Characterization of Two Isoforms of the Skeletal Muscle LIM Protein 1, SLIM1

LOCALIZATION OF SLIM1 AT FOCAL ADHESIONS AND THE ISOFORM SLIMMER IN THE NUCLEUS OF MYOBLASTS AND CYTOPLASM OF MYOTUBES SUGGESTS DISTINCT ROLES IN THE CYTOSKELETON AND IN NUCLEAR-CYTOPLASMIC COMMUNICATION*

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We have cloned and characterized a novel isoform of the skeletal muscle LIM protein 1 (SLIM1), designated SLIMMER. SLIM1 contains an N-terminal single zinc finger followed by four LIM domains. SLIMMER is identical to SLIM1 over the first three LIM domains but contains a novel C-terminal 96 amino acids with three potential bipartite nuclear localization signals, a putative nuclear export sequence, and 27 amino acids identical to the RBP-J binding region of KyoT2, a murine isoform of SLIM1. SLIM1 localized to the cytosol of Sol8 myoblasts and myotubes. SLIMMER was detected in the nucleus of myoblasts and, following differentiation into myotubes, was exclusively cytosolic. Recombinant green fluorescent protein-SLIM1 localized to the cytoplasm and associated with focal adhesions and actin filaments in COS-7 cells, while green fluorescent protein-SLIMMER was predominantly nuclear. SLIMMER truncation mutants revealed that the first nuclear localization signal mediates nuclear localization. The addition of the proposed nuclear export sequence decreased the level of exclusively nuclear expression and increased cytosolic SLIMMER expression in COS-7 cells. The leucine-rich nuclear export signal was required for the export of SLIMMER from the nucleus of myoblasts to the cytoplasm of myotubes. Collectively, these results suggest distinct roles for SLIM1 and SLIMMER in focal adhesions and nuclear-cytoplasmic communication.

The LIM domain is a double zinc finger motif that mediates the protein-protein interactions of transcription factors, signaling, and cytoskeleton-associated proteins (1–4). LIM is an acronym of the three transcription factors, Lin-11, Isl-1, and Mec-3, in which the motif was first identified (5). The LIM domain contains 50–60 amino acids with the consensus sequence (CX3CX17–19HX2CX2/XCX16–20CX2(H/D/C)). The conserved cysteine and histidine residues form two zinc-binding pockets stabilizing the tertiary structure of the protein (6, 7). Despite their structural resemblance to GATA-1 zinc fingers, there is no evidence that LIM domains bind DNA directly. Instead, an increasing number of studies implicate LIM domains in protein-protein interactions that regulate development, cellular differentiation, and the cytoskeleton (8, 9).

It has been proposed that LIM proteins form a scaffold upon which the coordinated assembly of proteins occurs (8, 10). No single LIM domain-binding motif has been identified. LIM domains can associate with other LIM domains to form homo- or heterodimers (8, 11). In addition, LIM domains also bind tyrosine-containing motifs, PDZ domains, ankyrin repeats, and helix-loop-helix domains in proteins lacking LIM domains including tyrosine and serine/threonine kinases, cytoskeletal proteins, and transcription factors (12–16).

LIM proteins have been demonstrated in both the nucleus and cytoplasm. LIM homeodomain and LMO proteins are nuclear proteins, which regulate transcription by forming complexes with other transcription factors (2, 10). Members of the cysteine-rich protein family (CRP1,1 CRP2, and CRP3/MLP (muscle LIM protein), each expressed in different muscle cell types) have a joint localization in the nucleus and along the actin cytoskeleton, probably reflecting dual roles (9). In the nucleus, MLP interacts with the myogenic transcription factor MyoD to regulate transcription (17). The importance of MLP in regulating the cytoskeleton has been demonstrated in MLP knockout mice, which develop marked disruption of the cardiac cytoarchitecture, resulting in a dilated cardiomyopathy and cardiac failure (18). In the cytoskeleton, CRP interacts with the focal adhesion-associated LIM protein zyxin, via a LIM-LIM interaction (8). CRP and zyxin also bind the actin-binding protein α-actinin (19, 20). These overlapping CRP-zyxin-α-actinin interactions may either stabilize or regulate the cytoskeleton. It has recently been proposed that zyxin, which contains a nuclear export sequence, shuttles between focal adhesions and the nucleus (21). The LIM protein, paxillin, also associates with focal adhesions, and this association is mediated via its LIM domains (22). LIM2 and LIM3 of paxillin are serine phosphorylated by an associated serine/threonine kinase, and this phosphorylation increases the association of paxillin with focal adhesions (23).

Three homologous skeletal muscle LIM proteins designated...
SLIM1, SLIM2, and SLIM3 have been identified by partial cDNA cloning and sequence data base analyses (24, 25). Each SLIM contains an N-terminal zinc finger followed by four complete LIM domains and no other signaling or DNA binding motifs. SLIM1 has also been cloned from a human heart library, designated “Four and a Half LIM protein 1,” and the gene has been localized to chromosome Xq27 (26). An alternatively spliced murine isoform of SLIM1, called KyoT2, has recently been identified as a binding partner of the DNA-binding protein, RBP-J (27). KyoT2 comprises the N-terminal two and a half LIM domains of SLIM1 followed by 27 novel C-terminal amino acids. These 27 C-terminal amino acids mediate binding to RBP-J, displacing RBP-J from DNA and thereby inhibiting transcription. SLIM1 does not bind RBP-J.

We report here the cloning and characterization of a novel alternatively spliced human isoform of SLIM1, designated SLIMMER (for SLIM1 with extra regions). We demonstrate that SLIM1 is localized at focal adhesions, while SLIMMER, with functional nuclear import and export sequences, localizes to the nucleus of myoblasts and the cytoplasm of myotubes. The discrete subcellular locations of the two isoforms suggest distinct roles in the cytoskeleton and nuclear-cyttoplasmic communication.

**EXPERIMENTAL PROCEDURES**

**Materials**

| Materials | Details |
|-----------|---------|
| [α-32P]dCTP | 1000 Ci/mmol from NEN Life Science Products. |
| [γ-32P]dATP | To label DNA for autoradiography. |
| T4 DNA Ligase | From Promega. |
| T7 DNA polymerase | From Clontech. |
| pCR 2.1 cloning vector | From Invitrogen. |
| Thermosequenase | From Amersham Pharmacia Biotech. |
| DNA fragments | Labeled with [α-32P]dCTP and [γ-32P]dATP and hybridized at 42 °C using standard conditions (29). |
| DNA sequencing primers | For DNA sequencing. |
| Enhanced Chemiluminescence | From Amersham Pharmacia Biotech. |

**Methods**

**Isolation of Human SLIM1 and SLIMMER cDNAs—a** Human bone marrow cDNA library (1 × 10^6 recombinants) was screened using a synthetic oligonucleotide “GCACCATGGCGGGGAAG” derived from anti-peptide antibodies were obtained by chromatography of premune or immune sera on specific peptide coupled to thiopropyl-Sepharose resin. Following extensive washing, specific antibodies were eluted from the column with 0.1 M glycine-HCl, pH 2.5.

**Immunoblot Analysis—** Proteins were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting utilized affinity-purified anti-peptide serum. The secondary antibody was horseradish peroxidase-conjugated sheep anti-rabbit IgG (Amersham Pharmacia Biotech) diluted to 1:10,000. Immunoblots were developed using Enhanced Chemiluminescence (Amersham Pharmacia Biotech).

**Preparation of Skeletal Muscle Samples—** Human skeletal muscle was obtained from tissue routinely divided at elective hip surgery (Box Hill Hospital, Medical Ethics Approval). Muscle was weighed and suspended five times (v/v) in 30 mM Tris-HCl, pH 7.4, 2 mM EDTA, 50 μg/ml phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 50 μM leupeptin, and 0.83 mM benzamidine. Lysates were homogenized on ice immediately for 3 × 20 s (LABSONIC 1510), and a sample of total cell lysate was removed and analyzed by SDS-PAGE and immunoblot analysis.

**Intracellular Localization of SLIM1 and SLIMMER in Sol8 Cell Lines—**The Sol8 mouse skeletal muscle cell line (31) was grown in Dulbecco’s modified Eagle’s medium (DMEM) plus 20% fetal bovine serum on gelatin-coated plates. Cells were replaced before they reached 50% confluence. For indirect immunofluorescence, myoblasts were grown on glass coverslips treated with 3% formaldehyde and 2% Triton X-100. Preimmune or affinity-purified anti-SLIM1 or anti-SLIMMER sera were added. The cells were washed and then placed in 1:400 FITC-anti-rabbit IgG antibody (Silense). Co-localization with propidium iodide was visualized by fluorescence microscopy. The co-localization was visualized by confocal microscopy.

**Transient Transfection of HA-tagged SLIM1 and SLIMMER in Sol8 Cells—** The cDNAs encoding the open reading frames of SLIM1, SLIMMER, and truncated constructs were amplified by PCR and cloned into the eukaryotic expression vector pSVTf (30). The two constructs were linearized by restriction enzyme digestion, and using the TNT Coupled Wheatgerm Extract systems (Promega) translated in vitro in the presence of [35S]methionine. Translation products were analyzed by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography.

**Antipeptide Antibodies—** Antipeptide antibodies were generated to synthetic peptides conjugated to keyhole limpet hemocyanin or diphtheria toxoid. The first peptide represented amino acids 261–272 (NKRFVFHQEQVY) unique to LIM4 of SLIM1. The second peptide represented amino acids 231–246 (KRTVSVSSHVPVSKAR) unique to the insert sequence of SLIMMER. Each conjugated peptide was injected intramuscularly into two New Zealand White rabbits. Affinity-purified anti-peptide antibodies were obtained by chromatography of preimmunization sera on specific peptide coupled to thiopropyl-Sepharose resin. Following extensive washing, specific antibodies were eluted from the column with 0.1 M glycine-HCl, pH 2.5.

**In Vitro Protein Translation—** The probe used was the 200-base pair (bp) fragment of SLIMMER, designated “Four and a Half LIM protein 1.” The construct was subcloned into the eukaryotic expression vector pSVTf (30). The two constructs were linearized by restriction enzyme digestion, and using the TNT Coupled Wheatgerm Extract systems (Promega) translated in vitro in the presence of [35S]methionine.

**Northern Blot Analysis—** The probe used was the 200-base pair (bp) insert of SLIMMER (nucleotides 784–968), which was obtained by SmaI/XhoI restriction digestion of the 2.1 kb cDNA encoding SLIMMER. DNA fragments were labeled with [α-32P]dCTP by random priming (Prime-a-gene, Promega) and hybridized overnight at 42 °C to a commercial human multisite mRNA membrane and washed using standard procedures (29). The membrane was allowed to decay and subsequently rehybridized to an actin probe.

**In Vivo Protein Localization—** Myoblasts were grown in Cos-7 cells, grown in DMEM plus 20% fetal bovine serum for 24 h or were induced to differentiate into myotubes as described above. The transfected myoblasts or myotubes were fixed, and the expression of HA-tagged protein was detected by a monoclonal anti-HA antibody diluted 1:50 (AMRAD).

**Transient Expression of SLIM1 and SLIMMER with Green Fluorescent Protein in COS-7 Cells—** The cDNAs encoding the open reading frames of SLIM1, SLIMMER, and truncated constructs were amplified by PCR and cloned in frame into the XbaI site of the pEGFP-C2 vector (Clontech). The nucleotide sequence of all GFP constructs was confirmed by dyeoxy sequencing. All constructs were transiently transfected into COS-7 cells, grown in DMEM with 10% fetal calf serum. Transfections were performed using a DEAE-dextran technique and grown for 2 days (29). Cells were washed, fixed, and permeabilized as outlined for Sol8 cells. Co-localization was performed using 50 μg/ml mouse monoclonal anti-paxillin antibody (Transduction Laboratories) or 1.500 tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma). Anti-paxillin was detected with a secondary Texas Red-conjugated anti-mouse IgG antibody. Transfected cells were visualized by confocal microscopy.

**Southern Blot Analysis—** The probe used was the 200-base pair (bp) insert of SLIMMER (nucleotides 784–968), which was obtained by SmaI/XhoI restriction digestion of the 2.1 kb cDNA encoding SLIMMER. DNA fragments were labeled with [α-32P]dCTP. Following transfection, the myoblasts were grown in DMEM plus 20% fetal bovine serum for 24 h or were induced to differentiate into myotubes as described above. The transfected myoblasts or myotubes were fixed, and the expression of HA-tagged protein was detected by a monoclonal anti-HA antibody diluted 1:50 (AMRAD).
RESULTS

Isolation of cDNA Clones Encoding Isoforms of SLIM1—Two cDNAs encoding isoforms of SLIM1 were incidentally isolated during attempts to clone the 43-kDa inositol polyphosphate 5-phosphatase (5-phosphatase) (28, 32). An oligonucleotide representing nucleotides 95–111 of the Type I 5-phosphatase (28) (GenBank™ accession number X77567) was used to screen 1.3 × 10^6 plaques of a λgt11 human bone marrow library. Two pos-

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**Fig. 1.** cDNAs and translated proteins of SLIM1 isoforms. A, diagram of the human cDNAs encoding SLIM1, SLIMMER, the corresponding translated proteins for each, and murine KyoT2. The predicted open reading frame is represented by the open box, and the untranslated region is represented by the line. The 200-bp insert cDNA of SLIMMER is represented by the hatched box labeled insert. Specific amino acid domains are represented as follows: single zinc finger (single shaded box labeled z-), LIM domains (double shaded boxes labeled LIM1–4), nuclear export signal (underlined), and RBP-J binding region (shaded box labeled R). The lines labeled A and B indicate the amino acid sequences against which A (the anti-SLIM1 antibody) and B (the anti-SLIMMER antibody) are directed. The open reading frames of SLIM1, SLIMMER, and various truncated constructs were cloned in frame into pEGFP-C2 and pCGN vectors. The domains of SLIM1 or SLIMMER contained in each construct are indicated. The number at the end of each construct indicates the C-terminal amino acid.

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**B** NLS2

![Graph showing NLS2 with corresponding amino acid sequences and domains.]
tive clones (1.9 and 2.1 kb) were identified, purified, and subcloned into the pcR2.1 vector. DNA sequencing of each clone revealed that they were identical, apart from a 200-bp insert present in the 2.1-kb cDNA (Fig. 1A). There was no homology in either clone to the 43-kDa 5-phosphatase, except for 16 out of 17 nucleotides from position 80 to 96, in both the 1.9- and 2.1-kb cDNA, which were identical to the oligonucleotide used to screen the library.

Sequence analysis revealed that the 1.9-kb cDNA is 99% identical to that of SLIM1 (25), which was derived by analysis of 36 overlapping sequences from a skeletal muscle cDNA library and various sequence data bases, except that our cDNA lacks 400 bp of the 3′-untranslated region. The 1.9-kb clone is 100% identical to the “Four and a Half LIM protein 1” cloned from a human heart library (26). Translation of the cDNA sequence reveals that SLIM1 is composed almost entirely of four LIM domains preceded by a single N-terminal zinc finger, with a predicted unmodified molecular mass of 32 kDa. Each LIM domain is separated by eight intervening amino acids, and there are no other known motifs, in particular homeodomain, or glycine-rich sequences (Fig. 1A). A comparison of SLIM1 with various protein sequence data bases reveals that it shows 47% overall identity with DRAL (SLIM3), a 32-kDa muscle protein that is down-regulated in rhabdomyosarcomas (33), and 45% with SLIM2 (25).

Sequence analysis of the 2.1-kb cDNA showed that it was identical to SLIM1 except for a 200-bp insert beginning at nucleotide 775. This insertion causes a shift in the reading frame such that it now terminates at nucleotide 1054, instead of 923 (Fig. 1, A and B). Translation of the open reading frame predicts a protein of 323 amino acids, with a predicted molecular mass of 34 kDa. Examination of various sequence data bases showed that the 2.1-kb isoform has not been previously described; therefore, we have designated this new isoform SLIMMER, for “SLIM1 with extra regions” (GenBank™ accession number AF063002). This acronym is also appropriate because, although larger in molecular mass, SLIMMER contains one less LIM domain than SLIM1. The SLIMMER cDNA encodes an N-terminal zinc finger followed by three LIM domains identical to SLIM1; however, the final 93 amino acids are different. These novel C-terminal 93 amino acids do not encode a fourth LIM domain but instead contain three overlapping sequences that each conform to a bipartite nuclear localization signal (NLS) amino acid sequence (34–36) (Fig. 1, A and C). Immediately following the NLS, there is a leucine-rich sequence, consistent with a nuclear export sequence (NES) (37–39) (Fig. 1C). The 27 C-terminal amino acids of SLIMMER are identical to the C-terminal amino acids described in KyoT2, a murine isoform of SLIM1 (Fig. 1, A and B). KyoT2 was identified from a yeast two-hybrid screen for binding partners of RBP-J, a DNA-binding protein (27). KyoT2 comprises the first two LIM domains of SLIM1 followed by 27 amino acids, not present in SLIM1 but present in SLIMMER, that mediate binding to RBP-J (Fig. 1A).

Expression Levels of SLIMMER mRNA in Various Tissues—A previous study by Morgan et al. (24) has shown very high levels of SLIM1 mRNA in skeletal muscle and only low levels in other sheep tissues tested. We demonstrated that human SLIM1 mRNA is highly expressed in skeletal muscle, with intermediate expression in heart and low level expression in prostate, colon, testis, ovary, small intestine, and placenta, a much wider expression than previously described (results not shown). However, the SLIM1 cDNA used as a probe in these studies probably detects both SLIM1 and SLIMMER transcripts. In order to determine whether SLIM1 and SLIMMER mRNAs are expressed in the same or different tissues, we performed a Northern blot analysis using a SLIMMER-specific probe, containing nucleotides 784–968 of the SLIMMER insert cDNA. High level expression of a 2.4-kb transcript was seen in skeletal muscle, with lower level expression in the heart, colon, prostate, and small intestine (Fig. 2). In addition, low level expression of a 4.4-kb transcript, possibly a pre-mRNA, was also observed in skeletal muscle and colon. In control studies, the Northern blot was reprobed with an actin probe, and equal loading of mRNA from each tissue was confirmed (results not shown).

In Vitro Translation of SLIM1 and SLIMMER—To confirm the molecular mass of the proteins predicted by the 1.9- and 2.1-kb cDNAs, the respective cDNAs were translated using a wheat germ expression system in the presence of [35S]methionine. Translation of the 2.1-kb SLIMMER cDNA resulted in a product that migrated as a 34-kDa polypeptide on reduced SDS-PAGE, while the 1.9-kb SLIM1 mRNA gave rise to a 32-kDa polypeptide, consistent with the respective molecular masses predicted by the cloning studies (Fig. 3).

SLIM1 and SLIMMER Protein Expression in Skeletal Muscle—To confirm the presence of the two SLIM1 protein isoforms in skeletal muscle, skeletal muscle lysates were immunoblotted with affinity-purified antipeptide antibody against either amino acids 261–272, specific to LIM4 of SLIM1 (anti-SLIM1), or amino acids 231–246, unique to SLIMMER (anti-SLIMMER) (Fig. 1A). Western blot analysis of human skeletal muscle, using the anti-SLIMMER antibody demonstrated a 34-kDa polypeptide, while the anti-SLIM1 antibody detected a 32-kDa polypeptide, consistent with their respective predicted molecular mass (Fig. 4).

Intracellular Localization of SLIM1 and SLIMMER in Differentiating Muscle Cell Lines—Given the high expression of both SLIM1 and SLIMMER in skeletal muscle upon Northern analysis, we investigated the intracellular distribution of SLIM1 and SLIMMER in the Sol8 skeletal muscle cell line, in both undifferentiated myoblasts and differentiated myotubes. Indirect immunofluorescence was performed using preimmune, affinity-purified anti-SLIM1, or anti-SLIMMER antisemur. In myoblasts, the anti-SLIM1 antibody detected diffuse cytoplasmic staining with low level nuclear expression (Fig. 5A). In multinucleate differentiated myotubes, SLIM1 was expressed exclusively in the cytoplasm (Fig. 5B). The anti-SLIMMER antibody detected prominent nuclear staining in myoblasts with faint cytoplasmic staining (Fig. 5C). The nuclear staining of SLIMMER in myoblasts was consistently much more prominent, and the cytoplasmic expression was much less than that observed using the SLIM1-specific antibody. In differentiated myotubes, SLIMMER was no longer detected in the nucleus but was localized exclusively in the cytosol (Fig. 5D). Upon serum deprivation-induced differentiation of myoblasts, two populations of cells, both myoblasts and myotubes, are
tubes (antibody (in vitro with affinity-purified anti-SLIM1 antibody (tubes (with either affinity-purified anti-SLIMMER, or anti-SLIM1 antibodies. This immunoblot is representative of three similar experiments.

Human quadriceps skeletal muscle was isolated, and skeletal muscle. Indirect immunofluorescence of Sol8 myoblasts (was grown as undifferentiated myoblasts, or differentiated into myotubes. The Sol8 skeletal muscle cell line in Sol8 myoblasts and myotubes. The Sol8 skeletal muscle cell line indicated on the visualized by fluorography. Migration of molecular weight markers is indicated on the left.

FIG. 3. In vitro translation of SLIM1 and SLIMMER. Expression of the human SLIMMER cDNA and SLIM1 cDNA translated in vitro. 35S-Labeled proteins were separated by reduced 12.5% SDS-PAGE and visualized by fluorography. Migration of molecular weight markers is indicated on the left.

anti-SLIMMER anti-SLIM1

kDa 78 - 78 -
48 - 48 -
34 - 34 -
28 - 28 -

Sk muscle Sk muscle

FIG. 4. Western immunoblotting of SLIM1 and SLIMMER in skeletal muscle. Human quadriceps skeletal muscle was isolated, and tissue lysates were analyzed by 12.5% SDS-PAGE and immunoblotted with either affinity-purified anti-SLIMMER, or anti-SLIM1 antibodies. This immunoblot is representative of three similar experiments.

FIG. 5. Indirect immunofluorescence of SLIM1 and SLIMMER in Sol8 myoblasts and myotubes. The Sol8 skeletal muscle cell line was grown as undifferentiated myoblasts, or differentiated into myotubes. Indirect immunofluorescence of Sol8 myoblasts (A and C), myotubes (B and D), or both myoblasts and myotubes (E) was performed with affinity-purified anti-SLIM1 antibody (A and B) or anti-SLIMMER antibody (C, D, and E). Preimmune serum showed no staining in myotubes (F) and myoblasts (not shown). Bars, 20 μm.

observed (40). In this dual population, SLIMMER was observed in the nucleus of myoblasts and cytoplasm of myotubes (5E). The preimmune serum showed no specific staining (Fig. 5F). Collectively, these studies implicate dual roles for SLIMMER in the nucleus of undifferentiated myoblasts and the cytoplasm of differentiated myotubes and indicate that SLIMMER may translocate from the nucleus to the cytoplasm.

The distinct intracellular localization of SLIM1 and SLIMM-
observed in myoblasts and myotubes. Although in muscle cell lines, localization to focal adhesions was not demonstrated, this may relate to the intense cytoplasmic staining, thereby masking the cytoskeleton.

GFP-SLIMMER was localized predominantly to the nucleus with low level cytoplasmic expression in the majority (76%) of transfected cells (Figs. 7B and 9A). This pattern of expression was observed in both high and low expressing cells. In addition, 13% of cells showed exclusive nuclear localization, and 12% demonstrated cytoplasmic expression alone (Fig. 7B). The intracellular distribution of recombinant GFP-SLIMMER correlates with the expression of SLIMMER observed in myoblasts. GFP-SLIM1-(spl), which contains the translated sequence common to SLIM1 and SLIMMER, up to the proposed splice site (Fig. 1, A and C), demonstrated a much lower level of nuclear expression (8% exclusively nuclear, 27% predominantly nuclear, and 65% cytoplasmic), than GFP-SLIMMER (Fig. 7B). This suggests that a NLS resides in the amino acid sequence unique to SLIMMER. We identified three possible overlapping bipartite NLS within the SLIMMER amino acid sequence, not present in SLIM1 (Fig. 1C). To evaluate the relative contribution of these proposed NLS, a series of recombinant proteins containing one, two, or three putative NLS were generated (Fig. 1A). GFP-SLIMMER-(NL1+) , which includes the first complete bipartite NLS, demonstrated a significantly greater level of nuclear expression (49% exclusively nuclear and 35% predominantly nuclear) than GFP-SLIM1-(spl) (p = 0.01) (Figs. 7B and 9B). These two recombinant proteins differ only by the presence of lysine 231 and arginine 232, which complete the first bipartite NLS in GFP-SLIMMER-(NL1+) (Fig. 1C). The addition of the second and third putative NLS (NLS2 and NLS3) to create recombinant proteins GFP-SLIMMER-(NL1,2+) or GFP-SLIMMER-(NL1,2,3+), respectively, did not further increase nuclear localization. Therefore, the first bipartite nuclear localization signal appears to be responsible for the higher nuclear and lower cytoplasmic expression of GFP-SLIMMER as compared with GFP-SLIM1.

GFP-SLIMMER-(NL1+), -(NL1,2+), and -(NL1,2,3+), which lack the C-terminal 69 amino acids, demonstrated a significant increase in the number of cells with exclusive nuclear expression, compared with the expression of GFP-SLIMMER (52 versus 13%) (Figs. 7B and 9B). A potential 11-amino acid leucine-rich NES was identified immediately following the bipartite NLS (Fig. 1C). The addition of this putative NES to create GFP-SLIMMER-(NL1,2,3,NE+) significantly decreased the level of exclusively nuclear expression (from 52 to 31%) (p = 0.04) and increased the proportion of cells with predominant

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FIG. 7. Expression of GFP-SLIM1 and GFP-SLIMMER. A, GFP-SLIM1 or GFP-SLIMMER, as indicated, were transiently transfected into COS-7 cells, and cell lysates were prepared and analyzed by 12.5% SDS-PAGE. Immunoblots were performed using polyclonal antibodies to green fluorescent protein. B, GFP-SLIM1, GFP-SLIMMER, and GFP fusion proteins of the constructs shown in Fig. 1A were transfected into COS-7 cells, and the cells were categorized without knowledge of the identity of the construct, according to the relative proportion of cytoplasmic versus nuclear expression. The expression patterns were categorized as follows: exclusively nuclear (nuclear, black box), predominantly nuclear but with cytoplasmic expression detected (nuclear > cyto, hatched box), and cytoplasmic (cyto, gray box). The total number of cells counted for each construct is indicated by n. The percentage of transfected cells localized to the indicated subcellular locations is shown in the bar graph (top) and in the table (bottom) and represents the mean of at least three separate transfections for each construct. The S.D. is indicated by the error bar.

FIG. 8. Intracellular localization of GFP-SLIM1. GFP-SLIM1 was transfected into COS-7 cells and visualized by confocal microscopy in A, B, and D. Cells were counterstained with antibodies to paxillin (C) or phalloidin (E). Bars, 20 μm.
nuclear expression and low level cytoplasmic expression (from 33 to 61%) (p = 0.03) (Figs. 7B and 9C). However, the addition of the potential NES did not result in exclusive cytoplasmic expression, since the NLS is still present. Collectively, these studies indicate that the leucine-rich motif functions as a nuclear export signal to translocate a proportion of SLIMMER from the nucleus to the cytosol.

The final C-terminal 27 amino acids present in SLIMMER are identical to 27 amino acids found in the C terminus of the murine LIM protein KyoT2 (Fig. 1, A and B). This domain mediates binding to the transcription factor RBP-J (27). We investigated the role these 27 amino acids play in regulating the subcellular location of SLIMMER by transiently expressing GFP-SLIMMER-(RBP-J minus), which lacks the 27 C-terminal amino acids common to SLIMMER and KyoT2 (Fig. 1A). GFP-SLIMMER-(RBP-J minus) had an almost identical distribution to full-length GFP-SLIMMER, indicating that the 27 C-terminal residues that mediate binding to RBP-J are not critical in determining the nuclear localization of SLIMMER (Fig. 7B). However, GFP-SLIMMER-(RBP-J minus) demonstrated a decrease in the number of transfected cells with exclusively nuclear (16 versus 31%) expression and a larger proportion of cells with predominantly nuclear and faint cytoplasmic expression (74 versus 61%), compared with SLIMMER-(NL1,2,3,NE+). This suggests that amino acids 266–296 may also contribute to nuclear export.

Function of Nuclear Localization and Export Sequences in Sol8 Myoblasts and Myotubes—To determine the role of the NLS and NES identified in SLIMMER, in its localization in the nucleus of myoblasts and cytoplasm of myotubes, C-terminal

Two Isoforms of Skeletal Muscle LIM Protein 1

DISCUSSION

We have presented the cloning and characterization of a novel isoform of the skeletal muscle LIM protein SLIM1, designated SLIMMER, which, unlike SLIM1, also contains novel sequences for nuclear import, nuclear export, and transcription factor binding. We have shown isoform-specific differences in the intracellular localization of SLIM1 and SLIMMER. SLIM1 associates with focal adhesions, whereas SLIMMER localizes to the nucleus in undifferentiated cells and the cytoplasm of differentiated myotubes. We have identified functional nuclear import and export sequences in SLIMMER, which may mediate nuclear-cytoplasmic transport.

Several lines of evidence support the contention that SLIMMER is a genuine isoform of SLIM1, not simply a cloning artifact. First, a search of expressed sequence tag data bases revealed the presence of a partial cDNA isolated from a human brain cDNA library that encoded the SLIMMER insert sequence and adjacent 3-prime SLIM1 sequence (expressed sequence tag yg79 h09.r1, GenBank accession number R52401). Second, anti-peptide antibodies derived from the novel amino acid sequence in SLIMMER immunooblotted a 34-kDa polypeptide, consistent with its predicted molecular weight, in skeletal muscle. Finally, the recent identification of...
another murine variant of SLIM1, KyoT2, is further evidence of differentially spliced isoforms of SLIM1 (27). Taken together, these data strongly suggest that the SLIM1 and SLIMMER cDNAs represent functionally significant alternatively spliced mRNA isoforms.

SLIM1 and SLIMMER are both abundant in skeletal muscle (25–27). We have shown that, in skeletal muscle-derived Sol8 myocytes, SLIM1 expression is predominantly cytosolic, using indirect immunofluorescence of myoblasts and myotubes, a finding confirmed by expression of recombinant HA-SLIM1. In addition, we have shown that GFP-SLIM1 is associated with focal adhesions and the actin cytoskeleton in COS-7 cells. We were unable to demonstrate SLIM1 in focal adhesions or along actin filaments using indirect immunofluorescence in Sol8 cells; however, the cytosolic staining under these circumstances was so intense that such localization was impossible. The LIM proteins paxillin and yixin also localize to focal adhesions. The association of paxillin with focal adhesions is mediated via its second and third LIM domains, which bind and are phosphorylated by serine threonine kinases, to increase paxillin’s association with focal adhesions (29). The SLIM1 binding partners in focal adhesions have yet to be delineated and are the subject of ongoing studies in the laboratory.

The results of this study suggest that SLIMMER may shuttle between the nucleus and cytoplasm. Firstly, we demonstrated that the addition of the first proposed NLS to a SLIM1 construct, which was expressed in the cytoplasm, significantly increased the level of nuclear expression. Two other overlapping NLS were identified in SLIMMER, but not SLIM1; however, these sequences did not further enhance nuclear expression. Second, the addition of the proposed leucine-rich NES to a mutant SLIMMER expressed predominantly in the nucleus resulted in decreased exclusive nuclear expression and an increased number of transfected cells, demonstrating dual expression in the nucleus and cytosol. Third, a variation in the level of nuclear versus cytoplasmic expression was consistently observed between transfected cells. Finally, the results of indirect immunofluorescence in Sol8 muscle cells indicate that SLIMMER localization in the nucleus and cytoplasm correlates with the differentiation status of the cell. The LIM-only protein MLP also has a differentiation-dependent intracellular location in muscle. MLP is exclusively nuclear in myoblasts but becomes progressively localized to the cytosol of differentiated myotubes (41).

There are at least two possible mechanisms that may serve to locate SLIMMER to the cytosol in myotubes: a cytoplasmic retention signal or a nuclear export mechanism by which a protein is expelled from the nucleus to the cytoplasm (39). Given that SLIMMER is highly expressed in the nucleus of myoblasts and is exclusively cytosolic in myotubes, cell cycle or differentiation-dependent nuclear export of SLIMMER must occur. Nuclear export has been well described in such proteins as the Rev protein of human immunodeficiency virus-1 and an inhibitor of cAMP-dependent protein kinase (PKI-α) (37, 38). These proteins contain an 11-amino acid sequence of repetitive leucines that bind to specific receptors. The leucine-rich motif found in SLIMMER is consistent with the previously described motifs found in Rev and PKI, although it lacks the C-terminal fourth leucine (corresponding to leucine 81 in Rev) that is often found in this motif. However, recent studies have shown that there is considerable diversity in the allowable residues at a number of positions, including each of the leucine residues previously reported essential; in particular, a serine can replace leucine 81, as is the case in SLIMMER (Fig. 1C) (42). We have some evidence that the sequence between the leucine-rich NES and the RBP-J binding domain may also contribute to nuclear export, although this amino acid sequence does not conform to one of the three types of NES that have been described. These include the leucine-rich NES, the glycine-rich export sequence found in M9, and the 24-amino acid signal found in hnRNPK (39). However, it is noteworthy that this additional region in SLIMMER that appears to contribute to nuclear export does contain strongly hydrophobic sequence, commencing at amino acid 279, consistent with the motifs that contribute to nuclear export.

The nuclear export of SLIMMER is subject to regulation, which may be dependent on the state of differentiation of the cell. The localization of SLIMMER was different in myoblasts as compared with myotubes. In myoblasts and transformed cells such as COS-7 cells, nuclear expression predominates over cytoplasmic localization. However, in highly differentiated cells such as myotubes, SLIMMER appears to be exclusively cytoplasmic. The leucine-rich export sequence was required for the export of HA-SLIMMER from the nucleus of myoblasts to the cytoplasm of myotubes. Prior to differentiation into myotubes, myoblasts permanently withdraw from the cell cycle (43). The relocation of SLIMMER from nucleus to cytoplasm may be dependent on the proliferative state of the myoblasts, withdrawal from the cell cycle, or myotubular differentiation. It should be noted that within the Sol8 population induced to differentiate by serum deprivation, SLIMMER remained localized to the nucleus in “reserve” myoblast cells, which, although they fail to differentiate, no longer proliferate due to temporary withdrawal from the cell cycle (40). Furthermore, expression of HA-SLIMMER in differentiated myotubes also showed localization of the recombinant protein in the nucleus of reserve myoblasts and the cytosol of myotubes. Expression of GFP-SLIMMER in serum-starved COS-7 cells retained a predominantly nuclear localization, suggesting that serum starvation-induced cell cycle withdrawal is unlikely to mediate nuclear-cytoplasmic translocation (results not shown). Collectively, these studies indicate that the localization of SLIMMER in the cytoplasm may indeed require myotubular differentiation. Several recent studies have shown that nuclear export may be initiated by stress, phosphorylation, or cellular adhesion; however, the stimulus to SLIMMER export has yet to be delineated (44–46).

We propose that SLIMMER is located in the nucleus of undifferentiated cells to regulate transcription. This contention is supported by the observation that the 27 C-terminal amino acids of SLIMMER are identical to the RBP-J binding region of KyoT2. KyoT2 complexes via this 27-amino acid domain with, and thereby regulates, RBP-J, a highly conserved DNA-binding protein present in embryonic and all adult tissues (47, 48). KyoT2 represses Notch-activated transcription by competing with Notch for binding to RBP-J and by displacing RBP-J from DNA promoter sequences (27). Notch is a large transmembrane receptor, which regulates ligand-dependent neural and muscle differentiation (49, 50). The intracellular domain of Notch interacts with RBP-J and inhibits MyoD-dependent myogenic differentiation (51). Potentially, therefore, KyoT2 and SLIMMER, by inhibiting Notch-activated RBP-J-mediated transcription, may block this inhibition of myogenic differentiation and promote muscle differentiation.

SLIM1, SLIMMER, and KyoT2 differ in their number of LIM domains. The addition or removal of a single LIM domain can have a critical effect on the distribution and function of LIM proteins (9). The addition of a LIM domain may either create new binding partners or stabilize existing associations. Since SLIM1 has four LIM domains and SLIMMER has three, multiple binding partners may be located in the nucleus, cytosol, or...
cytoskeleton, enabling a complex number of interactions and a means for communication between these compartments. In addition, KyoT2 and probably SLIMMER bind RBP-J via non-LIM C-terminal residues and thereby regulate transcription. Collectively, these studies indicate that the three isoforms of SLIM1 play distinct roles to regulate muscle cell function.

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