4A), and there was a linear relationship between the intensity of the signal and the proportion of each RNA (Fig. 5). Analysis of chick gizzard and brain mRNAs showed that transcripts encoding the smooth

**Fig. 2. Alignment of the EF-hand calcium-binding motifs of smooth and non-muscle α-actinins.** The sequences shown are from chick smooth muscle (CSM) α-actinin (Baron et al., 1987b), chick non-muscle (CNM) α-actinin (fibroblast sequence, Baron et al., 1987a; Arimura et al., 1988; brain sequence, this study), human smooth muscle (HSM) α-actinin (Millecchia et al., 1989), and D. discoideum (DAA) α-actinin (Noegel et al., 1987). Pad characters (−) are introduced to accommodate the 5 extra residues present in the CNM and HNM isoforms. The 27 residues in CNM which differ from the sequence in CSM are boxed, as is the equivalent region in HNM which differs from the sequence in HSM. Note the high degree of conservation between chick and human of this 27-residue region in the non-muscle (boxed) and smooth muscle (underlined) isoforms respectively. The position of the amino acids whose oxygen containing side chains are involved in calcium chelation are shown as X, Y, Z, −X, −Z. Positions where hydrophobic residues are generally present are also shown (n).
Alternative Splicing of the Chick α-Actinin Gene

Fig. 3. Organization of the chick α-actinin gene in the region encoding the EF-hand calcium-binding motifs. A, restriction map of two chick α-actinin genomic clones. Restriction endonucleases used were Sall (S), EcoRI (E), FstI (F), Accl (A), BamHI (B), HindIII (H), EcoRV (RV), Kpnl (K), and XbaI (X). Southern blot analysis of these digested clones with the chick smooth muscle isoform a-actinin cDNA C17 (Baron et al., 1987b) and a variety of probes designed to detect single exons revealed a pattern of hybridization identical to that found with chick genomic DNA and consistent with the existence of a single gene (data not shown). The approximate positions of exons (■) within these clones and intron sizes are denoted by the lower line. Only the 5′ boundary of the most 3′ exon is shown (▲). B, sequence of exons and intron/exon boundaries. The nucleotide sequence and the deduced amino acid sequence of the exons are boxed. Numbers at the beginning and end of each exon show the position of the encoded amino acids in the complete α-actinin sequence. Exons labeled EF1a, EF2, and 3′ exon are common to both smooth (SM) and non-muscle (NM) α-actinin. Exons EF1b NM and EF1b SM encode sequence unique to each isoform. Flanking intron sequences are shown in lowercase letters. The approximate size of each intron is indicated. Positions of primers used in RT-PCR are shown by broken arrows.

and non-muscle isoforms of α-actinin, respectively, predomi-
inate in these tissues, as expected (Fig. 4B and Table I). The
fact that the larger of the two RT-PCR products was derived from the non-muscle α-actinin mRNA was confirmed by taking advantage of a diagnostic EcoRI site within the 15 bp unique to the non-muscle α-actinin sequence (data not shown). In other tissues both isoforms were present (Fig. 4B), and densitometry scanning of the autoradiograph showed that kidney, heart, lung, stomach, and breast (skeletal) muscle contained about equal ratios of the two transcripts, and the non-muscle transcript predominated in liver and spleen (Table I).

α-Actinin is reported to be a homodimer, dimer assembly being dependent on the four spectrin-like repeats within the molecule (Imamura et al., 1988). It is now clear that smooth and non-muscle α-actinins are identical in this region of the molecule, and it is possible therefore that heterodimers might form if both isoforms were to be expressed in a single cell at the same time. To establish whether a single cell type can express both isoforms of α-actinin, we have analyzed the α-actinin transcripts in three human cell lines. The nucleotide sequence of human non-muscle α-actinin contains a 15-bp insert between EF-hands 1 and 2 as in the chick, which, in the latter, has no equivalent in the smooth muscle isoform. However, the sequence of the human smooth muscle isoform has not yet been published for comparison with its non-muscle counterpart. Mapping α-actinin transcripts by PCR using mRNA isolated from MRC-5 fibroblasts and HeLa cells grown in monolayer revealed the presence of two alternatively spliced mRNAs, one encoding the non-muscle isoform. The other species, which is about 15 nucleotides smaller, probably encodes the human smooth muscle isoform. This interpretation of the results was validated by sequencing the two RT-PCR products from MRC-5 fibroblasts (Fig. 2). This result, therefore, clearly established that a single cell type can express two alternatively spliced isoforms of α-actinin.

The precise roles of the smooth and non-muscle isoforms of α-actinin is unclear. One possibility is that the calcium-insensitive smooth muscle isoform might be required for the formation of stress fibers, which are thought to contain a muscle-like sarcomeric arrangement of cytoskeletal proteins (Langanger et al., 1986; Drenckhahn and Wagner, 1986). Consistent with this hypothesis, MRC-5 fibroblasts and HeLa cells, which are of epithelial origin, both contain extensive actin stress fibers when grown in monolayer (Fig. 6, A and C) and express mRNAs encoding the smooth and non-muscle isoforms of α-actinin (Fig. 7). On the other hand, Namalwa (lymphoblastoid) cells grown in suspension do not contain stress fibers (data not shown) and only express low levels of the mRNA encoding the non-muscle isoform of the protein (Fig. 7). There appears to be no simple relationship between the proportion of smooth muscle isoform (MRC-5, 25%; HeLa, 3%) and the abundance of actin stress fibers; both cell types contain large numbers of filaments. However, the filaments in MRC-5 cells appeared to be much more tightly organized than those in HeLa cells, suggesting that the more smooth muscle isoform present, the more organized and "muscle-like" the structure formed. When the distribution of α-actinin in MRC-5 and HeLa cells was examined by immunofluorescence using a monoclonal antibody, α-actinin was found to colocalize with the actin filaments and also at the ends of actin filaments where they terminate at cell-matrix junctions called adhesion plaques (Fig. 6, B and D), the latter being particularly prominent in HeLa cells. Namalwa cells showed only a diffuse distribution of α-actinin (data not shown). Since all antibodies to smooth muscle α-actinin cross-react with non-muscle α-actinin, we have been unable to determine the distribution of the individual isoforms within the cell. To investigate the possibility that the two isoforms of α-actinin might be targeted to different structures within the same cell, we have expressed cDNAs encoding the com-
FIG. 4. Analysis of transcripts encoding chick smooth and non-muscle α-actinins using reverse transcription and RT-PCR. A, RNA was transcribed, in vitro, from non-muscle and smooth muscle α-actinin cDNA clones and mixed in various ratios, with the total concentration of input RNA remaining constant. Following RT-PCR where one of the primers was labeled with 32P (see "Materials and Methods"), the products were resolved on a polyacrylamide gel and detected by autoradiography. The figure shows the resolution of the non-muscle (upper band) and smooth muscle (lower band) isoforms in ratios from 100 to 0% of the smooth muscle isoform. B, analysis of poly(A)⁺ RNA prepared from various chick tissues by RT-PCR using a ³²P-labeled primer. The autoradiograph, which was exposed to enhance the weaker bands, shows the relative amounts of transcripts encoding the non-muscle (upper band) and smooth muscle (lower band) α-actinin isoforms in each tissue. Table I shows a more accurate assessment of the relative amounts of each transcript by scanning densitometry.

FIG. 5. Linearity of RT-PCR analysis of α-actinin transcripts. The autoradiograph shown in Fig. 4A was analyzed by laser scanning densitometry. The relative intensity values obtained for the levels of the smooth muscle α-actinin RT-PCR product was plotted against the proportion of input smooth muscle RNA.

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TABLE I

The tissue distribution of α-actinin mRNA in the chick

Comparison of the complete deduced sequences of chick brain and smooth muscle α-actinins shows that they differ only in the first of the two EF-hand calcium-binding motifs. This conclusion is in agreement with the results obtained from peptide mapping and amino acid sequencing of the complete sequence of chick smooth and non-muscle α-actinins in monkey COS cells and used antibodies specific for chick α-actinin to detect the distribution of the expressed protein. However, we have found no evidence for differential distribution of the expressed proteins; both α-actinin isoforms localized to stress fibers as well as to adhesion plaques (Fig. 8).

The levels of mRNAs encoding a number of proteins associated with cell-extracellular matrix junctions have been shown to increase rapidly when quiescent cells are exposed to growth factors (Ryseck et al., 1989). To investigate the possibility that α-actinin mRNA levels would be similarly induced, chick embryo cells were made quiescent by reducing the serum content of the culture medium. After 48 h, cells were stimulated to enter the proliferative phase of the cell cycle by addition of 20% serum, and samples were taken at various times thereafter to determine the level of α-actinin mRNA, by Northern blotting. The amount of α-actinin mRNA in quiescent chick embryo cells was very low when compared with that in cells in the log phase of growth (Fig. 9). Within 2 h of the addition of serum to quiescent cells, there was a dramatic increase in the levels of α-actinin mRNA. The mRNA levels for the cytoskeletal protein vinvulin (Fig. 9) also increased, although to a lesser degree. Levels of the mRNA for glyceraldehyde-3-phosphate dehydrogenase did not increase under these conditions and served as a useful control for the amount of mRNA loaded in the Northern blots. Analysis of the relative levels of the smooth and non-muscle α-actinin transcripts in growing chick embryo cells by RT-PCR showed, as expected, that the major transcript encodes the non-muscle isoform of α-actinin, although some smooth muscle α-actinin transcript was present (85% non-muscle; 15% smooth muscle). The ratio was similar in quiescent cells, and the addition of serum to these cells did not produce a major change in this ratio.

DISCUSSION

Comparison of the complete deduced sequences of chick brain and smooth muscle α-actinins shows that they differ only in the first of the two EF-hand calcium-binding motifs. This conclusion is in agreement with the results obtained from peptide mapping and amino acid sequencing of the
alternative splicing

A

localization of F-actin and α-actinin in human cell lines as detected by fluorescence microscopy. MRC-5 fibroblasts (A and B) and HeLa cells (C and D) were stained with nitrobenzoxadiazole phallacidin to label actin filaments (A and C) and with a monoclonal antibody to human platelet α-actinin (B and D) (a generous gift from Dr. M. Wilkinson, Royal College of Surgeons, London, United Kingdom). MRC-5 fibroblasts found at the termini of actin filaments in adhesion plaques display extensive actin stress fibers. α-Actinin shows a periodic distribution along the actin fibers (MRC-5 (B); HeLa (D)) but is also found at the termini of actin filaments in adhesion plaques (arrows). Magnification × 1000.

Fig. 6. Localization of F-actin and α-actinin in human cell lines as detected by fluorescence microscopy. MRC-5 fibroblasts (A and B) and HeLa cells (C and D) were stained with nitrobenzoxadiazole phallacidin to label actin filaments (A and C) and with a monoclonal antibody to human platelet α-actinin (B and D) (a generous gift from Dr. M. Wilkinson, Royal College of Surgeons, London, United Kingdom). MRC-5 fibroblasts (A) and HeLa cells (C) display extensive actin stress fibers. α-Actinin shows a periodic distribution along the actin fibers (MRC-5 (B); HeLa (D)) but is also found at the termini of actin filaments in adhesion plaques (arrows). Magnification × 1000.

FIG. 7. Analysis of smooth and non-muscle α-actinin transcripts in human cell lines. Poly(A)* RNA was isolated from MRC-5 fibroblasts, HeLa cells, and Namalva Lymphoblastoid cells. The relative proportions of smooth and non-muscle α-actinin mRNAs was determined using RT-PCR and a 32P-labeled primer as described under "Materials and Methods." Control human non-muscle RNA was synthesized in vitro using the human placental α-actinin cDNA (Millake et al., 1989) as template (NM in vitro). The upper band seen in the autoradiograph from all three cell lines coincides with the position of the non-muscle isoform determined using in vitro synthesized RNA. This was confirmed by sequencing the PCR product from MRC-5 fibroblasts. The lower band seen in MRC-5 and HeLa cells is approximately 15 nucleotides smaller and was shown by sequencing to correspond to the smooth muscle isoform. The relative amounts of the two transcripts was determined by scanning the autoradiograph using a laser densitometer (see text).

Fig. 7. Analysis of smooth and non-muscle α-actinin transcripts in human cell lines. Poly(A)* RNA was isolated from MRC-5 fibroblasts, HeLa cells, and Namalva Lymphoblastoid cells. The relative proportions of smooth and non-muscle α-actinin mRNAs was determined using RT-PCR and a 32P-labeled primer as described under "Materials and Methods." Control human non-muscle RNA was synthesized in vitro using the human placental α-actinin cDNA (Millake et al., 1989) as template (NM in vitro). The upper band seen in the autoradiograph from all three cell lines coincides with the position of the non-muscle isoform determined using in vitro synthesized RNA. This was confirmed by sequencing the PCR product from MRC-5 fibroblasts. The lower band seen in MRC-5 and HeLa cells is approximately 15 nucleotides smaller and was shown by sequencing to correspond to the smooth muscle isoform. The relative amounts of the two transcripts was determined by scanning the autoradiograph using a laser densitometer (see text).

purified proteins. The first 11 residues of EF-hand 1 are identical in both isoforms, but these common residues are followed by a region of sequence divergence in which a stretch of 27 amino acids found in the brain α-actinin sequence is replaced by just 22 amino acids in the smooth muscle isoform of the protein. As a consequence, brain α-actinin has a five-amino acid spacer between EF-hands 1 and 2 which is not present in the smooth muscle isoform. Comparison of the brain with partial fibroblast α-actinin sequences (Baron et al., 1987; Arimura et al., 1988) shows that the proteins are identical in the region of overlap, suggesting that this isoform is widely expressed in non-muscle tissues. Whether or not it is the only non-muscle isoform remains to be established. Chick brain α-actinin shows 85 and 97% sequence identity at the DNA and protein level, respectively, with human non-muscle α-actinin (Millake et al., 1989; Nishiyama et al., 1990; Yousouffian et al., 1990), demonstrating the highly conserved nature of the protein. Most of the 24 residues which differ between the human and chick proteins are located within the spectrin-like repeats, and there are only two amino acid sequence differences in the EF-hand region of the proteins. Human placental α-actinin contains the 5 amino acid spacer.

Fig. 8. Localization of chick non-muscle and smooth-muscle α-actinins expressed in monkey COS cells. Full-length cDNAs encoding chick non-muscle (A and B) and smooth muscle (C and D) α-actinins cloned into the pECE eucaryotic expression vector (Ellis et al., 1986) were transfected into monkey COS-1 cells (Guzman, 1981) and the expressed proteins detected by immunofluorescence using a chick-specific α-actinin antiserum, as described previously (Jackson et al., 1989). A and C, cells stained with the chick-specific polyclonal α-actinin antibody. B and D, the same cells stained with nitrobenzoxadiazole-phallacidin to label actin filaments. Arrows indicate the co-alignment of α-actinin with actin filaments. Magnification × 1100.

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Fig. 9. The effects of serum stimulation of quiescent chick embryo cells on the mRNA levels for the cytoskeletal proteins α-actinin and vinculin. Poly(A)* RNA was isolated from growing chick embryo cells, serum-starved cells, and cells 1, 2, and 4 h after serum stimulation. 4 μg of poly(A)* RNA was separated in formaldehyde agarose gels (1%), the RNA transferred to Hybond N membranes, and the filters hybridized with the following [α-32P]dCTP-labeled probes. (a) a 1.4-kb EcoRI-BamHI fragment of the 2.89-kb chick vinculin cDNA (Price et al., 1987); (b) a 3.6-kb chick smooth muscle α-actinin cDNA C17 (Baron et al., 1987b); (c) a 1-kb cDNA encoding part of avian glyceraldehyde phosphate dehydrogenase (GAPdH) (kindly provided by Dr. D. Gillespie, Beatson Institute, Glasgow) was used as a control to normalize the amount of mRNA loaded.

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between EF-hands 1 and 2, although this sequence is not found in *Dictyostelium discoideum* α-actinin. The possible significance of this sequence remains to be established.

The fact that the sequence of EF-hand 1 differs between smooth and non-muscle α-actinins may account for the different calcium sensitivities of the two isoforms with respect to actin binding (Burridge and Fasman 1981; Duhaime and Bamburg 1984; Bennett et al., 1984; Landen et al., 1985). EF-hand calcium-binding sites are known to contain a helix-loop-helix motif, where coordination of calcium ions is partly dependent on the residues at the X, Y, Z, −Y, −X, and −Z vertices in the loop region (Tuft and Kretsinger, 1975; Babu et al., 1988; Marsden et al., 1990). Functional EF-hands typically contain amino acids with oxygen-containing side chains at the X, Y, Z, −X, and −Z positions. Inspection of the sequence of EF-hand 1 in smooth muscle α-actinin shows a lysine residue at the Y coordinate making it unlikely to be a functional calcium-binding site. In brain α-actinin, the only residue which deviates from the consensus is at the −X position. However, the glycine residue at this position is thought to allow a water molecule to coordinate to the calcium ion (Arimura et al., 1988). The second EF-hand is common to both isoforms and would be predicted to be nonfunctional, since there are methionine and alanine residues at the Z and −Z positions, respectively. It would, therefore, be predicted that non-muscle α-actinin would bind just two calcium atoms per dimer.

The difficulties in predicting whether EF-hands are functional or nonfunctional purely based on sequence comparisons are well recognized (da Silva and Reinach, 1991). The only case where the stoichiometry of calcium-binding to α-actinin has been determined is for rabbit alveolar macrophage α-actinin, which was reported to bind four calcium ions per dimer with a $K_d$ of $4 \times 10^{-5}$ M (Bennett et al., 1984). This result differs from predictions based on the sequence analysis of chick brain and fibroblast α-actinins, although we have not ruled out the possibility that there might be an additional non-muscle isoform of the protein which contains two functional EF-hand sequences per subunit. The sequences of human placental (Millake et al., 1989), liver (Nishiyama et al., 1990), and endothelial cell (Yousouffian et al., 1990) α-actinins, however, are all identical in the coding sequence. We have attempted to determine the stoichiometry of calcium binding to the limited amounts of chick brain α-actinin available using both a nitrocellulose blot assay (Way et al., 1989) and SDS-PAGE blot assay, but the protein did not bind calcium under these conditions (data not shown), even though calmodulin has been shown to do so (Krinke et al., 1988). The mechanism by which calcium inhibits actin-binding by non-muscle α-actinin has not been established. The antiparallel orientation of the subunits in the α-actinin dimer (Walrath et al., 1986) has led to speculation that calcium binding to one subunit controls actin binding by the adjacent subunit. However, there is no direct evidence in support of this hypothesis.

The structure of the gene encoding the region of difference between the two isoforms of α-actinin is significant. Mutually exclusive splicing of two adjacent exons with common flanking exons is a very uncommon strategy in non-viral genes, and we are not aware of its occurrence in transcripts other than those which are expressed primarily in muscle cells, or which exhibit an alternative muscle-specific pattern of splicing. The list includes most tropomyosin genes (Wieczorek et al., 1988; Goodwin et al., 1991; Helfman et al., 1986; Clayton et al., 1988; Macleod and Gooding, 1988; Hanke and Storti, 1988; Lindquester et al., 1989; Libri et al., 1989), troponin T (Medford et al., 1984), myosin light chain 1/3 (Periasamy et al., 1984), M-type pyruvate kinase (Noguchi et al., 1986), B-creatine kinase (Wirtz et al., 1990), chick α-actinin (this work), and, most elaborate of all, the *Drosophila* myosin heavy chain gene (George et al., 1989). However, in most of these examples splicing is modulated specifically in skeletal muscle, whereas we have described in this paper only the second example of smooth muscle-specific modulation, the first case being exon 2 of rat α-tropomyosin (Wieczorek et al., 1988). In the case of α-actinin, it is clear that there are lower but various levels of incorporation of exon EF1b SM in non-muscle tissues and cell lines. Although no corresponding systematic analysis has been reported for α-tropomyosin, it seems that the levels of incorporation of the muscle-specific exon in fibroblasts, skeletal muscle and brain are very low and that the regulation might be more stringent (Wieczorek et al., 1988).

An initial analysis of the sequences around the mutually exclusive exons of α-actinin shows no obvious cause for this pattern of splicing. The match of the 5′ splice site of the alternative exons with the consensus sequence, CAG/GTAAGT, can be expressed numerically by summing the frequency with which the observed nucleotides are found at these positions in splice sites, expressed relative to the value obtained for the consensus sequence (Lear et al., 1990). Using the “other vertebrate” frequencies compiled by Shapiro and Senapathy (1987), the values for EF1b NM and EF1b SM are 75 and 78%, respectively. These are low values, and based on our demonstration that there is a significant correlation between the score and the competitive performance of a splice site sequence, the sites would be expected to be intrinsically weak (Lear et al., 1990). The sites are very similar to two of those tested in our experimental hierarchy, TM5nm and MBP5, both of which were very weak (Lear et al., 1990). The 3′ splice sites of the two exons are both preceded by an extensive region rich in pyrimidines but interspersed with purines (data not shown); there are no sequences matching those of optimal branch sites (Reed and Maniatis, 1988; Noble et al., 1988; Zhuang et al., 1989), but neither is it obvious that the branch site would have to be as far upstream as has been found for the muscle-specific exon in tropomyosin genes (Smith and Nadal-Ginard, 1989; Goux-Pelletan et al., 1990; Helfman et al., 1990). We hope to be able to map the branch site in the near future.

There are three major problems in understanding mutually exclusive splicing: the two exons do not appear to splice to each other, one exon is preferred in the default mode, and this preference is inverted in the specific muscle tissue. In the case of exons 2 (smooth muscle-specific) and 3 (nonspecific) of rat α-tropomyosin, some simple answers are available: the exons do not splice together because the branch site for exon 3 is too close to the 5′ splice site of exon 2 (Smith and Nadal-Ginard, 1989); exon 3 is preferred in default cells because its long polypyrimidine tract confers a competitive advantage over exon 2 (Mullen et al., 1991), and sequences in and around exon 3 are required for it to be suppressed in smooth muscle cells (Nadal-Ginard et al., 1991). In contrast, the default pattern of the central exons of β-tropomyosin is influenced by a range of factors, including the order of splicing events, splice site sequences, exon sequences, the long polypyrimidine tract upstream of the muscle-specific exon, sequences between that tract and the 3′ splice site, or secondary structures around that site (Helfman et al., 1988, 1990; Libri et al., 1990, 1991; Goux-Pelletan et al., 1990; Clout d’Orval et al., 1991). The nature of the flanking exons and splice site sequences determined the outcome of experiments in which exons 3 and 4 of myosin light chain 1/3 were shown to be in competition (Gallego and Nadal-Ginard, 1990). In α-actinin, the size of
the intron between the exclusive exons and preliminary sequence data do not suggest that there is any steric hindrance to splicing, and it seems likely that the outcome will, as with β-tropomyosins, be determined by a number of factors. The range of ratios seen for the two exons in different cell lines and tissues suggests that a simple explanation of tissue specificity, involving an activator or repressor produced only in muscle or non-muscle cells, is unlikely to be adequate. It will be of great interest to determine whether there are common smooth muscle-specific regulators of splicing that affect the splicing of both α-tropomyosin and α-actinin.

The fact that various tissues contain mRNAs encoding both isoforms of α-actinin does not imply that both isoforms are expressed in a single cell type. However, we have clearly established that both MRC-5 fibroblasts and HeLa cells grown in culture can express both mRNAs. This result raises two questions. First, given the close sequence similarity between the two α-actinin isoforms, are there mechanisms which restrict assembly of subunits to the formation of homodimers? Clearly, translation of the mRNAs could be both spatially and temporally separated, although rigorous proof that heterodimers are not assembled in those cells that express both isoforms is lacking. Second, what are the functions of the two isoforms? In smooth muscle, α-actinin is localized in membrane-associated dense plaques and may serve to anchor F-actin to these sites (Fay et al., 1982; Geiger et al., 1986). mRNA encoding the non-muscle isoform of α-actinin may be most appropriate in this case. In non-muscle cells in vivo, a non-muscle calcium-sensitive isoform of α-actinin may serve to cross-link F-actin in a manner which can be regulated by local changes in calcium concentration. In certain non-muscle cells this might be important in the regulation of cell shape and cell motility, although it is puzzling that a D.discoideum mutant lacking a functional α-actinin gene apparently fails to display any abnormality in this respect (Noegel et al., 1989). In adherent cultured cells, α-actinin is frequently found in actin stress fibers and in cell-matrix junctions. Stress fibers are thought to be contractile and to contain a sarcomeric arrangement of cytoskeletal proteins, with α-actinin being antiparallel with respect to tropomyosin (Langanger et al., 1986). Again it is possible that a smooth muscle calcium-insensitive isoform may be required at this site to maintain the integrity of the filaments in the face of changes in intracellular calcium concentration. Perhaps the calcium-sensitive non-muscle isoform is localized to cell-matrix junctions and provides one of the mechanisms by which cell adhesion to the extracellular matrix can be regulated. This hypothesis receives some support from the observations reported here that MRC-5 fibroblasts and HeLa cells, which display numerous actin stress fibers, express mRNAs encoding the smooth and non-muscle isoforms of α-actinin, whereas Namalwa lymphoblastoid cells have no stress fibers and exclusively express the mRNA encoding the non-muscle isoform of α-actinin. In addition, the actin filaments in MRC-5 fibroblasts appeared to be much more tightly organized and muscle-like than those of HeLa cells. This observation may be a reflection of the proportion of the muscle-type α-actinin in MRC-5 cells (25%) when compared with HeLa cells (3%). Furthermore, immunofluorescent staining of α-actinin in HeLa cells, where the non-muscle isoform predominates (97%), was particularly prominent in adhesion plaques, whereas MRC-5 fibroblasts had less obvious adhesion plaques and contained relatively less non-muscle (75%) to smooth muscle α-actinin.

Although the concept of differential localization of the smooth and non-muscle α-actinin isoforms is an attractive one, the high degree of sequence identity between the two isoforms has made it impossible to raise polyclonal antibodies specific for each isoform, and we have been unable to test this hypothesis directly. However, experiments in which smooth (Feramisco, 1979) and non-muscle (Meigs and Wang, 1986) α-actininas have been fluorescently labeled and microinjected into fibroblasts do not support such a concept. All isoforms localized to both stress fibers and cell-matrix junctions. Since these experiments were carried out with α-actininas isolated from tissues which are likely to be a mixture of more than one isoform, we have expressed cDNAs encoding the complete sequence of the smooth and non-muscle isoforms of chick α-actinin in COS cells. Again, our results mirror those obtained with the proteins isolated from tissues. It remains possible that the high levels of intracellular α-actinin achieved by microinjection or transient expression leads to aberrant localization of the protein. Serum stimulation of quiescent chick cells leads to a rapid increase in the mRNA levels encoding both the smooth and non-muscle isoforms of α-actinin. The increase in α-actinin mRNA levels follows a similar course to that for vinculin mRNA (Ben-Ze‘ev, 1989, and this study), but the increase is substantially larger for α-actinin than vinculin. This result suggests that α-actinin is a member of the family of immediate early response genes which includes c-fos (Rollins and Stiles, 1989). However, formal proof of this awaits the demonstration that the increase in α-actinin mRNA levels is due to increased transcription which is independent of protein synthesis. Expression of several other components of cell-matrix junctions have also been shown to fall into this category including fibronectin, β-integrin, vinculin and α-tropomyosin, and β-actin (Blatti et al., 1988; Ryseck et al., 1989). It is unclear at present whether increased expression of these proteins is part of the programmed series of events required for transition from the G0 to G1 phase of the cell cycle or whether they fulfill some other role. Growth factors such as platelet-derived growth factor are chemotactic for fibroblasts (Seppa et al., 1982), and modulation of the expression of these proteins may be required to support increased cell motility. Genes whose transcription is responsive to serum-derived growth factors have been shown to contain a serum response element (Riviera and Greenberg, 1990). The CArG box, which is contained within this element, is found in the promoters of a number of genes expressed in muscle (Rosenhal, 1990). It will be interesting to see whether the α-actinin promoter contains such a regulatory element.

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