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Cloning and characterization of a muscle-specific protein

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Cloning and Characterization of a muscle-specific protein:ALP

by

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DISSERTATION

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of the

UNIVERSITY OF CALIFORNIA

San Francisco

this thesis is dedicated to my parents.

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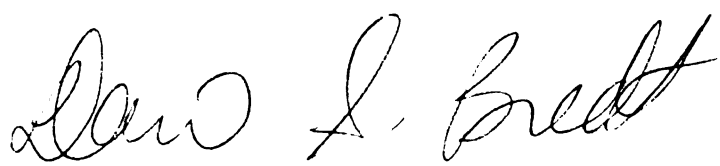
Cloning and Characterization of a muscle-specific protein: ALP

Houhui Xia

Abstract

PDZ motifs are protein-protein interaction domains that often bind to C-terminal peptide sequences. The two PDZ proteins characterized in skeletal muscle, syntrophin and neuronal nitric oxide synthase, occur in the dystrophin complex suggesting a role for PDZ proteins in muscular dystrophy. Here, I am describing the identification of a actinin-associated LIM protein (ALP), a novel protein in skeletal muscle that contains an N-terminal PDZ domain and a C-terminal LIM motif. ALP is expressed at high levels only in differentiated skeletal muscle, while an alternatively spliced form occurs at low levels in heart. ALP is not a component of the dystrophin complex, but occurs in association with a-actinin-2 at the Z-lines of myofibers. Biochemical and yeast two-hybrid analyses demonstrate that the PDZ domain of ALP binds to the spectrin-like motifs of a-actinin-2, defining a new mode for PDZ domain interactions. Fine genetic mapping studies demonstrate that ALP occurs on chromosome 4q35, near the heterochromatic locus that is mutated in fascioscapulohumeral muscular dystrophy.



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Introduction

The cytoskeleton is a complex protein network that provides cellular structure. By partitioning the cell, the cytoskeleton can also provide microdomains that allow specific responses to localized stimuli. The assembly and maintenance of the cytoskeleton is mediated, in large part, by high affinity interactions between modular consensus protein-binding motifs. These sites for protein-protein interaction are often multi-functional and the specific binding partners are determined by the variations in amino acid sequences between the individual domains. A recently identified motif, the PDZ domain, is an 80-120 amino acid domain that was first identified in the postsynaptic protein, PSD-95, which contains three PDZ domains in tandem (Cho, et al., 1992). Sequence analysis has subsequently demonstrated that PDZ domains are common protein motifs that occur in a variety of dissimilar proteins that interact with the cytoskeleton (Ponting and Phillips, 1995). Individual PDZ domains occur in neuronal nitric oxide synthase (nNOS), syntrophins, p55, dishevelled and CASK, while multiple PDZ domains occur in PSD-95, dlg, and ZO-1,2 and PTP-BAS.

Recent work indicates that PDZ domains are multifunctional protein-protein interaction motifs (Brenman and Bredt, 1997, Kornau, et al., 1997, Sheng, 1996). One mode for interaction of PDZ domains involves association with the C-terminus of target proteins. Thus the C-terminus of Fas binds to the third PDZ domain of PTP-BAS and this interaction participates in Fas-mediated apoptosis of T-cells (Sato, et al., 1995). Similarly, the first and second PDZ domains of PSD-95 bind to the C-termini of certain ion channels in brain and anchor these channels to synaptic sites at the plasma membrane (Kim, et al., 1995, Kornau, et al., 1995). PDZ-PDZ interactions have also been identified. The PDZ domain of nNOS binds to the second PDZ domain of PSD-95 (Brenman, et al., 1996). These multifunctional interactions of PDZ domains

assembles a complex of nNOS / PSD-95 / the N-methyl-D-aspartate receptor calcium channel at the postsynaptic density.

Functional roles for PDZ domains have been demonstrated in diverse tissues. Mutations in the PDZ domain of *Drosophila* INAD alter transduction of visual signals (Shieh and Zhu, 1996), while mutations in the *C. Elegans* PDZ proteins Lin-2 and Lin-7 result in abnormal vulval development (Hoskins, et al., 1996). In all of these cases, the PDZ domains are implicated in targeting intracellular proteins to appropriate multiprotein complexes at the plasma membrane.

To understand and further define the role of PDZ domains in cytoskeletal assembly, we have focused on skeletal muscle as a model system. The regular and defined structure of skeletal muscle makes it an ideal tissue for study. Previous studies demonstrated that the two known PDZ domain proteins in skeletal muscle, the family of sytrophins and nNOS, are both components of the dystrophin complex (Adams, et al., 1993, Brenman, et al., 1995). nNOS isoforms lacking the PDZ domain do not interact with the dystrophin complex and occur in the skeletal muscle cytosol. The PDZ domains of nNOS and syntrophin directly interact with each other and this linkage anchors nNOS to the dystrophin complex (Brenman, et al., 1996). The absence of dystrophin in Duchenne muscular dystrophy results in a loss of syntrophins and nNOS from the sarcolemma and these abnormalities may contribute to the disease process.

We hypothesized that other PDZ proteins in muscle may also occur in the cytoskeleton. Characterization of these proteins will help better understand the function of PDZ domains and may identify candidate genes for inherited muscular dystrophies. Here I will mainly describe the cloning of a novel cDNA encoding a protein of 39 kD that consists of a N-terminal PDZ domain and a C-terminal LIM domain. The protein is expressed at high levels only in skeletal muscle, where it occurs at the Z-lines in association with α-actinin-2. We therefore name this protein actinin-associated LIM protein (ALP). Biochemical and two-hybrid analysis indicate that the PDZ domain of

ALP binds to the spectrin-like repeats of α-actinin-2, establishing a novel mode of interaction for PDZ domains. Chromosomal mapping indicates that the ALP gene occurs at 4q35 within 7-10 Mb of the heterochromatic region that is deleted in fascioscapulohumeral muscular dystrophy (FSHD) (Altherr, et al., 1995).

Chapter I: Structure and function study of nNOS

Nitric oxide (NO) is a major endogenous mediator involved in diverse developmental and physiological processes (for review see Bredt and Snyder, 1994). In the brain, NO is suggested to be a neurotransmitter. In teh perrpheral nervous system, NO functions as a non-adrenergic-non-cholinergic transmitter in numerous pathways including the gastrointestinal and urogenital tracts (Bult et al., 1990). NO produced in endothelium is responsible for adjacent blood vessel relaxation. O formed in immunologically activated cells functions in a non-specific immune response that mediates certain cytotoxic and bacteriocidal actions of activated macrophages (Hibbs et al., 1987). Because NO is a short- lived free radical, regulation of signaling occurs largely at the level of NO biosynthesis. Three mammalian NO synthase (NOS) genes have been identified and all catalyze the production of NO by identical enzymatic mechanism. NO derives from the guanidino nitrogen of L-arginine in a unique cytochrome P_{450} type reaction which consumes NADPH. Regulation of NOS isoforms is very different. Endothelial and neuronal NOS enzymes (eNOS and nNOS, or types I and III respectively) are discretely expressed in specific tissues and rapidly transduce signaling events in a calcium dependent manner, while inducible NOS (iNOS or type III) enzymatic activity is not affected by calcium level in the cell.

In addition to controlling diverse cellular processes, NO also participates in certain pathophysiological conditions. Deregulation of nNOS in brain is associated with glutamate receptor overactivity and contributes to neuronal damage in animal models of stroke (Dawson et al., 1992). Mutant mice lacking nNOS are relatively resistant to neuronal damage following focal cerebral ischemia. iNOS activity

contributes to cellular damage in a variety of immunological disorders such as rheumatoid arthritis and septic shock (Huang et al., 1994).

Maintenance of physiological NO signaling in the face of this potential toxicity requires tight regulation of NOS at numerous points. In addition to transcriptional control, NOS proteins are all regulated by calmodulin (Bredt and Snyder, 1990; Cho et al., 1992), which links NO formation to increases in cellular calcium. A more complex level of regulation is reflected by targeting of NOS proteins to intracellular membranes. This subcellular targeting restricts NO signaling to specific targets within a limited microenvironment while minimizing aberrant toxic pathways. Membrane association of endothelial type NOS (eNOS) is mediated by two fatty acid modifications (Robinson et al., 1995). Co-translational N-terminal myristoylation of eNOS is necessary for membrane association. In addition, eNOS is post-translationally palmitoylated by a dynamic process regulated by a specific agonist, bradykinin. For nNOS, although it lacks consensus sequences for fatty acid modification, it occurs largely in particulate fractions (Hecker et al., 1994). The majority of nNOS immunoreactivity in neurons is associated with rough endoplasmic reticulum and specialized electron dense synaptic membrane structures while in skeletal muscle nNOS is associated with the sarcolemma (Kobzik et al., 1994). What factors might restrict nNOS protein to this subcellular localization? By comparing the primary sequence of nNOS with eNOS and iNOS, we found nNOS protein has an extra N-terminal fragment, which does not seem to be reguired for the enzymatic function of nNOS.

To analyze the role of the extended N-terminal region of nNOS, I constructed a deletion mutant, nNOSΔ1-226, lacking the first 226 amino acids. Expression vectors containing full length nNOS and nNOSΔ1-226 were transiently transfected into COS cells. Three days following transfection NOS catalytic activity was measured in tissue homogenates. Kinetic characteristics of NOS activity for the nNOSΔ1-226 mutant was essentially indistinguishable from the full length isoform. Both constructs displayed

similar V_{max}, K_m for arginine as well as regulation by calcium / calmodulin (Figure 1).

Rather than regulating catalytic activity we reasoned that the N-terminal domain of nNOS may instead target nNOS to skeletal muscle sarcolemma. Within this domain, Ponting and Phillips (1995) noted nNOS contains a 66 amino acid motif which bears homology to a heterogeneous family of signaling enzymes that share the property of being localized to specialized cell-cell junctions (Figure 3b). Proteins containing this motif, which is named "GLGF" for a conserved tetrapeptide (Cho et al., 1992), include: *dlg*-1, the product of the lethal discs-large tumor suppressor gene that localizes to the undercoat of the septate junction in *Drosophila* (Bryant and Woods, 1992); disheveled *(dsh)*, a gene required for planar cell polarity in *Drosophila*; PSD-95, a brain-specific protein that localizes to the post-synaptic density in mammals (Cho et al., 1992); ZO-1, 2, peripheral membrane proteins that localizes to tight junctions (zona occludens) of epithelial and endothelial cells (Jesaitis and Goodenough, 1994; Willott et al., 1993); and certain protein tyrosine phosphatases, such as PTP1E, which are thought to localize at the junction between the plasma membrane and the cytoskeleton. Later on, this "GLGF" repeat was renamed as PDZ, short for PSD95, Dlg, ZO-1, 2. The PDZ motif in nNOS was found to be responsible for nNOS localization to cell membrane through or partially through interaction with PSD95 in neurons or syntrophins in skeletal muscle (Brenman JE et al).

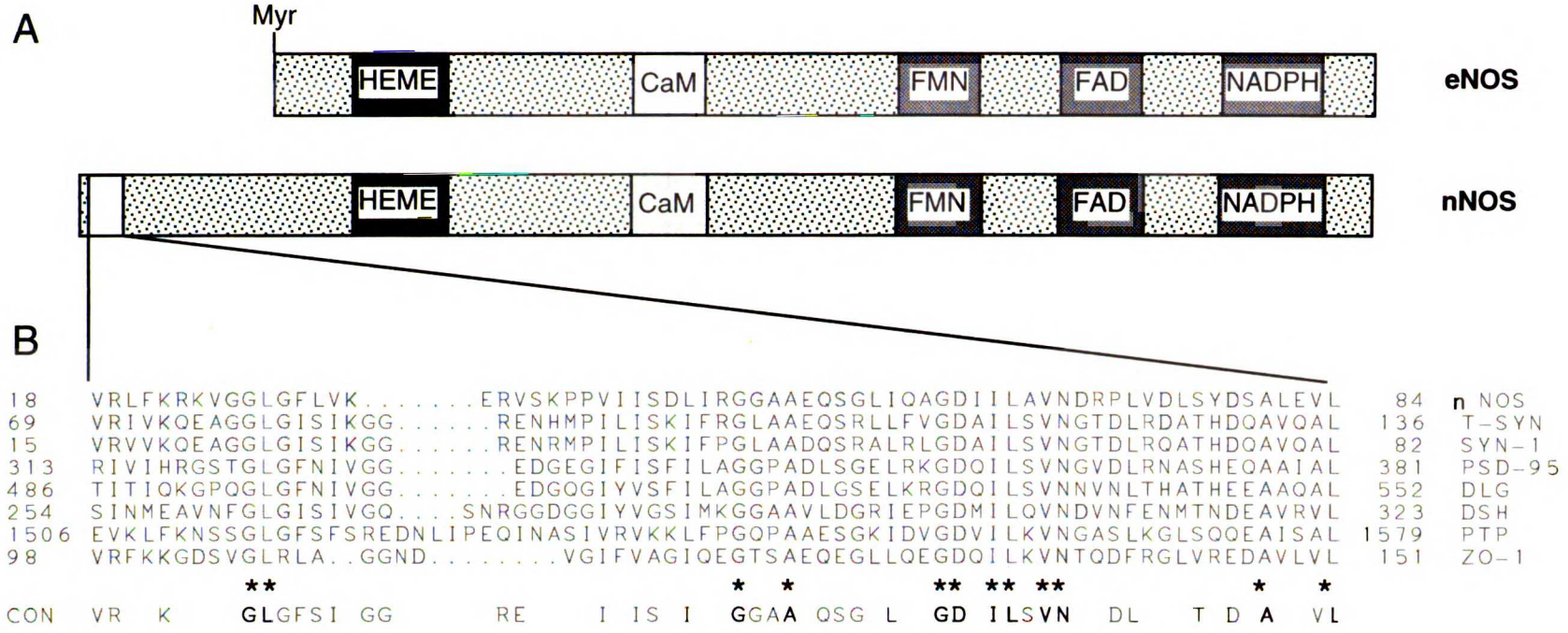
A
Myr
HEME
CaM
FMN
FAD
NADPH
eNOS
HEME
CaM
FMN
FAD
NADPH
nNOS
B
18 VRLFKRKVGGLGFLVK.....ERVSKPPVIISDLIRGGAAEQSGLIQAGDIILAVNDRPLVDLSYDSALEVL 84 n NOS
69 VRIVKQEAGGLGISIKGG.....RENHMPILISKIFRGLAAEQSRLLFVGDAILSVNGTDLRDATHDQAVQAL 136 T-SYN
15 VRVVKQEAGGLGISIKGG.....RENRMPILISKIFPGLAADQSRALRLGDAILSVNGTDLRQATHDQAVQAL 82 SYN-1
313 RIVIHRGSTGLGFNIVGG.....EDGEGIFISFILAGGPADLSGELRKGDQILSVNGVDLRNASHEQAAIAL 381 PSD-95
486 TITIQKGPQGLGFNIVGG.....EDGQGIYVSFILAGGPADLGSELKRGDQILSVNNVNLTHATHEEAAQAL 552 DLG
254 SINMEAVNFGLGISIVGQ....SNRGGDGGIYVGSIMKGGAAVLDGRIEPGDMILQVNDVNFENMTNDEAVRVL 323 DSH
1506 EVKLFKNSSGLGFSFSREDNLIPEQINASIVRVKKLFPGQPAAESGKIDVGDVILKVNGASLKGLSQQEAISAL 1579 PTP
98 VRFKKGDSVGLRLA..GGND.....VGIFVAGIQEGTSAEQEGLLQEGDQILKVNTQDFRGLVREDAVLVL 151 ZO-1
CON VR K GLGFSI GG RE I IS I GGAA QSG L GD ILSVN DL T D A VL

Figure 1 (A & B)

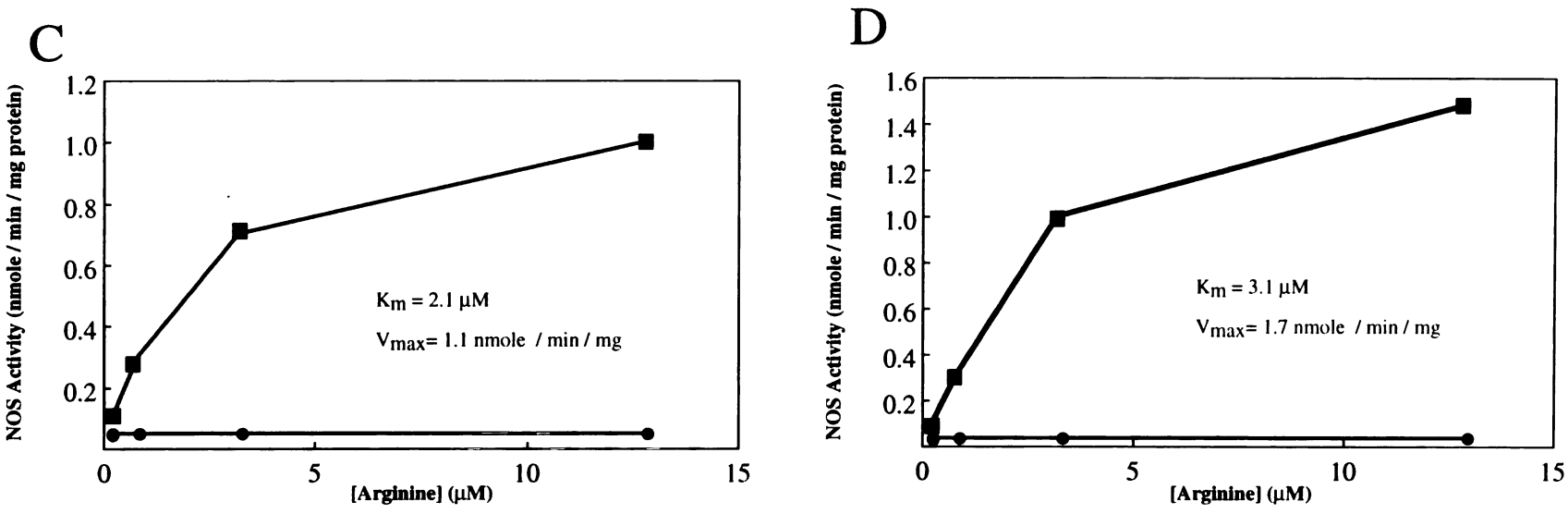

Figure 1. The extended N-terminus of nNOS contains a GLGF domain that is not required for enzyme activity.

a) Schematic alignment of cofactor binding domains of eNOS and nNOS indicating N-terminal myristoylation of eNOS and extended N-terminus of nNOS. The jagged line indicates the region deleted for the nNOSΔ1-226 mutant. b) Alignment of the GLGF / dystrophin binding domain of nNOS with syntrophins and a family of other cytoskeletal associated proteins. c, d) COS cells were transfected with 10 μg of expression vector using a CMV promoter to drive expression of either (c) full length nNOS or (d) a truncation mutant nNOSΔ1-226. NOS activity was measured in cell homogenates three days following transfection in the presence of either 200 μM free calcium(solid squares) or 2 mM EDTA (solid circles). Kinetic constants (Vmax and Km) were calculated by Scatchard plot analysis. Data are means of triplicate determinations which varied by less than 10 %. This experiment was replicated twice with similar results.

Chapter II: Molecular Cloning of ALP and Characterization of mRNA Expression

To identify novel PDZ proteins expressed in skeletal muscle, we performed RT-PCR with degenerate primers that amplify amino acids GLGF (sense) and GDXIL (antisense) (see Materials and Methods for experimental details). These sequences are conserved amongst many PDZ proteins. A library of these cDNA fragments was constructed and processed to remove the syntrophins (see experimental procedures) leading to the identification of novel cDNAs encoding PDZ sequences. Northern analysis was then used to identify genes products that were enriched in skeletal muscle. The mRNA for one of the products, SK-2 (hereafter called ALP), migrated at 1.6 kb and occurred at high levels in skeletal muscle tissue, at low levels in heart and was undetectable in other tissues examined (Figure 2A). Human ALP showed a similar tissue-specific expression pattern (Figure 2B.) To better understand mechanisms that regulate ALP during muscle development, we evaluated expression in myogenic cultures. ALP expression was dramatically induced following myotube fusion in culture (Figure 2C).

In situ hybridization of embryonic day (E15) mouse embryo with antisense ALP probe demonstrated highest levels of ALP expression in developing skeletal muscle and heart (Figure 2D). ALP mRNA also occurred in embryonic intestine. No specific hybridization was observed using sense RNA control (Figure 2E).

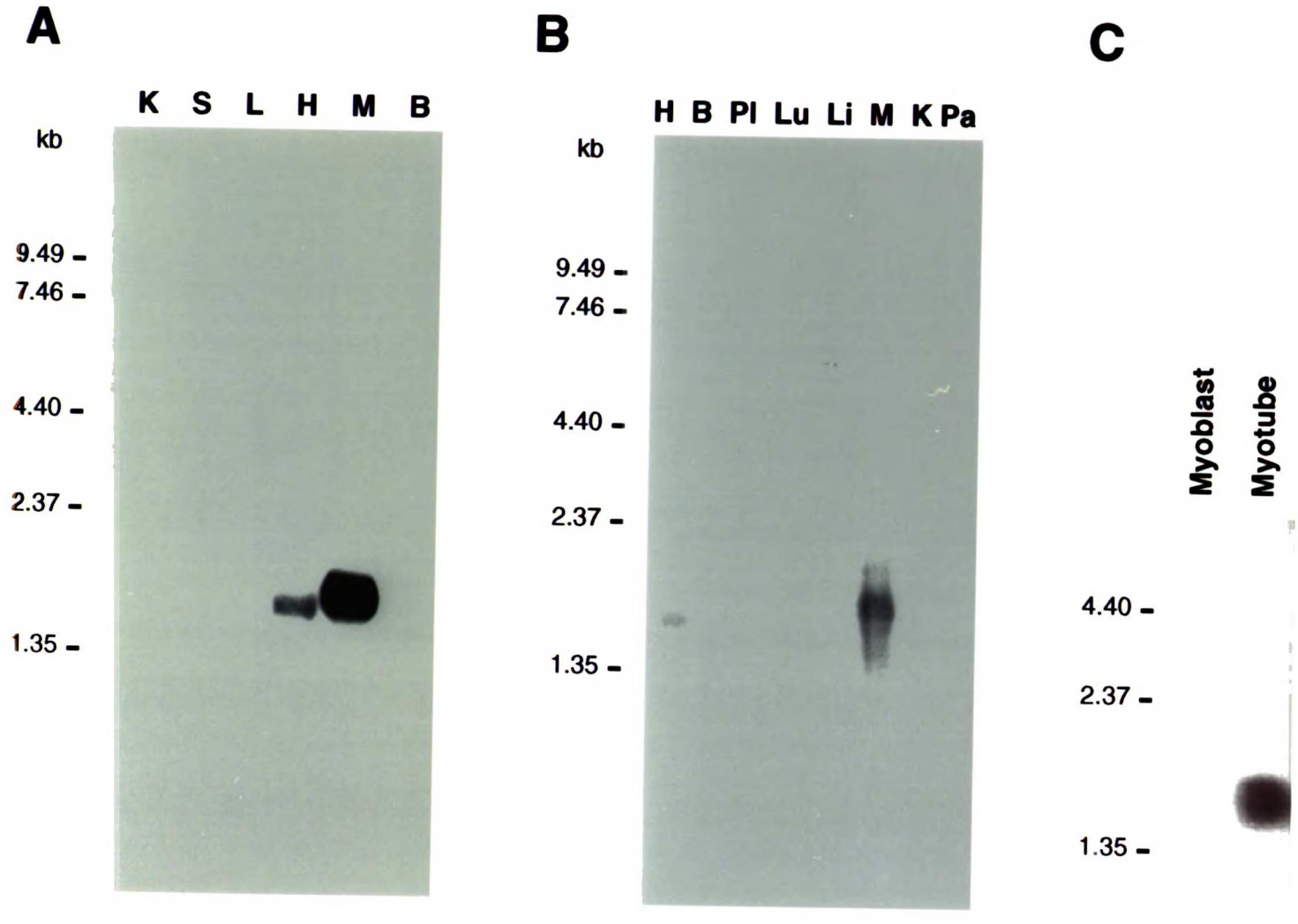
A
K S L H M B
kb
9.49
7.46
4.40
2.37
1.35
B
H B Pl Lu Li M K Pa
kb
9.49
7.46
4.40
2.37
1.35
C
Myoblast
Myotube
4.40
2.37
1.35

Figure 2 (A, B & C)

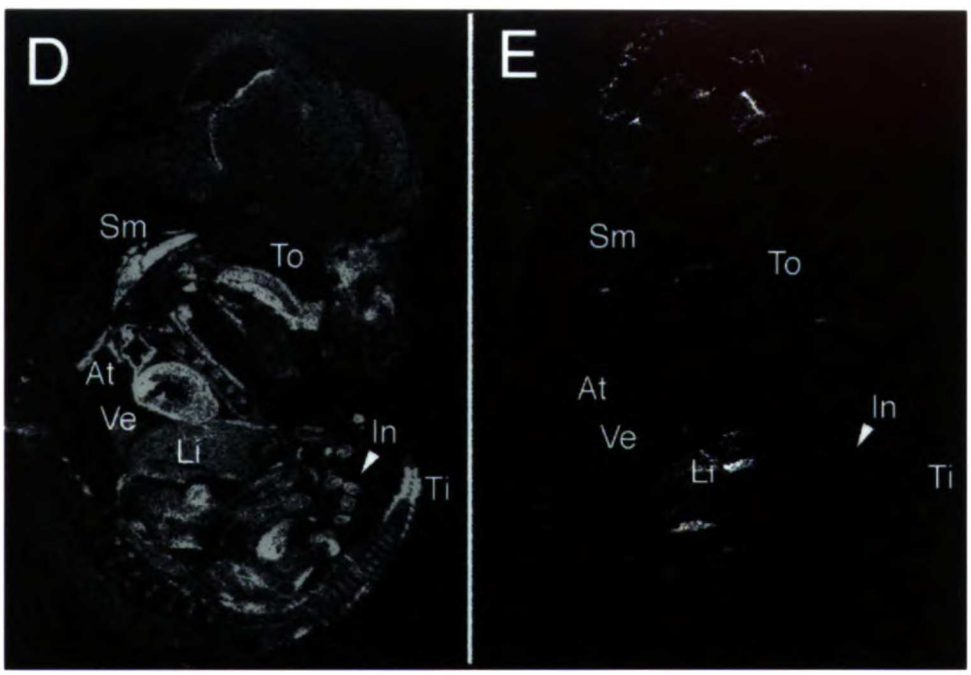

Figure 2 (D & E)

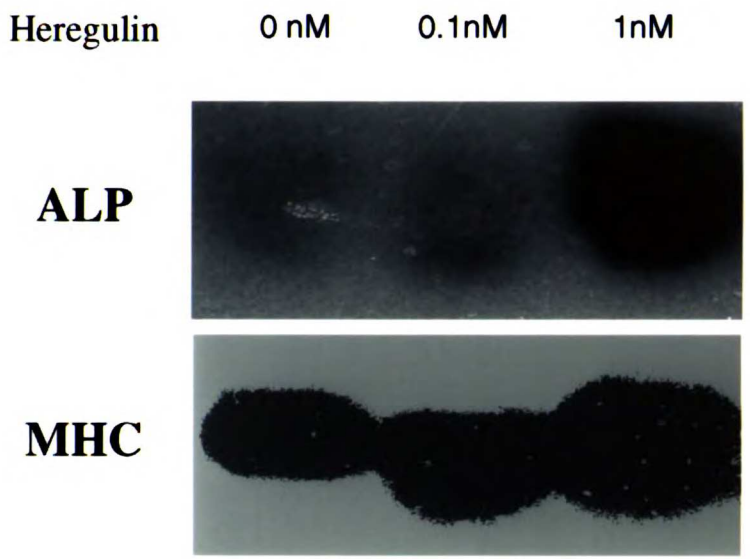

Figure 2f

Figure 2 . ALP mRNA expression in skeletal muscle, heart and other tissues and ALP mRNA upregulation by heregulin. (a) Northern blot of poly(A+) RNA (10 µg per lane) from rat kidney (K), spleen (Sp), liver (L), heart (H), skeletal muscle (M) and brain (B) was probed with 32P-labeled ALP. (b) Human multiple tissue northern blot purchased from Clontech (7760-1) was probed with hALP. Each lane contains approximately 2 µg of poly(A+) RNA from human heart (H), brain (B), placenta (Pl), lung (Lu), liver (Li), skeletal muscle (M), kidney (K) and pancreas (Pa). (c) ALP expression is induced following fusion of C2-myotubes. Each lane in (c) contains 2 µg of total RNA. (d) In situ hybridization of E15 rat embryo shows highest levels of ALP in developing skeletal muscles including tongue (To), sternocephalic (St), and tail (Ta). Hybridizing signal is also seen in heart atrium (At) and ventricle (Ve) and in a circular pattern in intestine (In). (e) No specific hybridization is seen using sense control probe. Hybridization in liver (Li) was considered non-specific as it was detected with sense and antisense probes. (f) ALP expression is upregulated by heregulin treatment. Muscle specific gene myosin heavy chain (MHC) transcript is not affected by heregulin.

Chapter III: Structural Features of ALP and Comparison with Its Homologues

cDNA clones encoding the full 1.6 kb mRNA were obtained from a rat skeletal muscle cDNA library. The mRNA contains a single open reading frame of 1086 base pairs encoding a protein of 39 kD. Sequence analysis shows that ALP has a homology at the N-terminus to PDZ domains. Alignment of ALP with other PDZ domains is shown (Figure 3A). The presence of histidine at amino acid 62 suggests that the PDZ domain may be in group I (Brenman and Bredt, 1997, Songyang, et al., 1997).

The central region of ALP showed no homology to any other cloned gene while the C-terminus encodes a LIM domain. Though ALP has not previously been reported, other proteins with a similar domain structure have been described. A database homology search with BLAST, indicated that ALP shares high homology to a number of newly identified transcripts including CLP-36, RIL and enigma (Figure 3B). CLP36 was identified as a cDNA whose expression in heart is down regulated by hypoxia (Wang, et al., 1995). RIL, short for reversion induced LIM protein, is down regulated in H-ras transformed cells (Kiess, et al., 1995). Enigma was identified as a insulin-receptor interacting protein (Wu, et al., 1996). These investigators however, did not recognize the homology of the N-terminal regions of CLP-36, RIL or enigma with the PDZ domain. The PDZ domain of ALP shares 55, 48, and 45% amino acid identity with the PDZ domains of CLP36 RIL and enigma, respectively. The LIM domain of ALP shares even stronger homology (67% identity) with CLP36 and RIL. While ALP, CLP 36 and RIL all only have one LIM domain, enigma has three LIM domains. The sequence homology indicates that ALP, CLP36 and RIL constitute a new family of proteins containing an N-terminal PDZ domain and a C-terminal LIM domain.

Recent analysis of the EST database showed that overlapping cDNAs corresponding to human ALP have been deposited. Human ALP is 91% identical to the rat sequence. We noted that EST clones from human heart libraries were consistently different in the central region from those in human skeletal muscle libraries (Figure 3C). Exons encoding the central 112 amino acids of skeletal muscle ALP are likely spliced out in heart and replaced by exons encoding 64 different amino acids. To confirm this differential expression, we amplified the region that was unique to heart transcripts and reprobed the northern blot. As expected we found heart specific expression of this region of ALP (data not shown). We therefore define two subtypes ALP_{SK} and ALP_{H} for the alternative transcripts that occur in skeletal muscle and heart, respectively.

```
   ALP-PDZ     5 VVLPGPASWG FRLSGGID..  ...FNQPLVI TRITPGSKAE AAN.LCPGDM
 CLP36-PDZ     6 IVLQGPGPWG FRLVGGKD..  ...FEQPLAI SRVTPGSKAA IAN.LCIGDL
 PSD95-PDZ   162 KLIKGPKGLG FSIAGGVGNQ HIPGDNSIYV TKIIEGGAAH KDGRLQIGDK
 α1SYN-PDZ    84 VRKADAGGLG ISIKGGRENK .....MPILI SKIFKGLAAD QTEALFVGDA
   nNOS-PDZ   20 LFKRKVGGLG FLVKERVSKP ......PVII SDLIRGGAAE QSGLIQAGDI
   INAD-PDZ  367 VQVRKEGFLG IMV...IYGK HAEVGSGIFI SDLREGSNAE LAG.VKVGDM

                              *                 #
   ALP-PDZ   ILAIDGFGTE SMTHADAQDR IKAASYQLCL KI       80
 CLP36-PDZ   ITAIDGEDTS SMTHLEAQNK IKGCVDNMTL TV       81
 PSD95-PDZ   ILAVNSVGLE DVMHEDAVAA LKNTYDVVYL KV      243
 α1SYN-PDZ   ILSVNGEDLS SATHDEAVQA LKKTGKEVVL EV      160
   nNOS-PDZ  ILAVNDRPLV DLSYDSALEV LRGIASETHV VL       95
   INAD-PDZ  LLAVNQDVTL ESNYDDATGL LKRAEGVVTM IL      444
```

Figure 3A

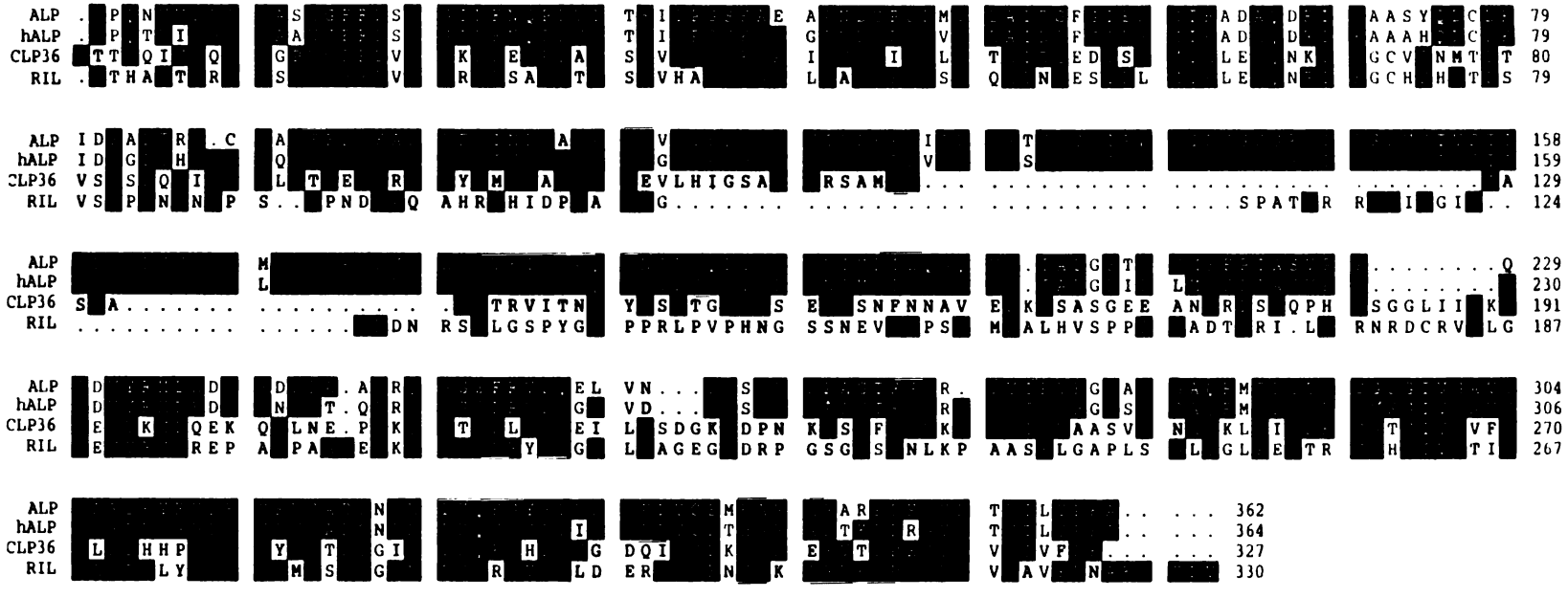

Figure 3B

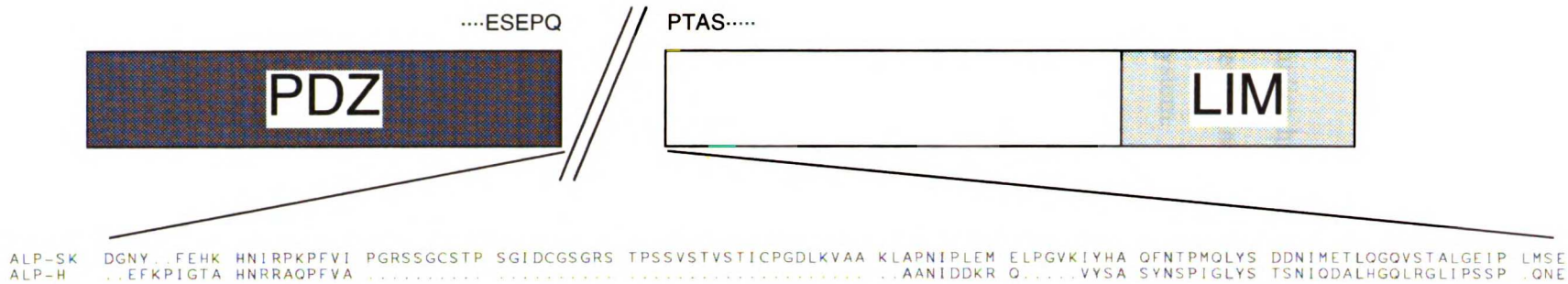
····ESEPQ
PTAS·····
PDZ
LIM
ALP-SK DGNY..FEHK HNIRPKPFVI PGRSSGCSTP SGIDCGSGRS TPSSVSTVSTICPGDLKVAA KLAPNIPLEM ELPGVKIYHA QFNTPMQLYS DDNIMETLQGQVSTALGEIP LMSE
ALP-H ..EFKPIGTA HNRRAQPFVA ....AANIDDKR Q.....VYSA SYNSPIGLYS TSNIQDALHGQLRGLIPSSP .QNE

Figure 3C

Chapter IV: The PDZ Domain of ALP Binds α-actinin-2

Previous studies have shown that PDZ domains participate in protein-protein interactions. To determine potential targets for the PDZ domain of ALP we used the yeast two hybrid system. We screened 10^6 clones from a adult skeletal muscle library (Clontech) and obtained 128 positive clones, 35 of which were recovered and analyzed. All positive clones encoded fragments of α-actinin-2, a muscle specific cytoskeletal protein that contains an N-terminal actin binding domain, four central spectrin-like repeats and a C-terminal region homologous to calcium binding EF-hands (Beggs, et al., 1992). ALP interacts only with the spectrin-like repeat region of α-actinin-2, and all interacting clones encodes repeat 3 (Figure 4).

Interaction of a PDZ domain with spectrin-like repeats is unprecedented. We therefore asked whether this interaction was specific. We found that the PDZ domains of nNOS, α1-syntrophin and the 3 PDZ domains of PSD-95 did not interact with α-actinin-2 in the yeast two hybrid system. We previously identified a point mutation that universally disrupts all known types of PDZ interactions (K. Christopherson, W. Lim and D. S. B, submitted). This change corresponds to a mutation found in *inactivation no after depolarization* (INAD) (Shieh and Niemeyer, 1995), a PDZ protein in *Drosophila* that binds to the *transient receptor potential* (TRP) calcium channel (Shieh and Zhu, 1996). This mutation corresponds to position Leu78 in ALP. Mutating Leu78 of ALP to Lys abolished the interaction with α-actinin-2 (Figure 4) demonstrating specificity of the PDZ domain interaction with α-actinin-2.

To further confirm this interaction we expressed a bacterial fusion protein linking glutathione-S-transferase (GST) to the PDZ domain of ALP. We found that this fusion protein specifically retained α-actinin-2 from solubilized skeletal muscle

cytoskeleton (Figure 5), but did not retain α1-syntrophin. By contrast, an analogous column containing the PDZ domain of nNOS "pulled-down" α1-syntrophin but not α-actinin-2.

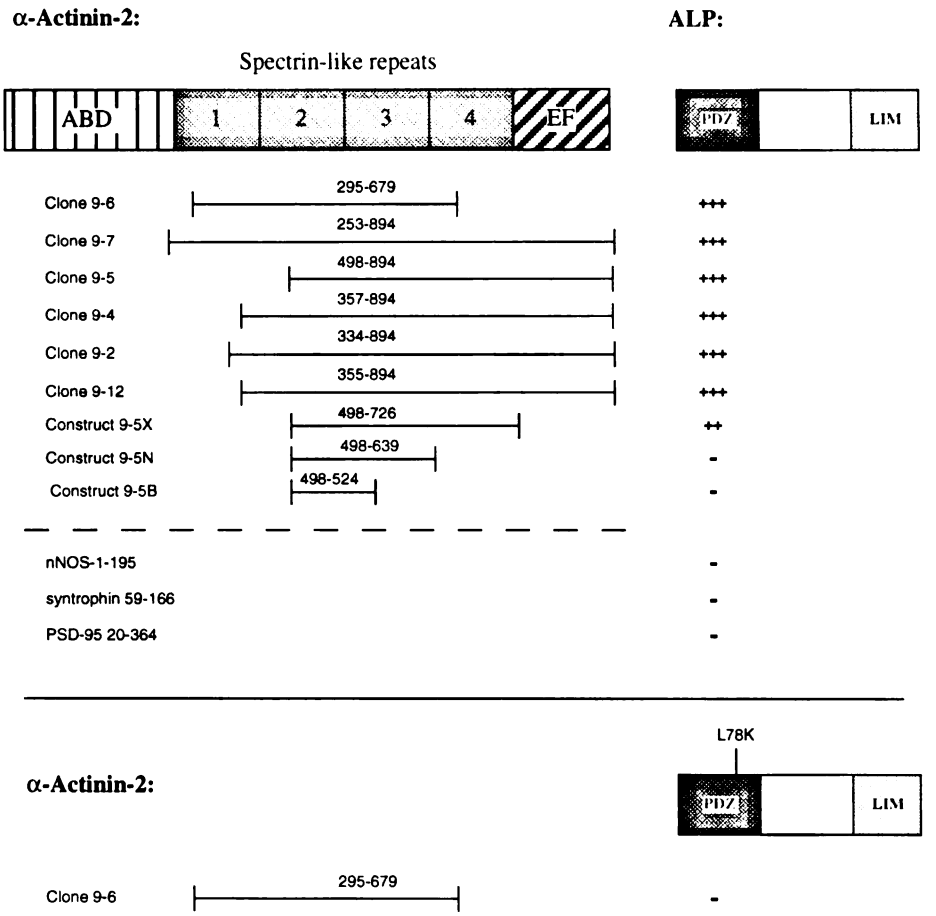
α-Actinin-2:
ALP:
Spectrin-like repeats
ABD
1
2
3
4
EF
PDZ
LIM
Clone 9-6
295-679
+++
Clone 9-7
253-894
+++
Clone 9-5
498-894
+++
Clone 9-4
357-894
+++
Clone 9-2
334-894
+++
Clone 9-12
355-894
+++
Construct 9-5X
498-726
++
Construct 9-5N
498-639
-
Construct 9-5B
498-524
-
nNOS-1-195
-
syntrophin 59-166
-
PSD-95 20-364
-
α-Actinin-2:
L78K
PDZ
LIM
Clone 9-6
295-679
-

Figure 4

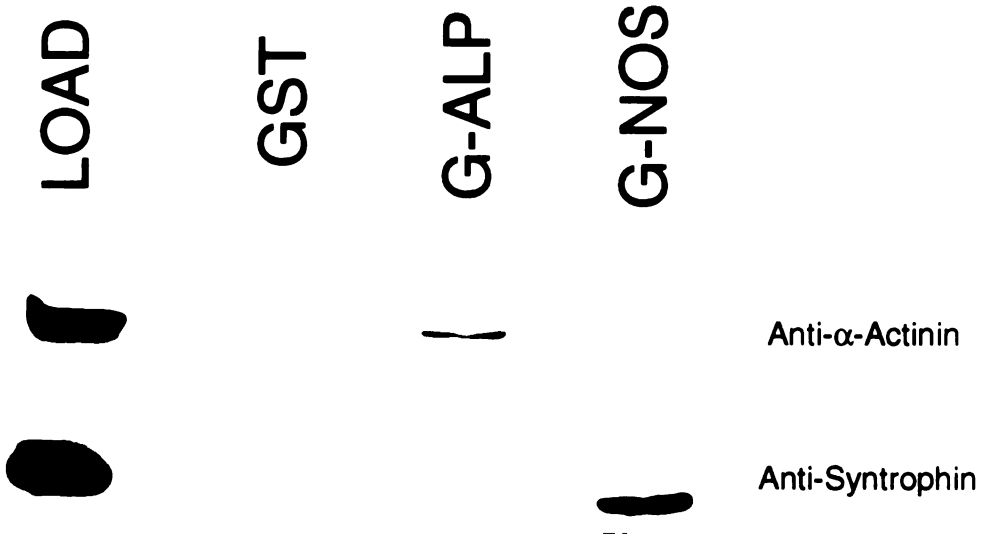

Figure 5

Figure 4

The PDZ domain of ALP binds to the spectrin repeats of a-actinin-2. The sequence encoding amino acids 1-128 of ALP was fused to GAL4 DNA binding domain. Clones 9-2, 4, 5, 6, 7 and 12, which were rescued from a yeast 2-hybrid screen of a rat skeletal muscle library, encode different fragments of a-actinin-2. Clone 9-5 was truncated with restriction enzymes to yield clones 9-5X, N, B. All ALP interacting clones encoded at least two complete spectrin-like repeats one of which was third repeat. nNOS, PSD-95 and a1-syntrophin did not interact with a-actinin-2. Mutation of ALP leucine 78 to lysine abolished interaction with a-actinin-2.

Figure 5

Biochemical association of ALP and a-actinin-2 and specificity of the PDZ-spectrin-like repeat interaction. Affinity chromatography demonstrates that a-actinin-2 is selectively retained by an immobilized ALP fragment (amino acids 1-128) fused to GST, not by GST-NOS (amino acids 1-299) fusion protein, which selectively brings down syntrophin. The load is 20% of the input used for affinity chromatography experiment.

Chapter V: ALP Colocalizes with α-actinin-2 at the Z-lines in Skeletal Muscle

To determine whether the interaction with α-actinin-2 is physiologically important in localizing ALP to specific cytoskeletal domains, we developed an affinity purified antiserum for immunofluorescent localization studies (see experimental procedures.) We evaluated the specificity of the serum by western blot analysis of crude tissue extracts. As expected, the antiserum recognized a prominent band of 39 kD in skeletal muscle and a much less intense band of 35 kD in heart (Figure 6A). No immunoreactive bands were noted in spleen, kidney, brain or liver. Immunofluorescent staining of longitudinal sections of adult skeletal muscle showed that ALP co-localized with α-actinin-2 on the Z-lines (Figure 6C). No ALP immunoreactivity was found at the sarcolemma, nucleus or other structures of the myofiber. Western blotting of myogenic cell (C2C12) extracts showed that ALP is absent from myoblasts but is induced within three days following myotube fusion (Figure 6B).

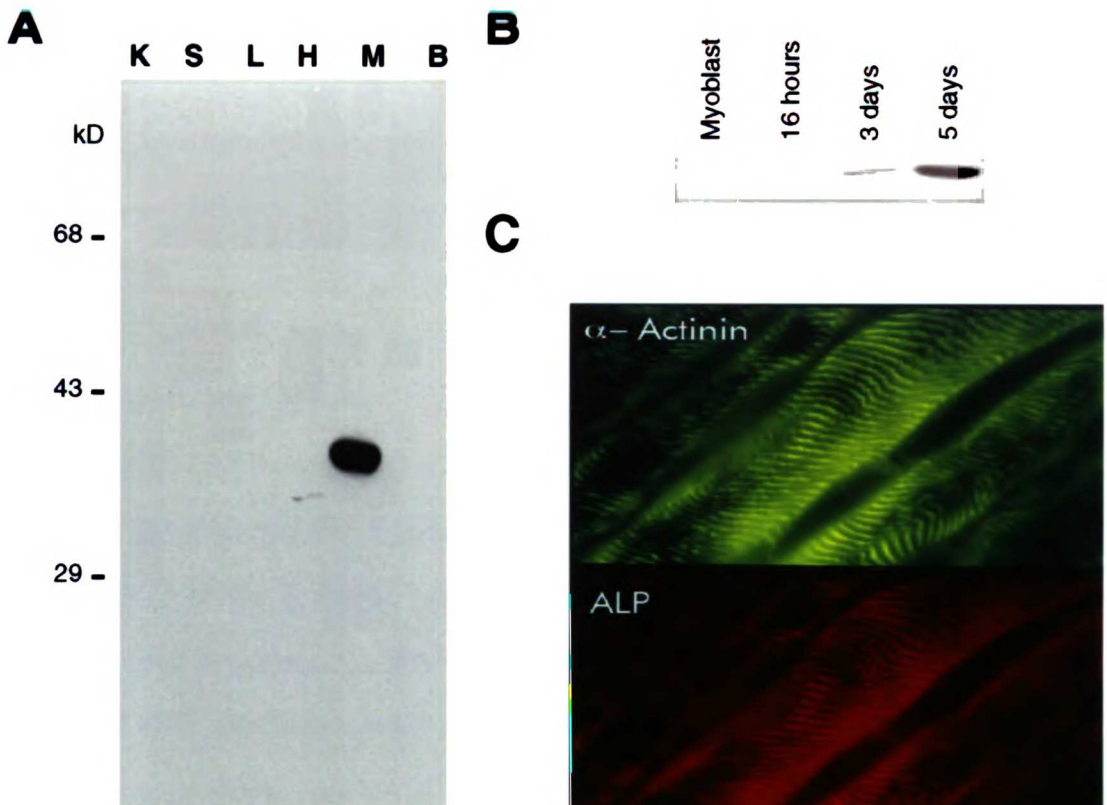
A
K S L H M B
kD
68 -
43 -
29 -
B
Myoblast
16 hours
3 days
5 days
C
α– Actinin
ALP

Figure 6

Figure 6

ALP protein is enriched in skeletal muscle and colocalizes with a-actinin-2 at the Z lines. (a) Rat tissue extracts (100 μg per lane) from rat kidney (K), spleen (S), liver (L), heart (H), skeletal muscle (M) and brain (B) was run on SDS-PAGE gel and transferred to a PVDF membrane and then probed with a polyclonal antibody against GST-ALP fusion protein. (b) Western blotting of protein extracts from C2 myogenic cultures shows that ALP is absent from myoblasts and is present in myotubes 3 and 5 days following fusion. (c) Immunofluorescent staining of rat skeletal muscle longitudinal sections shows that ALP (red) occurs at the a-actinin-2 (green) rich Z-lines.

Chapter VI: ALP expression is upregulated by Heregulin

To test if ALP expression is subject to growth factor regulation, we used C2C12 cell line as a model system. C2C12 myoblast cells were grown to 90% confluency before switching to differentiation medium (low serum). On the third day, the differentiated myotubes were treated with 0.1 or 1 nM heregulin (Genetech) for 24 hours before they were harvested for mRNA preparation. The resulting northern blot was probed with full length ALP. Compared with untreated myotubes, ALP expression was upregulated more than three folds by 1 nM heregulin treatment. A control band, myosin heavy chain (MHC), was not affected by heregulin treatment (Fig. 2f).

What is the functional significance of this upregulation? After muscle cell differentiation, growth factors promote muscle maturation by modulating muscle specific gene expression. Heregulin (also called neuregulin) is such a growth factor secreted by motor neuron nerve terminals. It acts on receptor tyrosine kinase (erb2 and erb3) at neuromuscular synapses to activate a signaling pathway which results in new sets of gene expression in the myotube nucleus (Gassmann M; Lemke G.). Heregulin increases the expression of acetylcholine receptor (AChR) subunits α and δ, switching the expression of foetal type subunit γ to adult subtype ε (Jo SA et al). The switch from γ to ε subunits will change the kinetics of acetylcholine receptor activation by acetylcholine, thereby promoting myotube maturation.

So the upregulation of ALP transcripts here could shift the balance between the γ and ε expression pathways, hence mediates the AChR subtype switching by heregulin. The possible molecular mechanism could involve interaction of ALP LIM motif with transcription factors resposible for AChR subunit expression. Another

Chapter VII: The *ALP* Gene Maps to Human Chromosome 4q35

To determine whether ALP might map close to any known genetic mutations that are associated with muscle disease, we determined the chromosomal location of human ALP. We performed FISH with two independent digoxigenin labeled genomic ALP probes on normal human metaphase chromosomes. Hybridization with these probes resulted in specific labeling of only chromosome 4. The location of the probes was determined by digital image microscopy following FISH and localized by the fractional length from the p-terminus (FLpter) as described previously (Sakamoto, et al., 1995, Stokke, et al., 1995). Both clones mapped to the most telomeric region of chromosome 4, at 4q34-qter with FLpter values of 0.962+/- 0.004 and 0.959 +/- 0.005.

Interestingly, fascioscapulohumeral muscular dystrophy, the most common autosomal dominant muscular dystrophy, maps to the telomeric region of chromosome 4, at 4q35. As FSHD is associated with deletions of a subtelomeric repeat sequence (van Deutekom, et al., 1993), the localization of ALP relative to the 4q telomere was of interest. We therefore utilized somatic cell and radiation hybrid panels to map ALP within chromosomal band 4q35. PCR analysis of the somatic cell hybrids revealed that ALP maps within the proximal portion of this band (Figure 7B). ALP is not present in HHW 1372, which contains only the telomeric 2 Mb of chromosome 4q distal to the locus D4S187. Analysis of the radiation hybrid panel DNAs which contain independent fragments of 4q35 localizes ALP to the interval between D4S171 and the Factor XI gene (Figure 7C). Thus, the approximate distance of ALP from the telomere is 7-10 Mb.

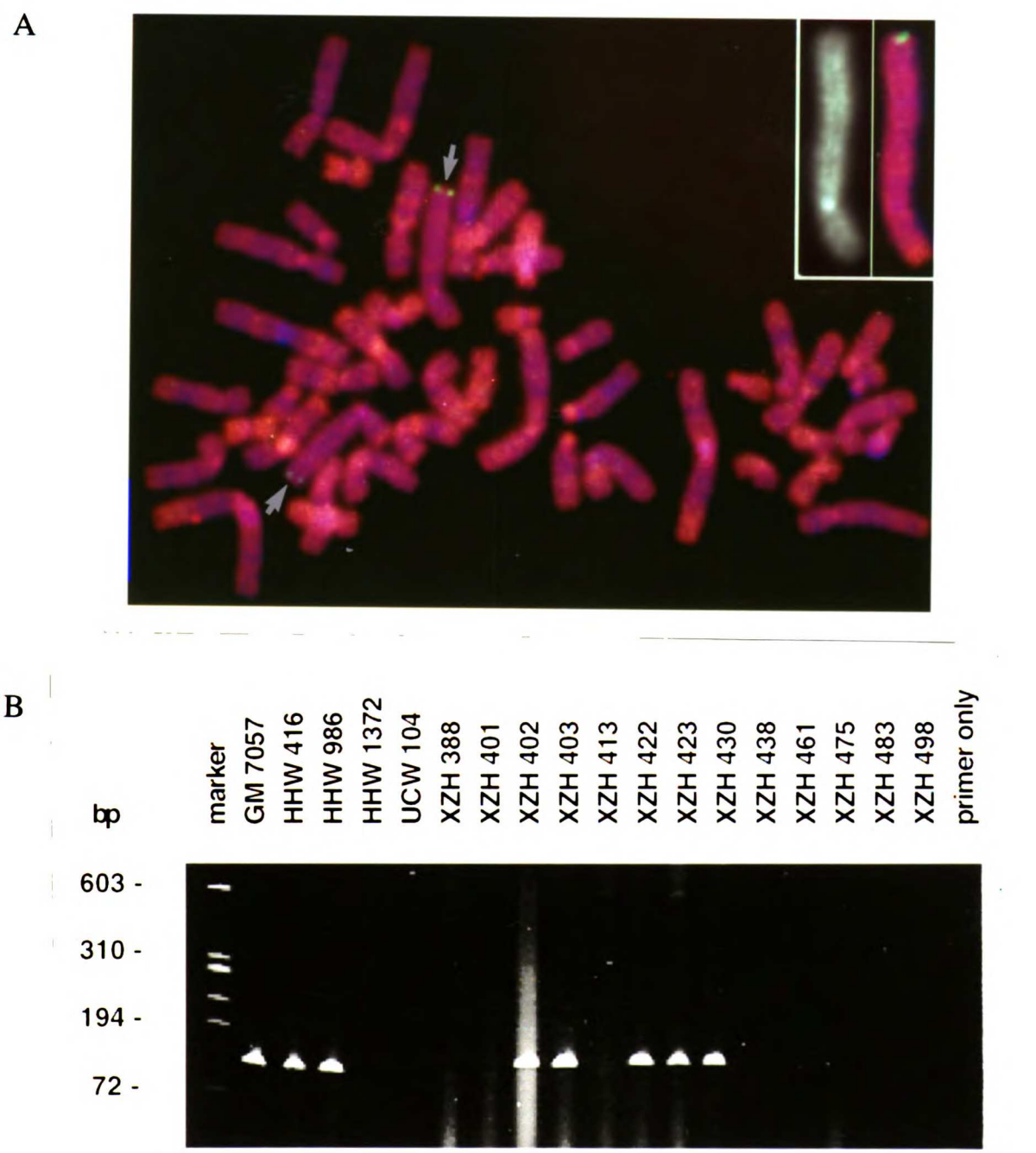
A
B
bp
marker
GM 7057
HHW 416
HHW 986
HHW 1372
UCW 104
XZH 388
XZH 401
XZH 402
XZH 403
XZH 413
XZH 422
XZH 423
XZH 430
XZH 438
XZH 461
XZH 475
XZH 483
XZH 498
primer only
603 -
310 -
194 -
72 -

Figure 7 (A & B)

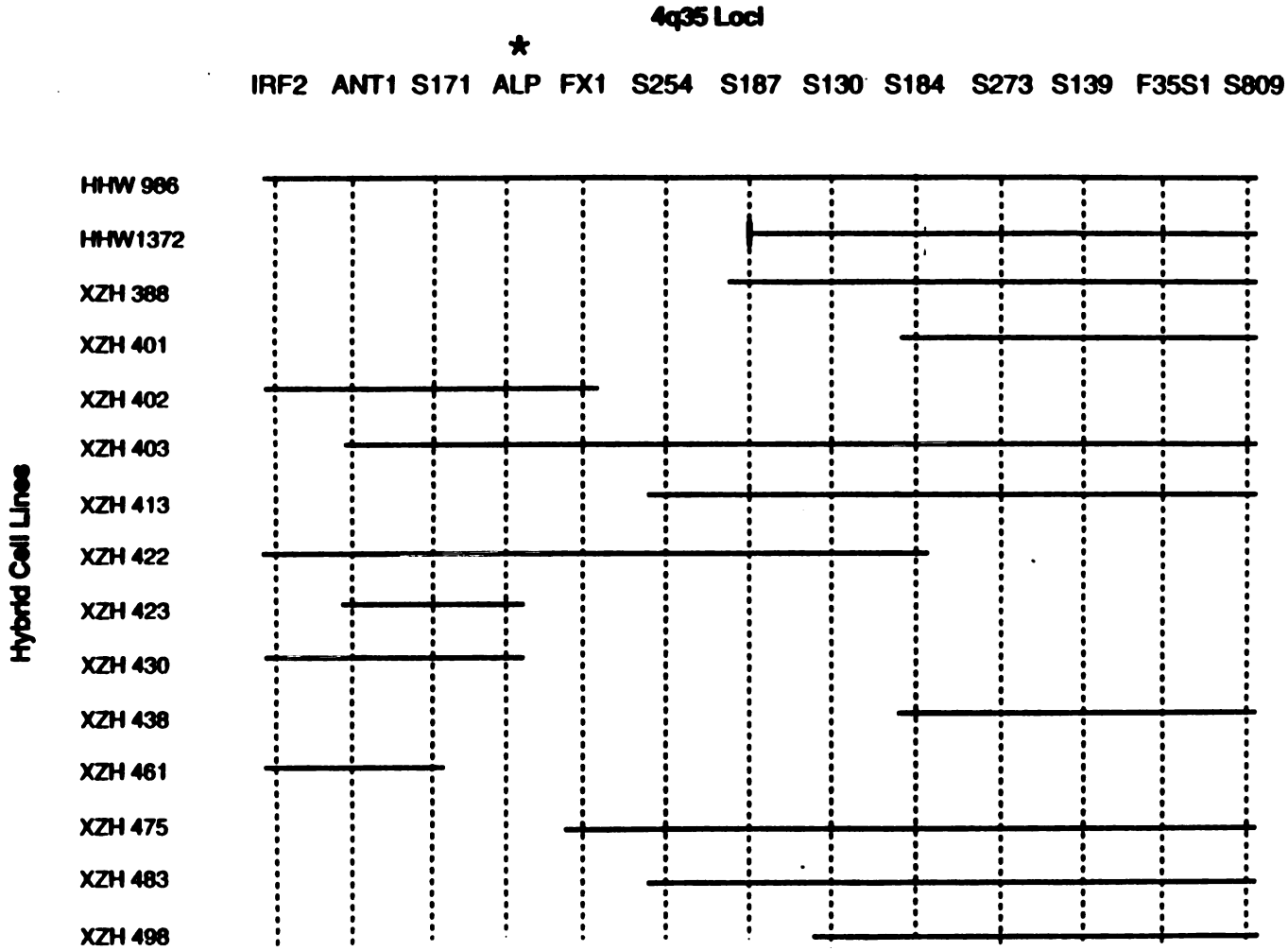
4q35 Locl
IRF2
ANT1
S171
ALP
FX1
S254
S187
S130
S184
S273
S139
F35S1
S809
Hybrid Cell Lines
HHW 986
HHW1372
XZH 388
XZH 401
XZH 402
XZH 403
XZH 413
XZH 422
XZH 423
XZH 430
XZH 438
XZH 461
XZH 475
XZH 483
XZH 498

Figure 7C

Figure 7

Human ALP maps to chromosome 4q35. (a) Fluorescence in situ hybridization of P1 clones to human metaphase chromosomes. Hybridizing signals (arrows) were detected by FITC (green) and the chromosomes were counterstained with propidium iodide and DAPI (purple, combined color). Inset on the lower left corner shows chromosome 4 with P1 hybridization aligned with a black and white image of DAPI-stained chromosome 4. (b) PCR analysis of human hamster somatic cell and radiation hybrids containing various portions of chromosomal band 4q35. The 12X bp amplification product from the ALP gene is present only in somatic cell hybrids containing the portion of 4q35 proximal to D4S187. Only those radiation hybrids that contain a portion of the interval between D4S171 and FXI were positive for ALP. (c) Schematic of the 4q35 locus contained within each somatic cell and radiation hybrid. The order and retention of the 12 loci between IRF2 (centromeric and D4S809 (telomeric) in the radiation hybrids were previously determined (Winokur, et al., 1993)

.

DISCUSSION

Here, we identified a functional interaction between a PDZ domain and the spectrin-like repeats of α-actinin-2. This association targets a novel LIM-protein, ALP, to the actinin-rich Z-lines of skeletal muscle fibers. PDZ domains are recently recognized protein-protein interaction motifs that are implicated in protein association with the cytoskeleton (Marfatia, et al., 1996) and in signal transduction (Brenman and Bredt, 1997, Sheng, 1996). Previous studies demonstrated that the two PDZ proteins in skeletal muscle, nNOS and the syntrophins are constituents of the dystrophin complex (Adams, et al., 1993, Brenman, et al., 1995). Our work here shows that the PDZ protein ALP does not associate with the dystrophin complex but instead binds to α-actinin-2, which is in the dystrophin superfamily of cytoskeletal proteins.

Interaction with the spectrin-like repeat represents a new mode of binding for a PDZ domain. Previous work has shown that PDZ domains of the postsynaptic density protein, PSD-95, bind to certain glutamate receptors and K+ channels in brain that terminate with a consensus of E-T/S-X-V/I (Cohen, et al., 1996, Kim, et al., 1995, Kornau, et al., 1995). These interactions appear to anchor ion channels to synaptic sites in neurons. Interaction with specific C-terminal peptides may be a general property of PDZ domains and two recent studies demonstrate that distinct PDZ domains bind to specific C-terminal peptide sequences (Songyang, et al., 1997, Stricker, et al., 1997). Certain PDZ domains can also associate with each other in a homotypic type interaction (Brenman, et al., 1996). The PDZ domain of nNOS binds to the second PDZ domain of PSD-95 in brain and to the PDZ domain of α1-syntrophin in skeletal muscle.

The binding interface between the PDZ domain of ALP and the spectrin-like repeats of α-actinin-2 represents a third mode for protein interactions mediated by PDZ

domains. We suspect that this type of interaction is not unique to ALP and may explain cytoskeletal interactions of diverse PDZ proteins. Zona occludens-1 protein of epithelial tight junctions contains three PDZ domains and associates with spectrin in the cell cortex (Willott, et al., 1993). Electron micrographic studies indicate that ZO-1 forms a complex with the central rod-like repeat domains of spectrin. It is not yet clear whether the PDZ domains of ZO-1 mediate this interaction. A complex ternary interaction between the spectrin-like repeats of dystrophin and the PDZ domains of nNOS / syntrophin may occur at the skeletal muscle sarcolemma (Chao, et al., 1996). Thus, *in vitro* assays demonstrate that nNOS binds directly to syntrophin, but not to dystrophin. However, the nNOS / syntrophin interaction in skeletal muscle requires that certain spectrin-like repeats of dystrophin be intact. And, nNOS is selectively absent from skeletal muscle sarcolemma in patients with Becker muscular dystrophy who have mutations within the spectrin like repeats of dystrophin (Chao, et al., 1996).

We find that ALP expression is normal in Duchenne and Becker muscular dystrophies (H.X. and D.S.B., unpublished). On the other hand, certain inherited muscular dystrophies are due to mutations in cytoskeletal proteins that do not interact with the dystrophin complex (Hoffman, et al., 1995). Plectin, a cytoskeleton-membrane anchorage protein of hemidesmosomes, links intermediate filaments to the sarcolemma and also occurs at the Z-lines in skeletal muscle (Wiche, et al., 1983). Mutations in plectin, do not effect the dystrophin complex, but cause an autosomal recessive muscular dystrophy associated with skin blistering (Smith, et al., 1996). It will be important to assess ALP expression in a variety of inherited muscular dystrophies to determine whether it may play a role in any of these diseases.

Our chromosomal mapping studies indicate that ALP occurs on human chromosome 4q 35. Interestingly, the location is approximately 7-10 Mb from the subtelomeric region that is mutated in fascioscapulohumeral muscular dystrophy (FSHD), an autosomal dominant disease (Wijmenga, et al., 1992). The specific

genetic defect in FSHD disease appears to be a deletion of heterochromatin (Lyle, et al., 1995, Winokur, et al., 1994). How this mutation results in muscular dystrophy is not clear. It is postulated that the telomeric mutation mediates a "position effect" which alters the expression of a nearby muscle-specific gene (reviewed in Altherr et al., 1995). Genes separated by genomic distances greater than 2 Mb from heterochromatin have been reported to be affected by position effect variegation in *Drosophila* (reviewed in, Bedell et al., 1996). Therefore, ALP should be considered a candidate gene for FSHD. In preliminary studies, we have not detected consistent changes in ALP expression in muscle biopsies from FSHD tissues (H. X., J. Mendell, J. Kissel, D. S. B., unpublished). However, the muscle samples from FSHD patients analyzed for expression of ALP may not have been from the critically affected muscle groups or from appropriate developmental stages.

What might be the normal function of ALP? Determining the role of the of LIM motif in ALP remains a critical question. LIM motifs were first identified in protein products from 3 different genes, lin11 (Freyd, et al., 1990), isl1 (Karlsson, et al., 1990) and mec3 (Way and Chalfie, 1988), which all contain two LIM domains in association with a homeodomain DNA binding motif. These transcription factor LIM proteins participate in cell fate determination. Many distinct classes of LIM proteins have now been identified that do not have a homeodomain, yet still participate in cell fate determination (Sanchez-Garcia and Rabbitts, 1994). At the biochemical level, LIM motifs are implicated in protein-protein interactions. LIM motifs have been shown to interact with basic helix-loop-helix transcription factors (Wadman, et al., 1994), protein kinase C (Kuroda, et al., 1996), receptor tyrosine kinase tight turn structure (Wu, et al., 1996), as well as with other LIM motifs (Schmeichel and Beckerle, 1994). In striated muscle, the LIM-only protein, MLP, occurs at the Z-lines and is an essential regulator of myogenic differentiation (Arber, et al., 1994). Targeted disruption of MLP results in a disorganization of the myocyte cytoarchitecture (Arber, et al., 1997). It will

be interesting to determine whether the LIM domain of ALP interacts with MLP or other LIM proteins at the Z-lines. To decisively determine the role of ALP in cellular physiology and muscular dystrophy, it will be important to delete the function of ALP by knockout and dominant negative approaches.

Materials and Methods

mRNA Isolation and cDNA Analysis

RNA was isolated using the guanidine isothiocyanate / CsCl method, and mRNA was selected with oligo dT sepharose. For degenerate reverse transcriptase-polymerase chain (RT-PCR), rat skeletal muscle mRNA was reverse transcribed with RTth polymerase using random hexamer primers. PCR, using a 54° C annealing temperature, was then performed using the following primer pair: GGGGGATCCGGXGGXCTXGGXHT and, GCGGAATTCAGDARXATRTCXCC. Amplified products were resolved on agarose gels. Bands of 120-160 bp were isolated and subcloned into pBluescript vectors (Stratagene) for sequence analysis. Before transformation, ligation products were digested with Bgl II to remove syntrophin cDNA clones. Clones encoding ALP were isolated from a rat skeletal muscle cDNA library (Stratagene) by plaque hybridization.

For Northern blotting, RNA was separated on a formaldehyde agarose gel and transferred to a Nylon membrane. A random primed ^{32}P probe was generated using the 160 bp ALP cDNA obtained from RT-PCR as a template. The filter was washed at high stringency, 63^{0}C, 0.1% sodium chloride / sodium citrate, 0.1% SDS and exposed to X-ray film for 2 hours at -70^{0}C.

Antibodies, Immunohistochemistry and Western Blotting

A glutathione-S-transferase fusion protein encoding amino acids 1-207 of ALP was expressed and purified from *E. coli* as described (Brenman, et al., 1995). Rabbits were immunized with the GST-ALP fusion protein emulsified in complete and

incomplete Freund's adjuvant. Serum bleeds were evaluated by ELISA. For affinity purification, ALP antiserum was applied to a column of GST-ALP fusion protein immobilized on Reacti-Gel (Pierce). The column was washed with 20 volumes of buffer containing 500 mM NaCl, 10 mM Tris, pH 7.5. ALP antibody was eluted with 10 bed volumes of 100 mM Glycine, pH 2.5. Monoclonal antibodies to syntrophin (Peters, et al., 1994) and α-actinin (Sigma, clone EA 53) were also used.

For immunofluorescent staining, skeletal muscle samples were flash frozen in liquid nitrogen-cooled isobutane, sectioned on a cryostat (10 μm), and melted directly onto glass slides. Sections were then postfixed in 2% paraformaldehyde-phosphate-buffered saline (PBS). Tissues were blocked in PBS containing 1% normal goat serum. Monoclonal antibodies to α-actinin (1:100) and polyclonal ALP antibody were applied to sections overnight at 4^0C. For indirect immunofluorescence, secondary goat anti-mouse fluorescence isothiocyanate (FITC) or donkey anti-rabbit Cy-3 conjugated antibodies were used according to the specifications of the manufacture (1:200, Jackson Laboratories).

For western blotting, protein extracts were resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Primary antibodies were diluted in block solution containing 3% bovine serum albumin, 0.1% Tween 20 in Tris-buffered saline and incubated with membranes overnight at 4^0C. Labeled bands were visualized using enhanced chemiluminescence (Amersham).

Myogenic Cell Culture

C2 myogenic cell line was grown on gelatin coated dishes in Ham's F-10 nutrient mixture plus 0.8 mM extra $CaCl_2$ supplemented with 15% horse serum (Life Technologies, Inc.) and 2.5 ng/ml basic fibroblast growth factor (Promega).

Myoblasts were fused in media containing DME (Gibco) supplemented with 2% horse serum. Protein and RNA was extracted from cultured cells as described above.

GST Fusion Protein Affinity Chromatography

To extract cytoskeletal proteins from skeletal muscle, tissue was homogenized in 10 volumes of buffer containing 2 mM Tris-HCl (pH 9) and 1mM EGTA. After incubation at 37^0C for 30 minutes with gentle agitation, tissue extracts were titrated to pH 7.5 prior to centrifugation at 15,000 x g for 30 minutes. For "pull down" assays, the solubilized tissue samples were incubated with control or GST-fusion proteins linked to glutathione sepharose beads for 1 hr. Beads were washed three times with buffer containing 0.5% Triton X-100 + 350 mM NaCl, and proteins eluted with SDS loading buffer. GST-nNOS fusion protein construct was constructed by PCR and fusion protein purified as previously described (Brenman et al., 1995).

In Situ Hybridization

In situ hybridization used ^{35}S-labeled RNA probes exactly as described (Sassoon and Rosenthal, 1993). Sense and antisense probes to ALP (full length) was synthesized from a pBluescript vector using T3 and T7 polymerases.

Human Chromosome Mapping

Two P1 clones corresponding to human ALP (Research Genetics) were used to determine the location of ALP on human chromosomes by fluorescence *in situ* hybridization (FISH). The hybridized signal was detected by anti-digoxigenin conjugated with FITC as described (Sakamoto, et al., 1995, Stokke, et al., 1995).

Fine mapping of ALP was performed by PCR amplification of human hamster somatic cell hybrids and radiation-derived hybrids containing all or portions of human chromosome 4q35. The somatic cell hybrids included HHW986, containing intact human chromosome 4 (Carlock, et al., 1986), HHW 986, retaining only 4q35 translocated to a derivative 5p, and HHW 1372, in which only the telomeric region of 4q35 (distal to D4S187) is retained on a derivative X (Bodrug, et al., 1990). The radiation hybrids were derived from HHW416 and retain varying fragments of 4q35 (Winokur, et al., 1993). Negative controls included a human lymphoblastoid cell line GM7057 (NIGMS) and a Chinese hamster fibroblast cell line UCW 104.

Hybrids were screened by PCR for the presence of the ALP gene. The oligonucleotides (sense: GGGAGCTGTACTGCGAAA) and (antisense: CGATTGTTTTCTCGTGTA) amplify a 150 bp product within the 3' UTR of ALP. Amplifications with a 51°C annealing temperature were done in 25µl containing 250 ng genomic DNA, 50 µl each oligonucleotide, 1.25 mM each dNTP, 16.6 mM ammonium sulfate, 67 mM Tris-HCl, pH 8, 6.7 mM MgCl2, 10 mM β-mercaptoethanol and 2 units *Taq* polymerase.

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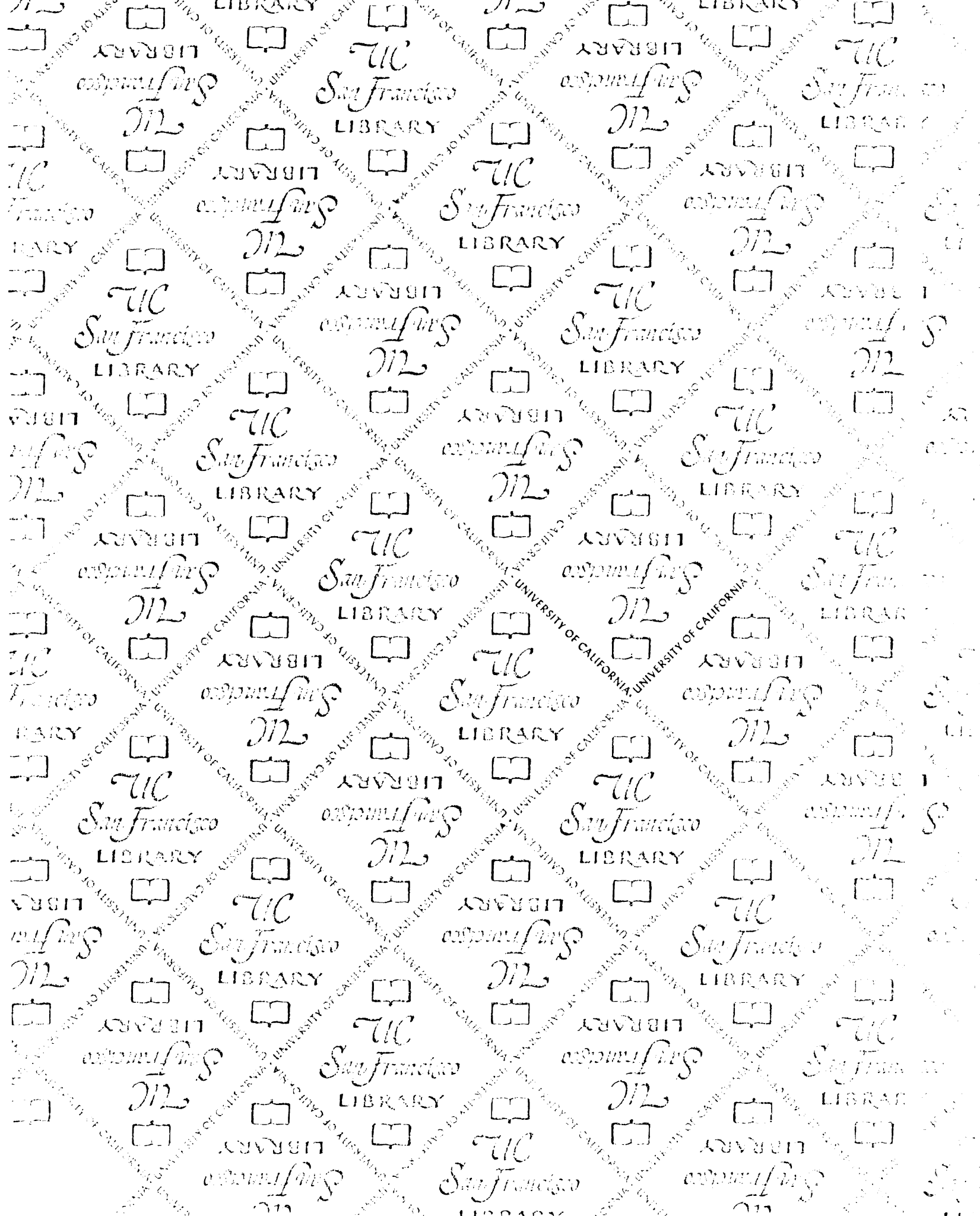
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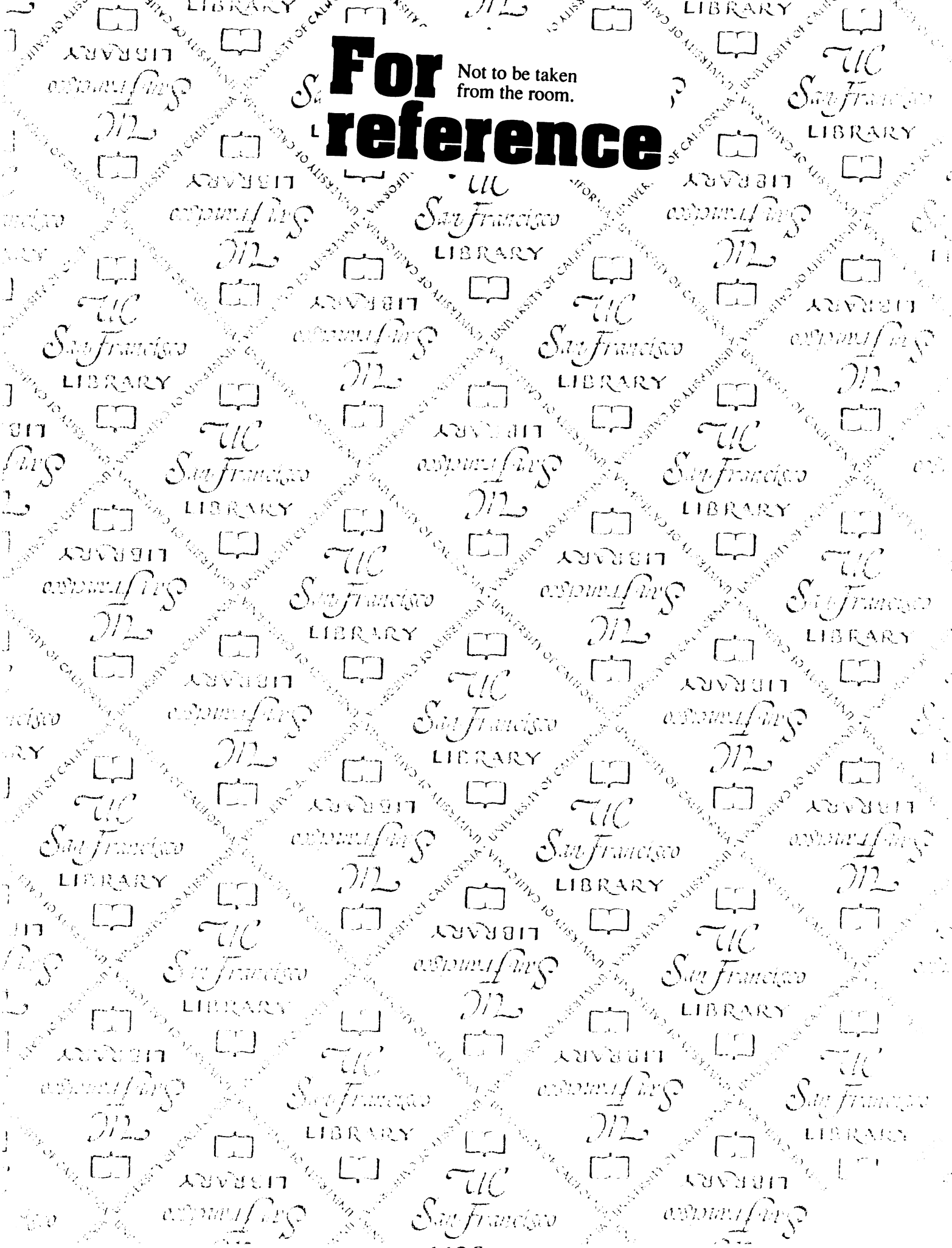
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