Transglutaminase-Induced Cross-Linking between Subdomain 2 of G-Actin and the 636–642 Lysine-Rich Loop of Myosin Subfragment 1

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ABSTRACT G-actin was covalently cross-linked with S1 in a bacterial transglutaminase-catalyzed reaction. The cross-linking sites were identified with the help of fluorescent probes and limited proteolysis as the Gln-41 on the DNase I binding loop of subdomain 2 in G-actin and a lysine-rich loop (residues 636–642) on the S1 heavy chain. The same lysine-rich loop was cross-linked to another region of G-actin in a former study (Combeau, C., D. Didry, and M-F. Carlier. 1992. *J. Biol. Chem.* 267:14038–14046). This indicates the existence of more than one G-actin-S1 complex. In contrast to G-actin, no cross-linking was induced between F-actin and S1 by the transglutaminase reaction. This shows that in F-actin the inner part of the DNase I binding loop, where Gln-41 is located, is not accessible for S1. The cross-linked G-actin-S1 polymerized upon addition of 2 mM $MgCl_2$ as indicated by electron microscopy and sedimentation experiments. The filaments obtained from the polymerization of cross-linked actin and S1 were much shorter than the control actin filaments. The ATPase activity of the cross-linked S1 was not activated by actin, whereas the K^+(EDTA)-activated ATPase activity of S1 was unaffected by the cross-linking. The cross-linking between G-actin and S1 was not influenced by the exchange of the tightly bound calcium to magnesium; however, it was inhibited by the exchange of the actin-bound ATP to ADP. This finding supports the view that the structure of the DNase binding loop in ADP-G-actin is somewhere between the structures of ATP-G-actin and F-actin.

INTRODUCTION

The ATP hydrolysis-coupled interaction of myosin with actin filaments (F-actin) during the cross-bridge cycle is the molecular basis of muscle contraction and has an essential role in the motility of eukaryotic cells. The myosin heads, called S1, interact with actin and are responsible for the ATPase activity. Actin filaments also have an active role in the cross-bridge cycle, and they are not just passive partners of the myosin motor. The description of the myosin-actin interface during the various stages of the cross-bridge cycle has an enormous importance for the understanding of the motile process. S1 also interacts with actin monomers (G-actin), which leads to actin polymerization and can be functionally significant in nonmuscle cells where actin polymerization can cause cell shape change and intracellular movement. It was shown by ethyl dimethylamino propyl carbodiimide cross-linking that G-actin binds to S1 by an electrostatic interaction that takes place between the 636–642 loop on S1 and the negatively charged residues on the actin N terminus (Combeau et al., 1992; Lheureux and Chaussepied, 1995). On the basis of the known atomic structures of G-actin (Kabsch et al., 1990), S1 (Rayment et al., 1993b), and the molecular model of F-actin (Lorenz et al., 1993), attempts were made to assign the interacting F-actin and S1 surfaces (Rayment et al., 1993a) by taking into account also the results of biochemical studies including cross-linking (Mornet et al., 1981; Sutoh, 1982; Sutoh, 1983). By a combination of various methods it was concluded that the lysine-rich 636–642 loop in S1 interacts electrostatically with the acidic N terminus of actin, whereas other stretches of residues on the 50-kDa domain of S1 are in hydrophobic interaction with several residues on the subdomain 1 of F-actin. The atomic model of the F-actin-myosin complex implicated also the His-40-Gly-42 site on the DNase I binding loop of subdomain 2 of actin as part of the F-actin-myosin interface (Rayment et al., 1993a). This assumption was indirectly supported by cross-linking studies between actin and myosin subfragment 1 involving Lys-50 on the DNase I binding loop. Bertrand et al. (1994) cross-linked Lys-50 in G- and F-actin to S1 with maleimidobenzoic acid-*N*-hydroxysuccinimide ester, whereas Bonafe et al. (1994) cross-linked the same lysyl residue in F-actin by glutaraldehyde to the 48-kDa central fragment of S1. On the basis of these results it was suggested that the DNase I binding loop is located on the myosin-actin interface in both the monomer and polymer forms of actin.

The subdomain 2 of actin, where the DNase I binding loop (residues 38–52) is located, is the most mobile region of actin (Strzelecka-Golaszewska et al., 1993; Muhlrad et al., 1994). According to the atomic model of F-actin, the DNase I binding loop is involved in actin-actin contacts (Holmes et al., 1990; Schutt et al., 1993; Lorenz et al., 1993). This assumption is consistent with the inhibition of actin polymerization by chemical modification of His-40 (Hegyi et al., 1974) and by the proteolytic cleavage of the loop with subtilisin (Schwyter et al., 1989) and *Escherichia coli* A2 strain protease (Khaitlina et al., 1993). It was shown

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by biochemical studies (Muhlrad et al., 1994) and three-dimensional reconstruction of electron microscopic images of the actin filaments (Orlova and Egelman, 1992; Orlova et al., 1995) that the orientation of subdomain 2 depends on the nature of the filament-bound nucleotides and divalent cations.

In the light of the functional importance of subdomain 2 both in G- and F-actin, it was of interest to study the possible involvement of the His-40-Gly-42 site on the DNase I binding loop in the binding of the myosin head by direct cross-linking. We took advantage of the finding that transglutaminase reacts specifically with Gln-41 at this site (Takashi, 1988; Hegyi et al., 1992).

It is well known that transglutaminase catalyzes the formation of an isopeptide bond in the reaction, R-$CONH_2$ + R′-NH_2 → R-CONH-R′ + NH_3, in which R-$CONH_2$, the acceptor, is a protein-bound glutamine, and R′-NH_2, the donor, is an alkylamine (Folk and Chung, 1985). The alkylamine can be a lysyl residue of a protein and in this case the enzyme catalyzes the formation of a zero-length inter- or intramolecular cross-link. Several eukaryotic transglutaminases were described (Greenberg et al., 1991). The activity of all is absolutely dependent on calcium with optimal activity at ~5 mM Ca. This introduces limitations for the use of eukaryotic transglutaminases in the study of monomeric G-actin because actin is polymerized by millimolar concentration of calcium. Recently, a bacterial transglutaminase was described (Ando et al., 1989) which is active in the absence of divalent cations and therefore is more suitable for work with G-actin. As this transglutaminase has been shown to use Gln-41 as a sole acceptor on actin (Kim et al., 1995), we attempted the cross-linking of both G- and F-actins to S1 with the help of this enzyme. We observed cross-linking between Gln-41 on G-actin and the lysine-rich 636–642 loop on the S1 heavy chain, whereas no cross-linking occurred between F-actin and S1 in the presence of bacterial transglutaminase. The extent of cross-linking between G-actin and S1 was affected by the nature of the actin-bound nucleotide.

MATERIALS AND METHODS

Chemicals

ATP, dithioerythritol (DTE), chymotrypsin, trypsin, *Staphylococcus aureus* strain V8 protease, soybean trypsin inhibitor, phenylmethanesulfonyl fluoride, and *N*-(iodoacetyl)*N*′-(5-sulfo-1-naphtyl)ethylenediamine (IAEDANS) were from Sigma Chemical Company (St. Louis, MO). Dansyl ethylenediamine (DED) was purchased from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade.

Proteins

Myosin and actin were prepared from back and leg muscles of rabbit by the methods of Tonomura et al. (1966) and Spudich and Watt (1971), respectively. S1 was obtained by digestion of myosin filaments with chymotrypsin at a 300:1 (by mass) ratio (Weeds and Taylor, 1975). The digestion was stopped using 0.1 mM phenylmethanesulfonyl fluoride. S1 isoforms, S1(A1) and S1(A2), were separated on a Fast Flow S-Sepharose cation exchange column. Bacterial transglutaminase was obtained and purified as previously described (Ando et al., 1989). Protein concentrations were obtained by absorbance, using an A (1%) at 280 nm of 7.5 for S1 and an A (1%) at 290 nm of 6.3 for actin. Molecular masses were assumed to be 115 and 42 kDa for S1 and actin, respectively.

Preparation of various forms of actin

Actin was stored as CaATP-G-actin in G-actin buffer (0.2 mM $CaCl_2$, 0.1 mM ATP, and 2 mM Tris-HCl, pH 7.6) plus 0.5 mM β-mercaptoethanol. Before use, β-mercaptoethanol was removed by dialysis against G-actin buffer. This actin was used in the experiments if not stated otherwise. F-actin was prepared from CaATP-G-actin by polymerization with 0.2 mM EGTA and 2 mM $MgCl_2$. MgATP- and MgADP-G-actin were prepared essentially according to the procedure of Drewes and Faulstich (1991) as described by Muhlrad et al. (1994).

Cross-linking reaction

For cross-linking, 8–12 μM G-actin were routinely incubated with 8 μM S1(A2) and 0.1–0.2 unit/ml bacterial transglutaminase in G-actin buffer at 0°C for 30–60 min. The reaction was terminated by addition of 2% sodium dodecyl sulfate (SDS) containing buffer and incubation in a boiling water bath for 3 min. The products of the reaction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In most of the experiments, IAEDANS-labeled S1 or actin was used.

Chemical modifications

Labeling of Cys-707(SH_1) thiol of S1 by IAEDANS

S1 was labeled with the fluorescent thiol reagent IAEDANS, which specifically reacts with the SH_1 thiol on the 20-kDa C-terminal fragment of S1 according to Takashi (1979).This was done by incubating 10 μM S1 in 100 mM NaCl and 30 mM Tris-HCl, pH 7.4, with 90 μM IAEDANS on ice for 4 h. The reaction was terminated by addition of 1 mM DTE. Finally, S1 was dialyzed against G-actin buffer at 4°C overnight.

Labeling of actin Cys-374 by IAEDANS

G-actin (60 μM) in G-actin buffer was incubated with 100 μM IAEDANS on ice overnight. The reaction was terminated by addition of 1 mM DTE. Excess of DTE was removed by dialysis against G-actin buffer overnight at 4°C.

Labeling actin Gln-41 by DED

This was done by the method of Kim et al. (1995). Briefly, G-actin (50 μM) in G-actin buffer was incubated with 100 μM DED and 0.5 unit/ml bacterial transglutaminase in G-actin buffer. The reaction was carried out for 2 h at 25°C and was stopped by removing excess DED on a Sephadex spin column equilibrated with G-actin buffer.

Limited proteolysis

Tryptic digestion of S1

IAEDANS-S1 (40 μM) in G-actin buffer was digested by trypsin with 50:1 S1 to trypsin w/w ratio at 25°C for 25 min. The reaction was stopped by addition of 1 μM phenylmethanesulfonyl fluoride, and the incubation at 25°C was continued for 20 min.

Digestion of S1 by V8 protease

IAEDANS-S1 (40 μM) in G-actin buffer was digested by V8 protease with 30:1 S1 to V8 protease w/w ratio at 25°C for 1 h. The digest was immediately added to the cross-linking mixture containing G-actin and transglutaminase.

Sedimentation

The polymerization of the cross-linked G-actin-S1 was studied by sedimentation. G-actin (12 μM) in G-actin buffer was incubated with 8 μM S1 and 0.1 unit/ml bacterial transglutaminase in G-buffer at 0°C for 60 min. This was immediately followed by addition of 2 mM $MgCl_2$ and incubation at 25°C for 30 min to polymerize actin. The polymerized mixture was sedimented at 75,000 rpm for 45 min in a Beckman TL-100 ultracentrifuge, and the resulting pellet and supernatant were analyzed by SDS-PAGE.

Electron microscopy

S1 and G-actin were cross-linked and then polymerized as described above. Phalloidin (12 μM) was added to the samples to stabilize filaments and decrease the critical polymerization concentration. The samples were diluted with F-actin buffer (2 mM $MgCl_2$, 2 mM Tris-HCl, pH 7.6) to a final concentration of 2.3 μM immediately before mounting on the electron microscope grid. Grids were prepared as described previously (Miller et al., 1988) using 1% aqueous uranyl acetate as the negative stain.

Grids were examined with a Hitachi H-7000 electron microscope operating at 75 kV with a condenser aperture of 200 μm and an objective aperture of 50 μm. A liquid nitrogen-cooled cold finger (anti-contamination device) was used throughout. Micrographs were taken at an apparent microscope magnification of 30,000. Magnification was calibrated using a diffraction grating replica (54,800 lines/inch).

SDS-PAGE

SDS gel electrophoresis was carried out according to Mornet et al. (1981). It was performed either on 7–18% polyacrylamide gradient or 10% linear slab gels. Molecular masses of the bands were estimated by comparing the electrophoretic mobility of the bands with that of markers of known molecular masses.

ATPase assays

Actin- and K^+(EDTA)-activated ATPase activities (micromoles of phosphate/micromoles of S1/sec) were calculated from the inorganic phosphate produced and measured according to Fiske and Subbarow (1925). The reaction was performed at 22°C on 1-ml aliquots taken at various time intervals. Incubation times were chosen so that no more than 15% of the ATP was hydrolyzed. For actin-activated ATPase, the assay contained cross-linked and polymerized 0.1 μM S1 and 0.15 μM actin (or an uncross-linked control) to which uncross-linked 5 μM F-actin, 2 mM $MgCl_2$, 20 mM HEPES buffer (pH 7.4), and finally 2 mM ATP was added. For K^+-(EDTA)-activated ATPase, the reaction mixture contained 0.1 μM S1, 0.15 μM F-actin (with and without cross-linking as in the actin-activated ATPase), 2 mM ATP, 6 mM EDTA, 600 mM KCl, and 50 mM Tris-HCl buffer, pH 8.0.

RESULTS

Cross-linking of G- and F-actin to S1

In order to reveal cross-link formation, S1(A2) labeled with the fluorescent thiol reagent IAEDANS was incubated at 25°C or 0°C in the presence of bacterial transglutaminase with CaATP-G-actin. At various time intervals, aliquots were removed and analyzed by SDS-PAGE (Fig. 1). The gels were first photographed under UV light to reveal the fluorescent bands and then stained with Coomassie Blue. A new fluorescent band with the apparent molecular mass of 180 kDa appeared on the gel after even a short 5 min incubation with transglutaminase, both at 0 and 25°C. We assume that this band is a cross-linked product of G-actin and S1 heavy chain because it appeared only upon incubation of the mixture of S1 and G-actin with transglutaminase and not when the enzyme was incubated with S1 or G-actin only. In addition to the actin-S1 cross-linked product, higher molecular weight species also appear on Fig. 1. This species is relatively minor when cross-linking is carried out at 0°C, and it is more prominent relative to the 180-kDa band at

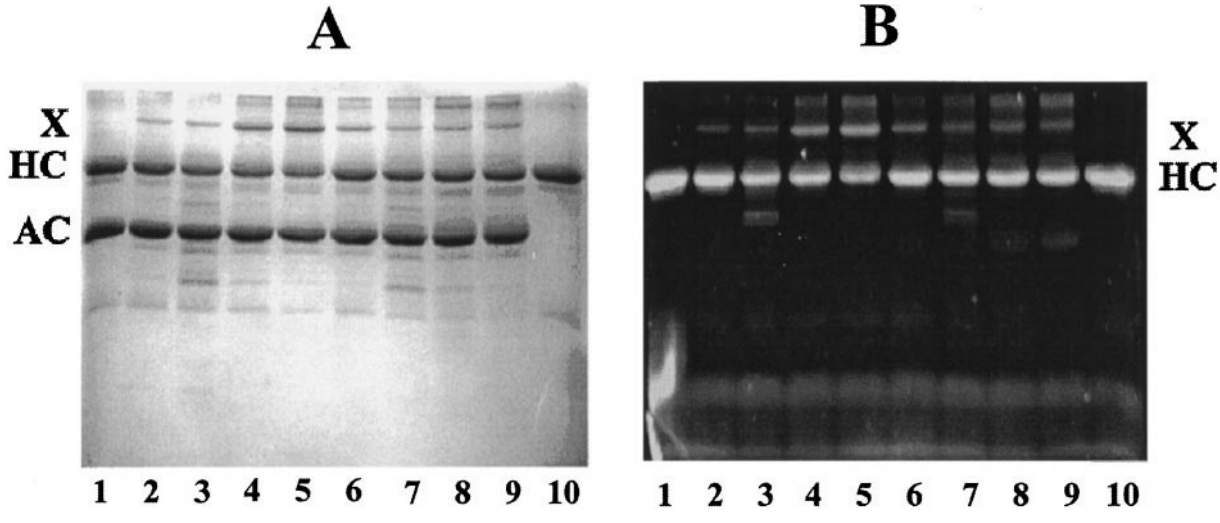

FIGURE 1 Time course of cross-linking 8 μM CaATP-G-actin to 8 μM IAEDANS-labeled S1(A2) at 25 and at 0°C. (*A*) Coomassie Blue-stained gel; (*B*) fluorescent gel. Vertical symbols: *X*, 180-kDa actin-S1 cross-linked product; *HC*, S1 heavy chain; *AC*, actin. *Lane 1*, S1(A2) plus actin; *lanes 2–5*, S1(A2) and actin treated with 0.2 unit/ml transglutaminase for 5, 15, 30, and 60 min at 0°C; *lanes 6–9*, actin and S1(A2) treated with 0.2 unit/ml transglutaminase for 5, 15, 30, and 60 min at 25°C; *lane 10*, S1(A2).

25°C. In order to reduce the amount of the high molecular weight species the cross-linking was routinely performed at 0°C. The experiment described in Fig. 1 was carried out with the S1(A2) isoform because this isoform does not polymerize actin under the conditions of the reaction (Chaussepied and Kasprzak, 1989). However, cross-link formation has been also observed in the presence of unseparated S1 that contains both the S1(A1) and S1(A2) isoforms, which indicates that the use of the nonpolymerizing S1(A2) isoform is not an essential requirement for the cross-link formation.

To test the assumption that the 180-kDa band is a cross-linked product of actin and the S1 heavy chain, the cross-linking was carried out also using IAEDANS-labeled CaATP-G-actin and unlabeled S1(A2) (Fig. 2). The fluorescent 180-kDa band appeared also in this case only when the two proteins were incubated together in the presence of transglutaminase, proving that the band is indeed a covalently coupled derivative of S1 and actin.

Transglutaminase-induced cross-linking between F-actin and IAEDANS-labeled S1(A2) was also attempted (data not shown). However, no cross-linked band appeared with F-actin, unlike in the case of G-actin in which the cross-linked band was very prominent.

Assignment of the cross-linking sites between G-actin and S1

In order to identify the cross-linking sites it was first necessary to assign that of the two proteins which supplies the glutamine acceptor. It is known that Gln-41 of actin can serve as glutamine acceptor in the transglutaminase reaction (Takashi, 1988; Hegyi et al., 1992; Kim et al., 1995), however, we could not exclude the possibility that S1 has a glutamine residue that can also serve as an acceptor in the reaction. To check this possibility we attempted to couple S1 by transglutaminase with the fluorescent amine DED, which proved to be an excellent amino donor in the transglutaminase reaction with G-actin (Kim et al., 1995). Since no labeling of S1 was observed with DED (results not shown) we could assume that ϵ-amino group of lysine(s) of S1 reacted with a glutamine residue of actin in the cross-linking reaction. As Gln-41 was found to be the only transglutaminase reactive glutamine residue in the former studies (Takashi, 1988; Hegyi et al., 1992; Kim et al., 1995), we supposed that this residue is the acceptor also in the cross-linking reaction with S1. To substantiate this claim we attempted to covalently couple S1(A2) to CaATP-G-actin whose Gln-41 had been blocked by DED. As no fluorescent cross-linked product appeared on SDS-PAGE (gel not shown), Gln-41 has been assigned as the acceptor site in this transglutaminase-catalyzed reaction.

The assignment of the donor cross-linking site on S1 was carried out by using trypsin-digested S1 in which the S1 heavy chain had been cleaved into three stable fragments, 27, 50, and 20 kDa, aligned in this order from the N terminus (Balint et al., 1978). The 50-kDa and the 20-kDa fragments are connected through a lysine-rich loop (residues 636–642), which is lost as a consequence of the tryptic digestion (Tong and Elzinga, 1990). No cross-linked band appeared on the SDS-PAGE upon incubation of tryptic-S1 with IAEDANS-labeled CaATP-G-actin and transglutaminase (Fig. 3). We assumed that the lack of cross-link formation between tryptic S1 and G-actin is due to the loss of the lysine-rich connector loop. This loop can either be directly involved in the transglutaminase catalyzed cross-linking by supplying the lysine donor for the reaction or indirectly by the loss of the connector region, which is the

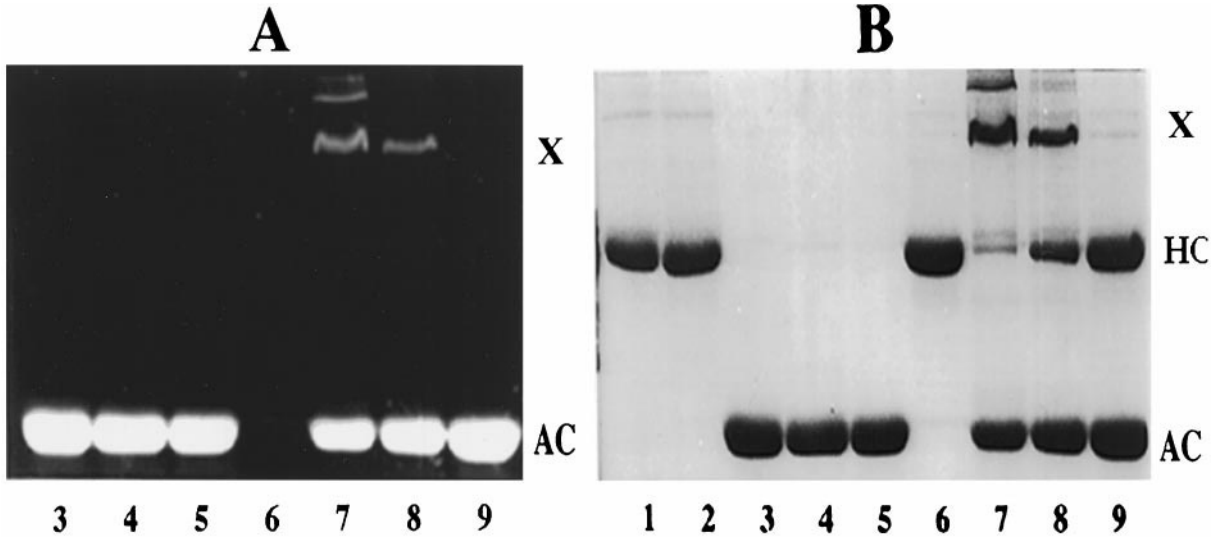

FIGURE 2 Cross-linking of 8 μM IAEDANS-labeled CaATP-G-actin to 8 μM S1(A2). (*A*) Fluorescent gel; (*B*) Coomassie Blue-stained gel. Vertical symbols as in Fig. 1. *Lanes 1* and *2*, S1(A2) treated with 0.2 unit/ml transglutaminase for 30 and 15 min; *lanes 3–5*, actin treated with 0.2 unit/ml transglutaminase for 30, 15, and 0 min; *lane 6*, S1(A2); *lanes 7–9*, S1(A2) and actin treated with 0.2 unit/ml transglutaminase for 30, 15, and 0 min as described in Materials and Methods.

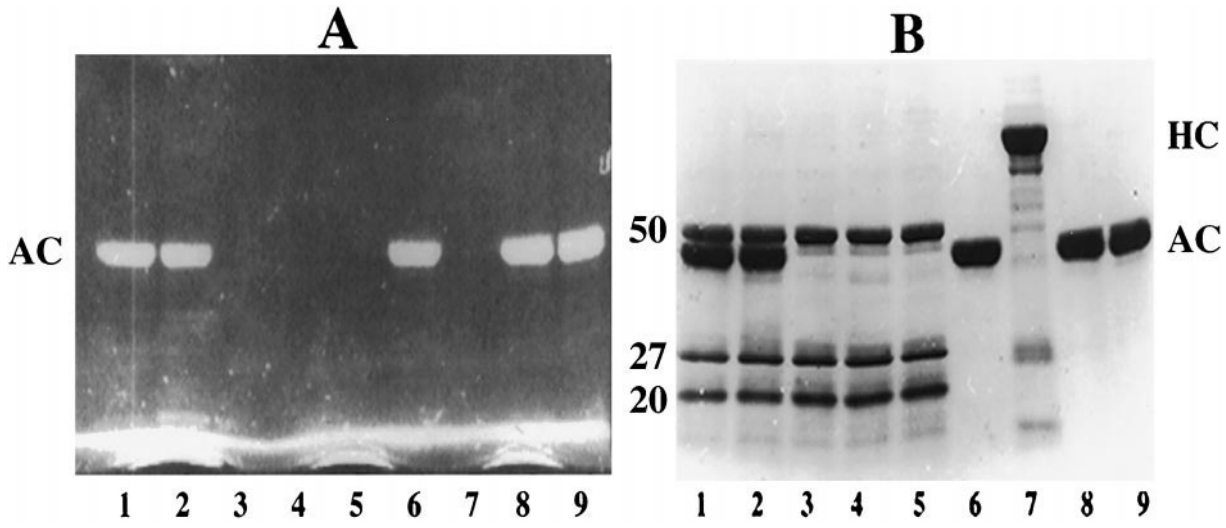

FIGURE 3 Attempt to cross-link 8 μM IAEDANS-labeled CaATP-G-actin to 8 μM trypsin-digested S1. (*A*) Fluorescent gel; (*B*) Coomassie Blue-stained gel. Vertical symbols: 50-, 27-, and 20-kDa tryptic fragments of the S1 heavy chain, respectively; *HC*, S1 heavy chain; *AC*, actin. *Lane 1*, tryptic S1 and actin; *lane 2*, tryptic S1 and actin treated with 0.2 unit/ml transglutaminase for 30 min; *lane 3*, tryptic S1; *lanes 4* and *5*, tryptic S1 treated with 0.2 unit/ml transglutaminase for 15 and 30 min; *lane 6*, actin; *lane 7*, undigested S1; *lanes 8* and *9*, actin treated with 0.2 unit/ml transglutaminase for 15 and 30 min as described under Materials and Methods.

main interaction site with G-actin (Lheureux and Chaussepied, 1995), eliminating cross-linking by decreasing actin-S1 affinity. To test these assumptions we attempted to cross-link the IAEDANS-labeled and V8 protease-digested S1 with CaATP-G-actin (Fig. 4). The V8 protease cleaves S1 into three stable fragments, 28, 48, and 22 kDa, aligned in this order from the N terminus (Chaussepied et al., 1983). The N-terminal residue of the 22-kDa C-terminal fragment is Gly-632, which means that in the case of V8 cleavage the C-terminal fragment retains the 636–642 lysine-rich connector loop. The SH_1 thiol (Cys-707), which is specifically labeled by the fluorescent thiol reagent IAEDANS, is also located on the C-terminal fragment. As a result of the incubation of the IAEDANS-labeled and V8-digested S1 with CaATP-G-actin and transglutaminase, a new fluorescent band appeared on the gel

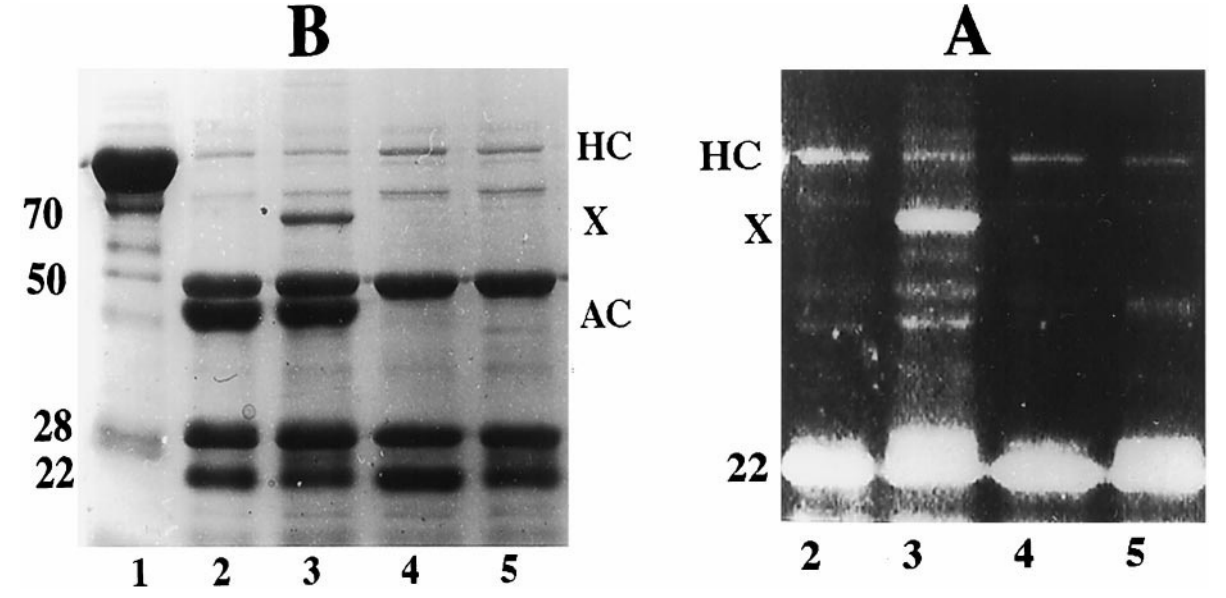

FIGURE 4 Cross-linking of 8 μM Ca-ATP-G-actin to 8 μM IAEDANS-labeled S1 digested by staphylococcus V8 protease. (*A*) Fluorescent gel; (B) Coomassie Blue-stained gel. Vertical symbols: 48-, 28-, and 22-kDa V8 fragments of the S1 heavy chain; (*X*) 68-kDa actin-22-kDa V8 S1 heavy chain fragment cross-linked product; (*HC*) S1 heavy chain; (*AC*) actin. *Lane 1*, S1; *lane 2*, V8-digested S1 plus actin; *lane 3*, V8-digested S1 and actin treated with 0.2 unit/ml transglutaminase for 30 min; *lanes 4* and *5*, V8-digested S1 treated with 0.2 unit/ml transglutaminase in the absence of actin for 15 and 30 min, respectively. The conditions for V8 digestion and cross-linking are described in Materials and Methods.

with a 68-kDa apparent molecular mass. Both the molecular mass and the fluorescence of the band indicate that it is a product of the covalent union of G-actin with the 22-kDa C-terminal fragment of S1. To locate more accurately the cross-linking site on the 22-kDa fragment, the IAEDANS-labeled S1 was subjected to limited proteolysis by V8 protease and cross-linked to G-actin. As the result of the cross-linking reaction, two new fluorescent bands appeared on the gel with apparent molecular masses of 180 and 68 kDa corresponding to the S1 heavy chain-actin and 22-kDa fragment-actin cross-linked products, respectively (Fig. 5, *lane 2*). This sample was further digested by trypsin. As a consequence of the trypsinolysis, which removes the 636–642 stretch from the N terminus of the 22-kDa fragment, both cross-linked bands disappeared (Fig. 5, *lane 3*). The most likely reason for the disappearance of these bands is that trypsin cleaves off the actin-bound 636–642 stretch from the S1 heavy chain, and the electrophoretic mobility of the resulting 636–642 peptide-actin complex is indistinguishable from the mobility of actin. Thus, the disappearance of the cross-linked bands following trypsinolysis unambiguously assigns the 636–642 lysine-rich loop as the donor cross-linking site.

Polymerization of the cross-linked product and the effect of pH, actin-bound divalent cation, and nucleotide on cross-linking

The polymerization of the G-actin-S1 cross-linked product by $MgCl_2$ was tested by electron microscopy and sedimentation experiments as described in Materials and Methods. The electron microscopic image of the cross-linked material (Fig. 6) showed actin filaments decorated with S1 molecules (*arrows*), which point to the polymerization of the cross-linked acto-S1. However, the cross-linked preparation yielded much shorter filaments than the control material. The polymerization of the cross-linked acto-S1 was also studied by sedimentation. Samples before and after sedimentation were analyzed by SDS-PAGE (not shown) and quantified by densitometry. According to the analysis, ~38% of S1 was cross-linked to G-actin, and all the cross-linked product sedimented together with the complex of uncross-linked F-actin and S1 in the ultracentrifuge. Electron microscopy of the cross-linked preparation did not reveal the presence of two kinds of filaments, which indicates that the cross-linked and uncross-linked actin may copolymerize. The actin-activated ATPase activity of the cross-linked acto-S1 was about 60% of the uncross-linked control, whereas the K^+(EDTA)-activated ATPases of the same two samples were essentially the same (Table 1). As the decrease in actin-activated ATPase (40%) caused by the cross-linking was similar to the extent of S1 cross-linking (38%), it seems likely that the attachment of the lysine-rich loop of S1 to the subdomain 2 of actin eliminates the actin activation of S1 ATPase. On the other hand, the S1 ATPase in the absence of actin is not affected by the cross-linking as shown by the measurement of the K^+(EDTA)-activated ATPase activity indicating that the active site of S1 is not impaired during the process.

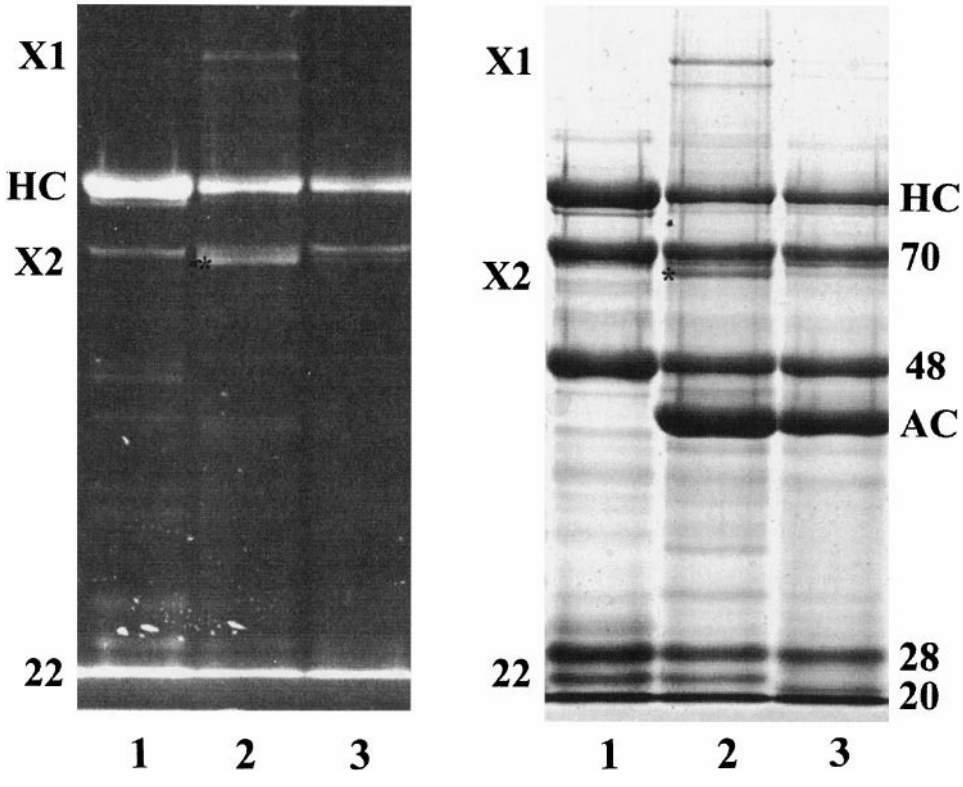

FIGURE 5 Tryptic digestion of V8-digested and IAEDANS-labeled S1 cross-linked to G-actin. IAEDANS-labeled S1 digested by V8 protease with 100:1 S1 to V8 w/w ratio at 22°C for 1 h and immediately added to the cross-linking mixture containing G-actin and transglutaminase (final concentrations 8 μM S1, 12 μM G-actin, 0.1 unit/ml transglutaminase). This mixture was incubated on ice for 1 h and then digested in the presence of 6 mM EDTA by trypsin at 50:1 S1 to trypsin w/w ratio at 22°C for 10 min. *Left panel*, fluorescent gel; *right panel*, Coomassie Blue-stained gel. Vertical symbols: 70-, 48-, 28-, and 22-kDa V8 fragments and 20-kDa C-terminal tryptic fragment of the S1 heavy chain; *X1*, S1 heavy chain-actin cross-linked product; *X2*, 68-kDa actin-22-kDa V8 S1 heavy chain fragment cross-linked product (*star*); *HC*, S1 heavy chain; *AC*, actin. *Lane 1*, V8-digested S1; *lane 2*, actin and V8-digested S1 treated with transglutaminase; *lane 3*, tryptic digest of the transglutaminase treated actin and V8-digested S1.

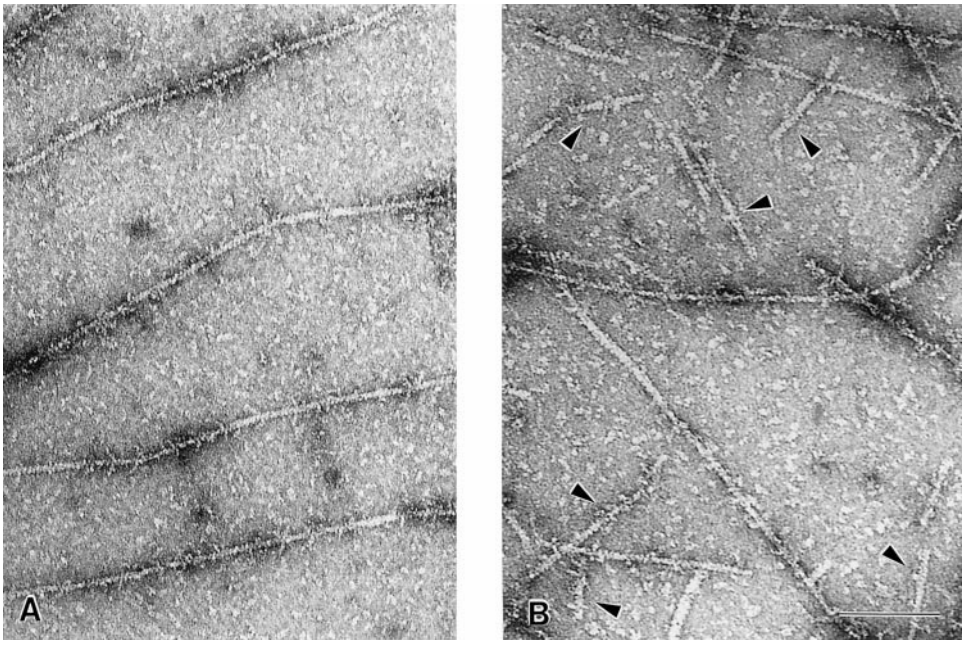

FIGURE 6 Electron microscopic examination of cross-linked and polymerized acto-S1. G-actin (12 μM) and S1 (8 μM) in G-actin buffer were incubated with 0.1 unit/ml transglutaminase for 1 h and polymerized with 2 mM $MgCl_2$. Samples were handled and examined as described in Materials and Methods. *A*, uncross-linked control; *B*, cross-linked sample. *Arrows*, S1 decoration of actin filaments. The *bar* in *B* represents 1000 Å.

The effect of pH on the formation of the G-actin-S1 cross-link was studied in the range of pH 6.7 to 8.0 (Fig. 7). The pH optimum of the reaction was found to be between pH 7.3 and 7.6 in which the extent of cross-linking was the highest, whereas at the higher and lower pH values the intensity of the cross-linked band decreased.

The nature of the tightly bound divalent cation is known to affect G-actin structure (Frieden et al., 1980), including that of subdomain 2 (Strzelecka-Golaszewska et al., 1993). Therefore, it was of interest to test the effect of Ca^{2+}-Mg^{2+} exchange on the cross-linking between CaATP-G-actin and IAEDANS-labeled S1 (Fig. 8). The analysis of the SDS-PAGE does not indicate any change in the extent of cross-linking upon Ca^{2+}-Mg^{2+} exchange, which may indicate that the vicinity of Gln-41, i.e., the inner part of the DNase I binding loop is not affected by the nature of the tightly bound cation, or the binding of S1 masks the difference between CaATP- and MgATP-G-actin at this site.

Under physiological conditions the tightly bound nucleotide of G-actin is ATP, which can be exchanged to ADP. The ATP-ADP exchange has a significant effect on the structure of the molecule (Frieden and Patane, 1985), which is prominently manifested in the structure of the DNase I binding loop (Strzelecka-Golaszewska et al., 1993; Muhlrad et al., 1994; Kim et al., 1995). We found that the nature of the bound nucleotide has a profound effect on the transglutaminase-catalyzed cross-linking between G-actin and S1 as a more intense cross-linked band is produced when MgATP-G-actin reacts with S1 than in the case of MgADP-G-actin (Fig. 9).

TABLE 1 ATPase activity of cross-linked and uncross-linked acto-S1 (sec^{-1}).

Assay system	Cross-linked acto-S1	Uncross-linked acto-S1
Actin activation	8.26	13.55
K^+ (EDTA) activation	8.57	9.19

ATPase assays were carried out as described in Materials and Methods.

DISCUSSION

G-actin has been successfully cross-linked to S1 heavy chain in a bacterial transglutaminase-catalyzed reaction. The transglutaminase-induced cross-linking has several advantages over other covalent cross-linking techniques. 1) The transglutaminase reaction is quite specific, and the acceptor of the reaction is always a glutamine residue, which in this case is Gln-41 of actin, whereas the donor is an alkylamine, which in proteins generally a lysine residue. 2) A zero-length covalent cross-link is formed during the reaction between the donor and acceptor residues. Thus, a very close proximity is an essential condition for successful cross-linking, which excludes possible artifacts. 3) No chemical modification of the participating proteins is needed for the reaction, and therefore undesired side reactions and changes in the biological activities of the proteins originating from the chemical modification can be avoided.

The results of the present cross-linking studies indicate a close proximity between Gln-41, located on the inner part of the DNase I binding loop in subdomain 2 of G-actin, and the 636–642 lysine-rich loop of S1, as Gln-41 is cross-linked to one of the five lysines of the 636–642 sequence. Cross-linking was observed only between G-actin and S1 but never between F-actin and S1. In most of the experiments we used the S1(A2) isomer of the rabbit skeletal S1 because this isomer does not promote actin polymerization under the conditions of the cross-linking reaction (Chaussepied and Kasprzak, 1989). However, we found that unseparated S1, which contains also the actin polymerizing isoform S1(A1),

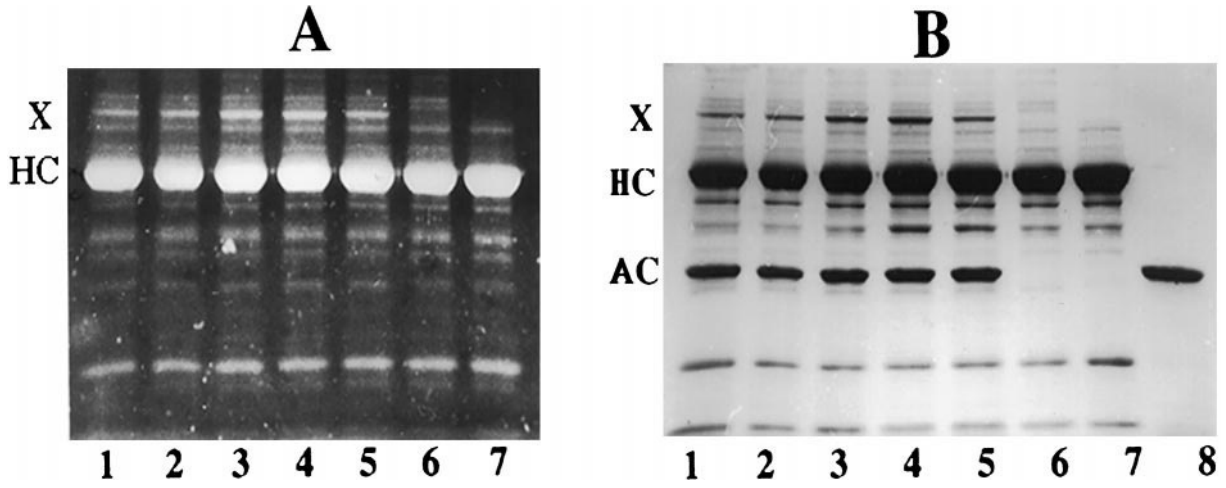

FIGURE 7 Effect of pH on the cross-linking between 8 μM Ca-ATP-G-actin and 8 μM IAEDANS-labeled S1. (*A*) Fluorescent gel; (*B*) Coomassie Blue-stained gel. Vertical symbols as in Fig. 1. *Lanes 1–5*, actin and S1 treated with 0.2 unit/ml transglutaminase at pH 6.7, 7.0, 7.3, 7.6, and 8.0, respectively; *lane 6*, S1 treated with 0.2 unit/ml transglutaminase at pH 7.6 as described in Materials and Methods; *lane 7*, S1; *lane 8*, actin.

can be also cross-linked to G-actin. This indicates that the rate of the cross-linking reaction is faster at 0°C (the temperature of the cross-linking) than the rate of the S1(A1)-induced actin polymerization.

The His-40-Gln-41-Gly-42 site on the DNase I binding loop was expected to be on the actin-myosin interface proximal to the Asn-552 to His-558 sequence of myosin according to the atomic model of the F-actin-myosin complex (Rayment et al., 1993a). The fact that no cross-link formation was observed between these sites on F-actin and S1 in the transglutaminase-catalyzed reaction should be taken into account in the modeling of the actin-myosin interface. The possibility that the His-40-Gly-42 site is not involved directly in myosin binding of F-actin was also indicated by the studies on the spectral characteristics of a fluorescent probe attached to Gln-41 (Kim et al,. 1996). However, Bertrand et al. (1994) and Bonafe et al. (1994) reported that Lys-50, residing on the outer part of the DNase I binding loop, can be successfully cross-linked by glutaraldehyde and maleimidobenzoic acid-*N*-hydroxysuccinimide ester, respectively, both in G- and F-actin to the central 50-kDa fragment of the S1 heavy chain. Both cross-linking reagents used in these studies label more than one residue, and therefore, the reaction in these cases is less specific than with transglutaminase, which labels only Gln-41 on actin. Moreover, the reagents in the former studies contain a spacer meaning that the reacting residues are probably less proximal to each other than in the case of the transglutaminase reaction in which only a zero-length cross-link is formed. These results together with our own observations

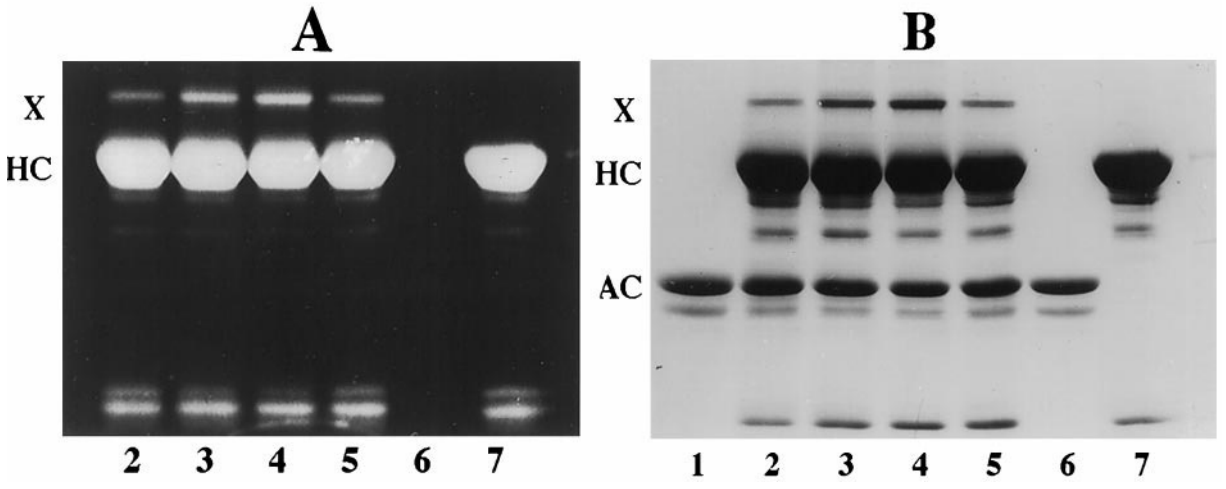

FIGURE 8 Cross-linking of 8 μM CaATP- or MgATP-G-actins to 8 μM IAEDANS-labeled S1(A2). (*A*) Fluorescent gel; (*B*) Coomassie Blue-stained gel. Vertical symbols as in Fig. 1. *Lane 1*, CaATP-G-actin; *lanes 2* and *3*, CaATP-G-actin and S1(A2) treated with 0.2 unit/ml transglutaminase for 15 and 30 min, respectively; *lanes 4* and *5*, MgATP-G-actin and S1 treated with 0.2 unit/ml transglutaminase for 30 and 15 min, respectively, as described in Materials and Methods; *lane 6*, MgATP-G-actin; *lane 7*, S1(A2).

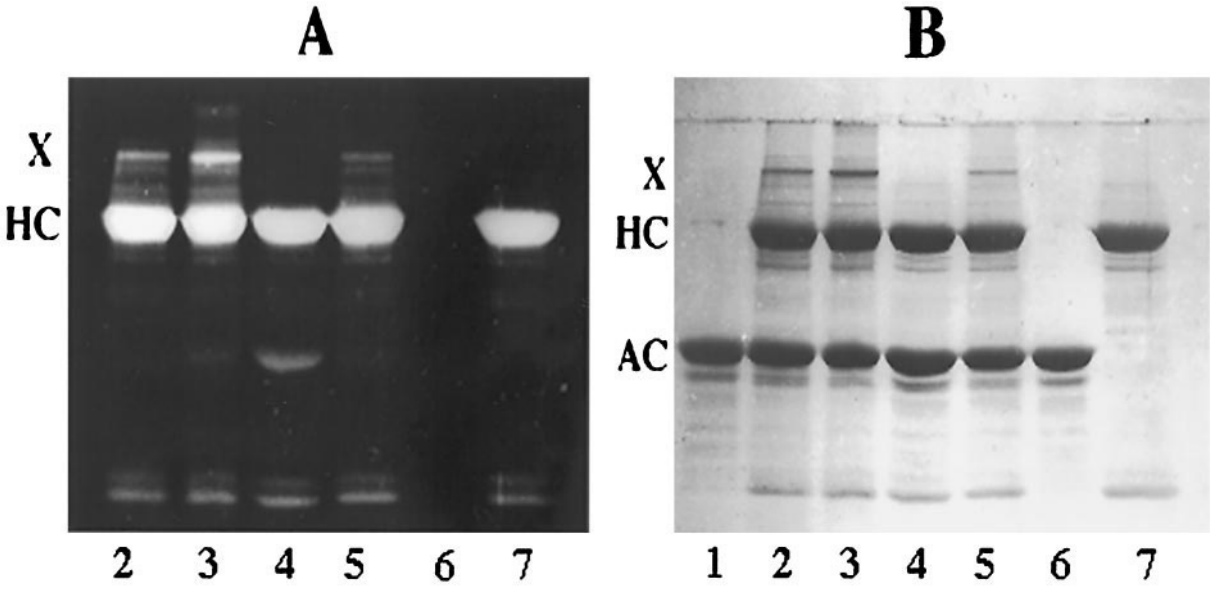

FIGURE 9 Cross-linking of 8 μM MgATP- or MgADP-G-actins to 8 μM IAEDANS-labeled S1. Vertical symbols as in Fig. 1. (*A*) Fluorescent gel; (*B*) Coomassie Blue-stained gel. *Lane 1*, MgATP-G-actin; *lane 2* and *3*, MgATP-G-actin and S1 treated with transglutaminase for 15 and 30 min, respectively; *lane 4* and *5*, MgADP-G-actin and S1 treated with transglutaminase for 15 and 30 min, respectively, as described in Materials and Methods; *lane 6*, MgADP-G-actin; *lane 7*, S1.

indicate that although the C-terminal region of the DNase I binding loop in F-actin can be a part of or near to the actin-myosin interface, the N-terminal region of the loop in which Gln-41 is located is not accessible for S1 in the actin filament. The lack of accessibility of Gln-41 in F-actin may be due to steric reasons, i.e., this part of the loop is covered by the adjacent protomer within the same filament strand in the long pitched helix (Hegyi et al., 1992), or the relatively large bulk of S1 prevents the binding of transglutaminase to the loop on F-actin. Another possibility is that the loop has a different structure in F-actin (Lorenz et al., 1993) than in G-actin, in which it is available for cross-linking with S1. We prefer this last possibility since cross-linking is also strongly inhibited in the case of MgADP-G-actin in which no steric hindrance exists.

It is rather unexpected that the G-actin-S1 cross-linked product copolymerizes with F-actin and S1 despite the fact that Gln-41, which is assumed to be on the actin-actin interface in long pitched helix (Hegyi et al., 1992), is blocked. It has been shown that polymerization of actin is inhibited by the modification of His-40 (Hegyi et al., 1974), which is next to Gln-41 in the sequence. However, it was possible to overcome the inhibition of polymerization upon His-40 modification by addition of phalloidin (Miki, 1988), which indicates that His-40 is not essential for polymerization. The modification of Gln-41 with dansyl ethylenediamine was found even to accelerate actin polymerization (Kim et al., 1995). This result together with those of the present work shows that Gln-41 is not essential for polymerization. However, the cross-linking of this residue perturbs F-actin structure as the polymerization of transglutaminase cross-linked acto-S1 results in significantly shorter actin filaments. The effect of this cross-linking on F-actin structure and function should be the subject of additional investigations.

Actin essentially cannot activate the ATPase activity of S1 in which lysine-rich loop has been cross-linked to Gln-41 of actin, despite of the fact that the cross-linking itself does not affect the K^+(EDTA)-activated ATPase activity of S1. It seems that the interaction of the lysine-rich loop of S1 with the acidic N terminus of actin is essential for the actin activation, and this is prevented by the cross-linking by rendering the lysine-rich loop unavailable for the interaction. The importance of the availability of the 636–642 loop of S1 for the actin-activated myosin ATPase was also shown by Chaussepied and Morales (1988) who found that covalent binding of this loop by a peptide dramatically inhibits the actin-activation of myosin ATPase.

The cross-linking of S1 to Gln-41 on G- but not on F-actin is not unique, as it has been reported by Combeau et al. (1992) that the penultimate C-terminal Cys-374 residue is cross-linked in G- but not in F-actin to S1 by paraphenylenedimaleimide or by benzophenemaleimide. In the same work the formation of two cross-linked products between G-actin and S1 was observed by using ethyl dimethylaminopropyl carbodiimide as cross-linking reagent. In the major product, the cross-linking took place between the acidic N-terminal residues of G-actin and the 601–696 fragment of the S1 heavy chain involving the lysine-rich 636–642 loop of S1, as suggested by Combeau et al. (1992) and shown by subsequent studies (Lheureux and Chaussepied 1995). In the minor product, an unidentified segment of actin is linked to the same region, i.e., to the 601–696 segment, of S1. In our work the 636–642 loop

was found to bind to the DNase I binding loop of G-actin. The fact that the same S1 loop, which has a significant role in actin activation of myosin ATPase and the electrostatic forces-based weak binding of actin to myosin (Chaussepied and Morales, 1988), can bind to different regions in G-actin indicates the existence of several structurally different G-actin-S1 complexes and points to the highly dynamic nature of both S1 and G-actin structures.

It is pertinent to ask what is the role of the DNase I binding loop and 632–646 loop contact in G-actin S1 interaction. We showed here by transglutaminase-catalyzed cross-linking that a G-actin-S1 complex having this contact exists in equilibrium solution containing the two proteins. Perhaps one of the reasons for the low activation of myosin ATPase by G-actin (Lheureux and Chaussepied, 1995) is the existence of this nonactivating complex. Another G-actin-S1 complex, with a 632–646 loop-actin N terminus contact, also exists under equilibrium conditions as shown by ethyl dimethylaminopropyl carbodiimide cross-linking (Combeau et al., 1992). This latter complex is probably the energetically preferred one for polymerization, and therefore, the first complex with the 632–646 loop-DNase I binding loop contact disappears during polymerization.

In this work, the effect of the exchange of the tightly bound cations and nucleotides on the cross-link formation between G-actin and S1 was also studied. The exchange of the tightly bound calcium to magnesium did not influence the extent of cross-linking, whereas the exchange of the actin bound ATP to ADP significantly inhibited the reaction. These results are in accordance with those of Strzelecka-Golaszewska et al. (1993) who showed by limited proteolysis and by fluorescence probes (Wawro et al., 1996) that the ATP-ADP exchange profoundly affects the structure of the DNase I binding loop whereas the Ca-Mg exchange hardly influences the loop structure. The findings that transglutaminase-catalyzed cross-link formation is most extensive in ATP-G-actin is partially inhibited in ADP-G-actin and completely prevented in F-actin support the assumption of Strzelecka-Golaszewska and her colleagues that the structure of the DNase binding loop in ADP-G-actin is somewhere in the middle between the structures of ATP-G-actin and F-actin.

In conclusion, we found that transglutaminase catalyzes the formation of a specific zero-length cross-link between the lysine-rich loop of S1 and Gln-41 in G- but not in F-actin. The exchange of the tightly bound calcium to magnesium does not affect the cross-linking, whereas the exchange of the bound ATP to ADP strongly inhibits the reaction. The cross-linked acto-S1 is polymerized into short filaments. The ATPase activity of S1, in which the lysine-rich loop is blocked by cross-linking, is not activated by actin. The results further prove the highly dynamic nature of the subdomain 2 of actin and show the existence of more than one G-actin-S1 complex. Moreover, the present findings should be taken into account in the construction of the atomic model of the actomyosin complex and have implications also in the mechanistic description of the molecular events of contraction.

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