Interaction of the N-Terminus of Chicken Skeletal Essential Light Chain 1 with F-Actin

O. A. Andreev,[#] L. D. Saraswat,[‡,⊥] S. Lowey,[‡,||] C. Slaughter,[§] and J. Borejdo*[,#]

Department of Biochemistry and Molecular Biology, University of North Texas, Fort Worth, Texas 76107, Rosenstiel Basic Med Science Research Center, Brandeis University, Waltham, Massachusetts 02254, and Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235

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ABSTRACT: Skeletal myosin has two isoforms of the essential light chain (ELC), called LC1 and LC3, which differ only in their N-terminal amino acid sequence. The LC1 has 41 additional residues containing seven pairs of Ala-Pro, which form an elongated structure, and two pairs of lysines located near the N-terminus. When myosin subfragment-1 (S1) binds to actin, these lysines may interact with the C-terminus of actin and be responsible for the isoform specific properties of myosin. Here we employ cross-linking to identify the LC1 residues that are in contact with actin. S1 was reconstituted with various LC1 mutants and reacted with the zero-length cross-linker 1-ethyl-3-[3-dimethyl-aminopropyl]-carbodiimide (EDC). Cross-linking occurred only when actin was in molar excess over S1. Wild-type LC1 could be cross-linked through the terminal α-NH_2 group, as well as via the two pairs of lysines. In a mutant ELC, where the lysines were deleted but two arginines were introduced near the N-terminus, the light chain could still be cross-linked via the terminal α-NH_2 group. When the charge was reduced in the N-terminal region while retaining the Ala-Pro rich region, the mutant could not be cross-linked. These results suggest that as long as the N-terminus contains charged residues and an Ala-Pro rich extension, the binding between LC1 and actin can occur.

Previous work suggested that the essential light chain 1 of skeletal myosin (LC1)[1] was able to interact with actin. Cross-linking studies revealed that under rigor conditions LC1, but not LC3, could be cross-linked to the C-terminus of actin (*1*, *2*); NMR studies showed that the N-terminal "difference peptide" of LC1, which is mobile in solution, became immobilized in the presence of F-actin (*3*), suggesting that this terminal peptide interacted directly with the C-terminus of actin (*4*). Involvement of the N-terminus of LC1 was further demonstrated by immunological work which showed that the ATPase activity of cardiac myofibrils (*5*) and skeletal myosin (*6*) decreased upon incubation with antibodies directed against the N-terminal part of LC1. More recent work showed that the enzymatic properties of acto-S1 (*7*, *8*), the motion of actin in an in vitro motility assay (*9*), and the speed of shortening of skeletal muscle fibers (*10*) were all modulated by LC1−actin interactions.

The binding between LC1 and actin most likely involves the positively charged N-terminus of LC1 (*2*, *10*) and the negatively charged C-terminus of actin (*1*). The N-terminal lysines of LC1 are attached to a segment containing seven pairs of Ala-Pro which are probably necessary for the lysines to reach actin. Here we asked whether the N-terminal lysine residues and the Ala-Pro segment are necessary and sufficient for bond formation, i.e., whether binding can occur without lysines and/or Ala-Pro and whether their presence is enough to ensure binding. The strategy was to prepare deletion mutants of LC1 in which 43, 29, 14, and 13 N-terminal residues were deleted, resulting in mutants containing no extension piece (Δ43), no Ala-Pro segment (Δ29), no N-terminal lysines (Δ14), and no lysines but having an additional charged amino acid appended to the Ala-Pro segment (Δ13), respectively. The mutants were hybridized into subfragment-1 (S1) and cross-linked to actin where possible with the zero-length reagent, and the amino acids involved in the formation of the cross-link were analyzed. We found that only wild type (WT) and Δ13 were cross-linked to actin, and only when actin was in molar excess over S1. The Δ13 (the same mutant used in ref *11*) cross-linked through the terminal α-NH_2 group, while WT was cross-linked either through the terminal α-NH_2 or through lysines 3, 4, 7, and 8. These results suggest that the presence of the N-terminal lysine residues is not essential and that the Ala-Pro region is necessary, but not sufficient, for the deletion mutant to form a bond with actin.

MATERIALS AND METHODS

Chemicals. 1-Ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) and *N*-chlorosuccinimide (NCS) were from

* To whom correspondence should be addressed.
[#] University of North Texas.
[‡] Brandeis University.
[⊥] Current address: L. D. Saraswat, ArQule, Inc., 303 Bear Hill Road, Waltham, MA 02154.
[||] Current address: S. Lowey, Dept of Mol Physiol & Biophysics, Univ of Vermont, Burlington, VT 05405.
[§] University of Texas Southwestern Medical Center.

[1] Abbreviations: EDC, 1-ethyl-3-[3-dimethyl-aminopropyl]-carbodiimide; ELC, essential light chain of skeletal myosin; LC1, essential light chain 1 of skeletal myosin; LC3, essential light chain 2 of skeletal myosin; WT, wild type; A, actin; S1, myosin subfragment-1; HC, heavy chain of S1; NCS, *N*-chlorosuccinimide; DNS, dansyl chloride.

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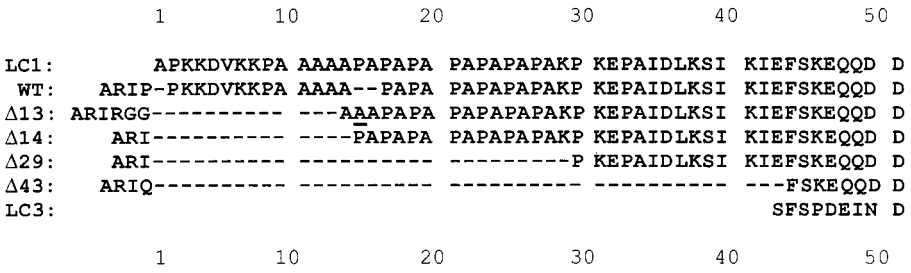

FIGURE 1: Sequences of different deletion mutants.

Sigma (St Louis, MO). Dansyl chloride (DNS) and [*N*-(5-aminopentyl)-5-(dimethylamino)-naphthalene-1-sulfonamide]-(dansyl-cadaverine) were from Molecular Probes (Eugene, OR).

Proteins. Chicken skeletal S1, S1 isoforms, and rabbit actin were prepared as described elsewhere (*12*, *5*, *13*). The concentrations of proteins were measured using the following values for the extinction coefficients. For S1, $A^{1\%}(280) = 7.5$ (using a molecular mass of 120 KDa for S1(LC1) and 111 KDa for S1(LC3)); for G-actin, $A^{1\%}(290) = 6.3$. The quality of the proteins was checked by SDS−PAGE.

Mutations. The cDNA obtained for chicken skeletal muscle LC1 was missing 45 bases from the 5′ end that encodes the first 15 residues of the light chain (*14*). Construction of wild-type LC1 from this cDNA is described in ref *15*. The deletion mutants were prepared by site-directed mutagenesis or by PCR and cloned into the EcoRI site of the expression vector pT7-7. The fusion proteins were expressed in *Escherichia coli* BL21 (DE3) and purified as described in ref *15*. The LC1s were exchanged into S1(A2), and the reconstituted S1s were separated from the starting material by ion-exchange chromatography (*15*).

Fluorescent Labeling of Actin. Modification of glutamine-41 of G-actin with dansyl-cadaverine was carried out as in ref *16*. The concentration of labeled actin was calculated by the absorbance at 290 nm after subtracting the absorbance of dansyl at this wavelength.

Cross-Linking. S1 and F-actin were mixed at different molar ratios and incubated for 1 h at room temperature with 50 mM EDC. Reactions were stopped by adding an equal volume of electrophoresis sample solution (4% SDS, 24% glycerol, 100 mM Tris, 4% mercaptoethanol, 0.02% Bromophenol Blue). Unless otherwise indicated, all cross-linking experiments were done in solutions containing 0.2 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl pH 7.5. The low concentrations of $MgCl_2$ and KCl were used to prevent formation of actin filament bundles (*17*, *18*). Light scattering measurements did not detect any bundle formation in this buffer solution.

Tricine Sodium-Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out according to Schagger and Jagow (*19*) using 8% polyacrylamide gels. After electrophoresis and staining, the slab was dried using a Novex gel dryer kit (Novex Co., San Diego, CA). Dry gels were scanned in transmission mode (transmission arm no. 01719) on a SM3+ Howtek scanner (Hudson, NH). The relative intensity of the various bands were measured by the Image Pro Plus program (Media Cybernetics, Silver Spring, MD) as described in ref *20*. A calibration of the scanner was done using Kodak stepped density filters.

Western Blots. After electrophoresis, the peptides were electroblotted onto nitrocellulose membrane (BioRad, CA). The membrane was incubated for 1 h with blocking solution, and then with the primary polyclonal antibodies to chicken muscle light chain 1 for 1 h, and finally with the horseradish peroxidase-conjugated secondary antibodies for 1 h. Luminescence was detected by X-ray films. The films were scanned as described above.

Sequencing. Protein samples containing myosin light chains covalently cross-linked to actin were subjected to SDS polyacrylamide gel electrophoresis in 10% gels, electroblotted to Immobilon Psq membranes (Millipore Corp., Bedford, MA), and stained with Coomassie Blue R-250. Two cross-linked species were resolved by electrophoresis, excised from the blot, and subjected to automated Edman degradation. Sequencing was performed on a Model 477A sequencer (Applied Biosystems Division of Perkin-Elmer, Foster City, CA) using the manufacturer's standard chemicals. The Pro-Blot program was employed, which provides extended coupling and cleavage to enhance reaction yields involving proline residues. Non-cross-linked myosin light chains from the same protein samples were excised as controls. Proteins were quantitated by visual comparison of Coomassie Blue staining intensity with known quantities of standard proteins (Bio Rad Labs, Hercules, CA).

RESULTS

Figure 1 shows the composition of various mutants in the order of decreasing molecular mass: The WT peptide is identical to native LC1 but begins with the sequence ARIP instead of alanine of native LC1, and a Pro-Ala pair is missing at positions 15 and 16. Δ13 has 13 aa missing and has a Pro to Ala change (the underlined Ala in the sequence) and a longer fusion peptide with two charged residues. Δ14 has 14 aa missing and has a shorter fusion peptide with one charged residue. Δ29 mutant is missing 29 residues from the N-terminus of LC1 and begins with the sequence ARI. Finally, Δ43 is similar to LC3 but has a fusion peptide followed by LC1 specific amino acids. The mutated light chains were exchanged into S1 and cross-linked to F-actin with EDC.

Identification of Products Indicative of Cross-Linking. Figure 2 shows the band pattern obtained when S1 isoforms are mixed with a 4× molar excess of actin and cross-linked with EDC. The results show that cross-linking of S1(LC1) and S1(WT) gives rise to the following major adducts (in order of decreasing molecular masses): 235, 210, 185, 150 + 160, and 66 KDa. Fluorescence patterns obtained using actin labeled at Gln-41 with dansyl-cadaverine (Figure 2, middle panels) and Western blots using antibodies against

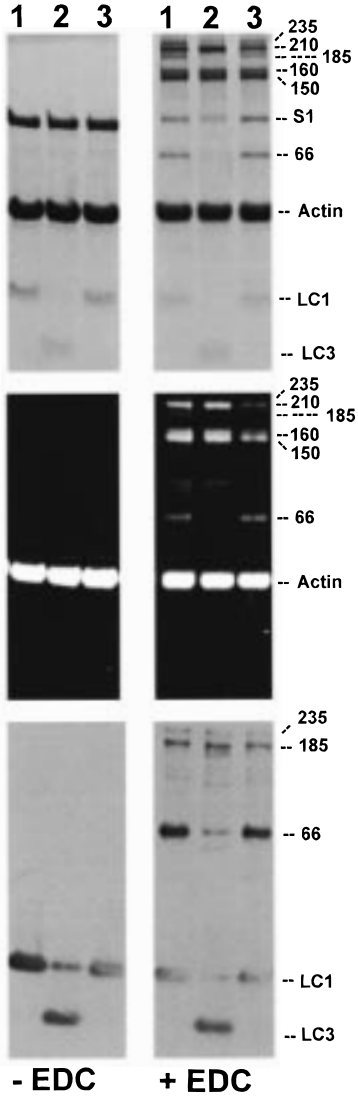

FIGURE 2: Cross-linking of S1(LC1) (lanes 1), S1(LC3) (lanes 2), and S1(WT) (lanes 3) to F-actin. Top: staining with Coomassie Blue. Middle: fluorescence of actin labeled with dansyl chloride. Excitation performed with variable intensity UV transilluminator (IBTIV-88, Fisher, PA). Bottom: Western blots using antibodies against LC1. SDS gel was identical to the one shown in Figure 2A. LC3 is stained with anti-LC1 because it is a polyclonal antibody. The reason that some 66 KDa is visible in Western blots of cross-linked S1(LC3) is that S1(LC3) is contaminated with S1-(LC1). Left column: control without EDC. Right column: after cross-linking with the EDC. Concentrations: 1 μM S1, 4 μM actin. Molecular mass of adducts is reported in kilodaltons.

LC1 (Figure 2, bottom panels) confirm our previous conclusion (*18*, *21*) that these bands correspond to the following complexes; the 235 KDa, HC_{S1} + A + A + LC1; the 210 KDa, HC_{S1} + A + A; the 185 KDa, HC_{S1} + A + LC1; the 160 KDa, HC_{S1} + A (cross-linked through a site on a 50 KDa tryptic fragment); the 150 KDa, HC_{S1} + A (cross-linked through a site on a 20 KDa tryptic fragment); the 66 KDa, A + LC1. Controls identified the 95 KDa band as the heavy chain of S1, the 43 KDa band as actin, and the 22 KDa band as LC1. Of all the complexes in which LC1 is associated with actin (the 235, 185, and 66 KDa adducts), we shall emphasize the 66 KDa complex. In agreement with earlier work (*21*) the formation of the 235, 210, 185, 160, and 66 KDa complexes were inhibited at a high molar ratio of S1/actin: neither LC1 nor WT formed a complex with actin when the molar ratio was greater than 1 (data not shown). Cross-linking of the S1(LC3) isoform at 1:4 molar ratio to actin does not lead to the production of light chain−actin complexes (Figure 2, lanes 2). This was true at any molar ratio (data not shown).

The Role of the N-Terminal Lys Residues. To determine the function of the N-terminal lysines, we engineered a mutant Δ13 which lacks Lys 3, Lys 4, Lys 7, and Lys 8 (Figure 1) and hybridized it with the heavy chain of S1. Figure 3 (lanes 3) shows the band pattern obtained when S1(Δ13) was mixed with a 4× molar excess of actin and cross-linked with EDC. The Coomassie stained gels (top panels), the fluorescence patterns obtained with dansyl-actin (middle panels), and Western blots (bottom panels) show that the S1(Δ13) gives the characteristic bands (235, 185, and 66 KDa) associated with the formation of a complex with actin (120 KDa band is a complex with the HC of S1). The formation of these complexes was inhibited at high molar ratios of S1/actin. Figure 4 (top) shows the cross-linking pattern at different molar ratios of S1/actin, and the bottom panel shows inhibition of the production of the 210 and Δ66 KDa adducts. In summary, the cross-linking pattern of the mutant and of S1(LC1) was identical, suggesting that the N-terminal lysine residues of LC1 are not essential for cross-linking.

Since Δ13 lacks the N-terminal lysines, the question arises, how does it cross-link to actin? To answer this we attempted to sequence the A-Δ13 complex (band Δ66 in the bottom of Figure 4A). We found that no residues were released when we tried to sequence this complex (data not shown), indicating either that the N-terminus was blocked by a cross-linker or that the Δ13 was cross-linked to actin through the terminal alanine. The former possibility is unlikely because it was possible to sequence Δ13 reacted with EDC. It seems therefore that Δ13 reconstituted with the heavy chain of S1 is cross-linked through the terminal alanine.

The Role of the Ala-Pro Segment. To find out whether the Ala-Pro segment is necessary for binding we examined the ability of the mutants Δ43 and Δ29 to form a cross-link with actin. Mutant light chains were reconstituted with the heavy chain of S1, mixed with a 4× molar excess of actin, and cross-linked with EDC. Figure 3 shows the band pattern obtained with S1(Δ43) (lanes 1), S1(Δ29) (lanes 2), and S1-(LC1) (lanes 4). The Coomassie stained gels (top panels), the fluorescence patterns obtained using dansyl-actin (middle panels), and Western blots (bottom panels) show that neither S1(Δ43) nor S1(Δ29) give bands (235, 185, and 66 KDa) characteristic of complexes of light chain and actin. This result suggests that the Ala-Pro segment is necessary for binding, in agreement with ref *7*.

We next determined whether the Ala-Pro segment is sufficient for binding. To this end we engineered a mutant Δ14 which is identical to Δ13 but has 14 aa missing and has a shorter fusion peptide with only one charged residue. Compared to Δ13 or WT which showed significant cross-linking at low molar ratios of S1/actin, Δ14 did not cross-link to actin (Figure 5) nor enhanced polymerization of G-actin (not shown). This was true at any molar ratio (not shown).

Amino Acid Residues of LC1 Involved in Cross-Linking. Since the presence of the N-terminal lysines is not essential

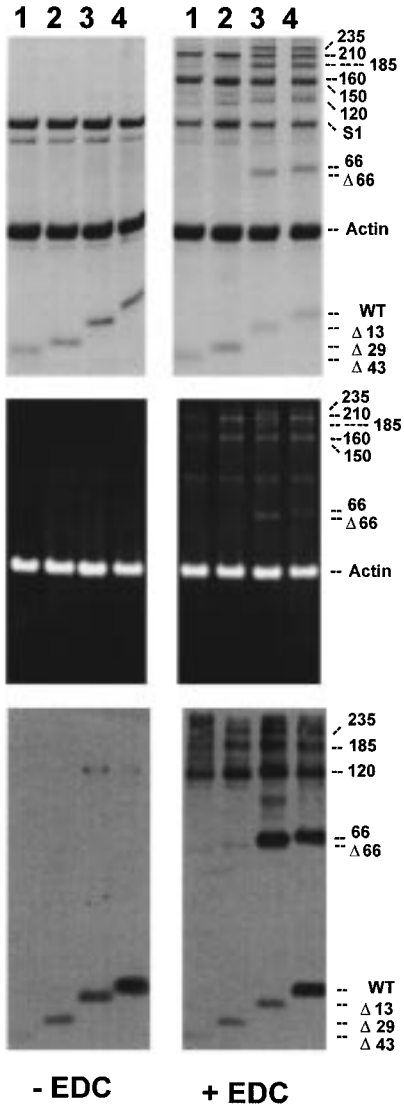

FIGURE 3: Cross-linking of S1(Δ43) (lanes 1), S1(Δ29) (lanes 2), S1(Δ13) (lanes 3), and S1(LC1) (lanes 4) to F-actin. Top: staining with Coomassie Blue. Middle: fluorescence of actin labeled with dansyl chloride. Bottom: Western blots using antibodies against LC1. SDS gel was identical to the one shown in A. Left column: control without EDC. Right column: cross-link with 50 mM EDC for 1 h at room temperature. Concentrations: 1 μM S1, 4 μM actin. Molecular mass of adducts is reported in kilodaltons.

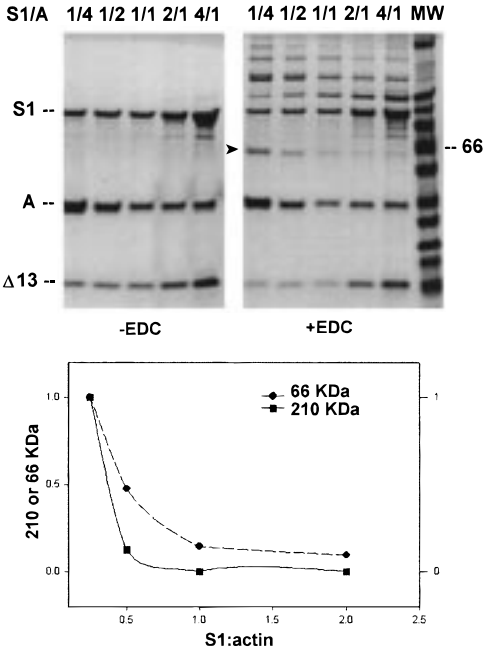

FIGURE 4: Cross-linking of S1(Δ13) to actin. Top: Cross-linking at different molar ratios (indicated at the top). Left column: control without EDC. Right column: after cross-linking with 50 mM EDC for 1 h at room temperature. Bottom: The dependence of the formation of the products of cross-linking of actin to S1(Δ13) on molar ratio. Squares, solid line: the 210 KDa product. Circles, dashed line: the Δ66 product.

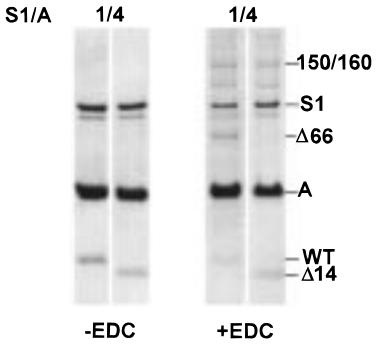

FIGURE 5: Comparison of cross-linking of A−S1(WT) (left lanes) with A−S1(Δ14) (right lanes) at the 1:4 molar ratio S1:actin.

for binding, it was of interest to find out to what extent these residues are involved in the interaction between light chain and actin. By determining the N-terminal sequence of the 66 KDa peptide formed in the A + WT complex (A + LC1 peptide could not be used because the N-terminus of native LC1 is blocked (*22*)), we took advantage of the fact that the N-terminus of actin is blocked. Automated Edman degradation was performed on five samples of the species of lower apparent molecular weight (66 KDa peptide migrated as a doublet in SDS−PAGE) and on two samples of the species of higher apparent molecular weight. Each sample contained 50−75 pmol of protein, estimated on the basis of the intensity of its staining with Coomassie Blue. The results are the averages of both sets of samples. They were compared quantitatively with a single control sample of approximately 400 pmol of non-cross-linked myosin light chain as a control. The absolute values for the cross-linked sequences have been normalized on the basis of their yields of Ile-3 to give values that can be compared directly to the un-cross-linked sample. The picomole yields were estimated by comparing the peak area of the PTH-(PTC)-lysine derived from each cycle of Edman degradation with a known quantity of standard PTH-(PTC)-lysine. The data were additionally corrected by applying the standard background subtraction and lag correction routines that are supplied by Applied Biosystems for analysis of sequence data. The results are shown in Figure 6. As expected, the cross-linked protein showed the myosin light chain sequence only, because the actin to which it was cross-linked is N-terminally blocked. The yield of amino acid residues was lower than that from comparable picomolar quantities of the non-cross-linked myosin light chain control, estimated on the basis of staining intensity with Coomassie Blue after electrophoresis. The average yield of Ile-3 from the non-cross-linked myosin light chain was estimated to be 0.35 pmol per pmol of protein, whereas the average initial yield of the cross-linked species was 0.20 pmol per pmol of protein. The difference may be further amplified by the ability of actin to take up Coomassie Blue 1.8 ± 0.4 better

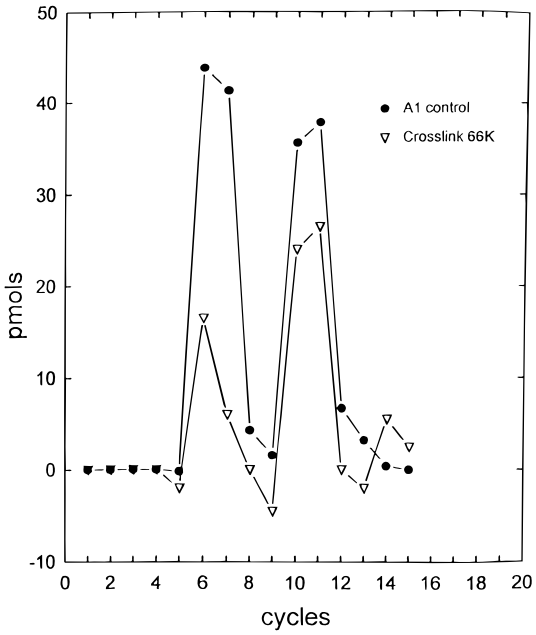

FIGURE 6: Amino acid sequencing of 66 KDa cross-link product. Open triangles: the yield of lysine residues in the first 15 cycles of automated Edman degradation of the 66 KDa cross-linked species of the actin−WT complex. Closed circles: data acquired from a control sample of a free wild type light chain derived from the same protein mixture. Picomole values for the cross-linked sequences have been normalized on the basis of their yields of Ile-3 to give values that can be compared directly to the control sample.

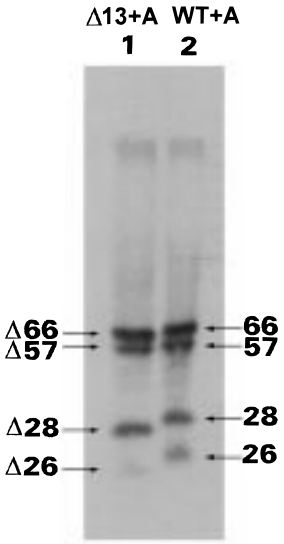

FIGURE 7: Western blot of cross-linking of Δ13 + A and WT + A. Antibodies against LC1 were used. Lane 1: pattern of digestion of Δ66 KDa band. Lane 2: digestion of 66 KDa band. Bands were cut out and digested with 20 mM NCS for 30 min at room temperature. Δ66, Δ57, Δ28, and Δ26 indicate complexes involving Δ13 mutant. Molecular mass of digestion products are reported in kilodaltons.

than LC1. This result suggests that some cross-linking occurred through the N-terminal α-amino group of WT.

In addition, the data provide evidence for the differences between the yields of lysines at any of the four positions compared to the yields in the non-cross-linked control. If the probability of cross-linking LC1 to actin through each lysine was equal, we would expect equal drop in yield of each peak. Figure 6 shows a more prominent drop in yield from the first two lysines. This suggests that cross-linking is more likely to occur through Lys 6 and 7 (Lys 3 and 4 in a native LC1) than through Lys 10 and 11 (Lys 7 and 8 in LC1).

In summary, the data suggest that the cross-linking occurred through the N-terminal α-amino group and, to varying extents, through lysines, in agreement with earlier studies (*2*, *10*, *23*).

Amino Acid Residues on Actin Involved in the Cross-Linking. To establish which residues on actin are involved in the formation of the cross-link, we digested the tryptophanyl peptide bonds of actin according to Lischwe and Ochs (*24*). Figure 7 is a Western blot of A + Δ13 and A + WT complexes after NCS cleavage. NCS cleaves at Trp 79, Trp 86, Trp 340, and Trp 356, yielding peptides (starting at the N-terminus) with molecular masses of 9, 29, 3.2, and 2.2 KDa. If LC1 was cross-linked to the fourth peptide, bands with molecular masses of 66, 57, 28, and 26 KDa would have been produced, consistent with the patterns observed with WT and Δ13. This result agrees with Sutoh (ref *1*) who suggested that Glu 361, Asp 363, and/or Glu 364 are involved in the EDC cross-link between LC1 and actin.

DISCUSSION

The contact between the N-terminal alanine of Δ13 mutant hybridized into the heavy chain of S1 could only be made when actin was in molar excess over S1 (Figure 4). The same is true for intact LC1 (*21*). The simplest explanation of this observation is that when the decoration of F-actin with S1 is incomplete, i.e., actin filament includes regions containing at least two consecutive unoccupied actin monomers, S1 binds to such regions in a slightly different orientation than to saturated filament (*18*, *25*). Alternatively, when filaments are completely saturated by S1, the C-terminus of actin may be blocked by an adjacent myosin head.

A surprising observation was that Δ13 hybrydized with the heavy chain of S1 was able to form a cross-link with actin although it lacks all 4 N-terminal lysines which are thought to be involved in contact formation (*2*, *10*). The present work suggests that the presence or the correct positioning of the lysines on the difference peptide relative to the acidic residues on the surface of actin is not necessary for the functional properties of the light chain. The Δ13 which is bound to actin through the N-terminal alanine retains most of the properties of intact LC1: S1 carrying Δ13 instead of LC1 is still able to slow in vitro motion of actin filaments and polymerize G-actin at a rate only slightly slower than LC1 (*11*). It is interesting to note that Δ13 forms just as good as or a better complex with actin than LC1 (compare the relative intensities of the 66 KDa band in Figure 2 with the Δ66 KDa band in Figure 3), although LC1 contacts actin through four residues whereas Δ13 binds it through only one residue.

Δ14, in contrast to Δ13, was unable to form a cross-link or enhance polymerization of G-actin. The difference in sequence between Δ13 and Δ14 is a minor one: Δ13 has three more amino acids and, most importantly, has a basic residue near the N-terminus. It is likely that the presence of the charged residue near the N-terminus is responsible for this difference. This is consistent with Timson et al. (*8*) who engineered a mutant devoid of all positive charges near the N-terminus and reported lack of cross-linking. Thus, the

presence of the specific lysine residues near the N-terminus is not essential for cross-linking.

The distance between LC1 and the surface of actin filament is about 8 nm (*26*, *27*). This work suggests that the Ala-Pro segment is necessary, but not sufficient by itself, to span this distance. Thus, removing the Ala-Pro segment abolished the formation of the contact altogether (Figure 3). Its presence alone, however, was not sufficient to ensure the contact. The fact that Ala-Pro repeat often plays a structural role (*28*, *29*) and that the formation of a contact required the addition of only a single basic residue suggests that the contact is not stereospecific, i.e., that it is made as long as there is an extension arm and a basic residue at the N-terminus.

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