Structural Connectivity in Actin: Effect of C-Terminal Modifications on the Properties of Actin

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ABSTRACT In this study, we use fluorescent probes and proteolytic digestions to demonstrate structural coupling between distant regions of actin. We show that modifications of Cys-374 in the C-terminus of actin slow the rate of nucleotide exchange in the nucleotide cleft. Conformational coupling between the C-terminus and the DNaseI loop in subdomain II is observed in proteolytic digestion experiments in which a new C-terminal cleavage site is exposed upon DNaseI binding. The functional consequences of C-terminal modification are evident from S-1 ATPase activity and the in vitro motility experiments with modified actins. Pyrene actin, labeled at Cys-374, activates S-1 ATPase activity only half as well as control actin. This reduction is attributed to a lower V_{max} value because the affinity of pyrene actin to S-1 is not significantly altered. The in vitro sliding velocity of pyrene actin is also decreased. However, IAEDANS labeling of actin (also at Cys-374) enhances the V_{max} of acto-S-1 ATPase activity and the in vitro sliding velocity by approximately 25%. These results are discussed in terms of conformational coupling between distant regions in actin and the functional implications of the interactions of actin-binding proteins with the C-terminus of actin.

INTRODUCTION

The in vivo function of actin requires a unique and dynamic structure that allows for interactions not only with myosin but also with other actin monomers and regulatory proteins. These complex interactions are designed for generation and regulation of force and cellular movements. To understand the interplay between actin and the components of motile apparatus, it is imperative to examine the structural and dynamic determinants of actin's function. The importance of one site on actin, its carboxy-terminus, to the intra- and intermolecular interactions of this protein has been highlighted in several recent studies.

Experiments performed with truncated actins devoid of the last two or three C-terminal residues (including Cys-374) have shed light on the importance of actin's C-terminus to intermonomer communication. Electron micrographs of filaments composed of actin$_{-3}$ (Mossakowska et al., 1993) or actin$_{-2}$ (O'Donoghue et al., 1992) indicate an increased flexibility and fragility of the polymer. The critical concentration for actin polymerization and the activation of S-1 ATPase are also affected by the removal of the C-terminal residues (Mossakowska et al., 1993; O'Donoghue et al., 1992). This is consistent with three-dimensional reconstructions of the actin filaments (Milligan et al., 1990) and NMR studies (Barden et al., 1989), which predict that Cys-374 is at or close to the intermolecular interface on actin. These data suggest that the C-terminal residues are crucial for filament strength and are involved in intermonomer communication.

Intramolecular interactions of actin's C-terminus and its coupling to other parts of the protein are indicated by the sensitivity of fluorescent probes (attached to Cys-374 on actin) to changes in the nucleotide cleft. Exchanges of Ca^{2+} for Mg^{2+} (Carlier et al., 1986; Valentin-Ranc and Carlier, 1989) and ATP for ADP (Frieden and Patane, 1985) were shown to affect the fluorescence of the IAEDANS probe on Cys-374. This demonstrates the transmission of structural perturbation from the nucleotide cleft to the C-terminus of actin. The reciprocal communication, from the C-terminus to the nucleotide cleft, has been suggested by the recent studies with *Drosophila melanogaster* Act88F mutants containing altered C-terminal sequences (Drummond et al., 1992). Interestingly, the substitutions and deletions in the C-terminus modulate not only the binding of ATP and profilin to actin but also that of DNaseI at a more distant part of actin.

The increasing evidence for structural coupling between the C-terminus and other parts of actin and the importance of this region for the binding of other proteins prompted our present investigation of the effects of Cys-374 labeling on actin. This study was also motivated by the fact that pyrene and IAEDANS (attached to Cys-374) actins are the most commonly used analogs of actin in experiments probing the structure, function, and interactions of this protein. In this work, we show that both the pyrene and IAEDANS probes on Cys-374 alter the nucleotide cleft environment and change, albeit differently from each other, the activation of myosin ATPase activity by actin and the in vitro motility of actin filaments. Moreover, using proteolytic digestion, we show that the C-terminal region on actin is coupled to a distant DNaseI site.

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Abbreviations used: Actin$_{-2}$ and Actin$_{-3}$, actin with the last 2 or 3 residues removed by trypsin; DNaseI, deoxyribonuclease I; F-actin, filamentous (polymerized) actin; G-actin, monomeric actin; HMM, heavy meromyosin; S-1, myosin subfragment-1.

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MATERIALS AND METHODS

Reagents

ATP, ATPγS, ϵ-ATP, β-mercaptoethanol, chymotrypsin, soybean trypsin inhibitor, and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO). Bradford reagent was purchased from Bio-Rad (Richmond, CA). *N*-(1-pyrenyl) iodoacetamide, 1,5-IAEDANS, and rhodamine-labeled phalloidin were obtained from Molecular Probes (Junction City, OR). Distilled and millipore-filtered water and analytical grade reagents were used in all experiments.

Preparation of proteins

Rabbit skeletal muscle α-actin was prepared in G-buffer (2 mM Tris, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, 0.2 mM ATP, and pH 7.6) by the procedure of Spudich and Watt (1971). Myosin subfragment-1 (S-1) was purified as described previously (Godfrey and Harrington, 1970; Weeds and Pope, 1977). Heavy meromyosin (HMM) was prepared by α-chymotrypsin digestion of myosin (Kron et al., 1991).

Labeling of actin

Control actin in all cases was processed exactly as the modified actin with the omission of fluorescent reagents. Actin was labeled at Cys-374 with *N*-(1-pyrenyl) iodoacetamide following the method of Cooper et al. (1983). The extent of labeling was measured by using a molar extinction coefficient of $E_{344} = 2.2 \times 10^4\ M^{-1}\ cm^{-1}$ for the protein-dye complex (Kouyama and Mikashi, 1981). The labeling stoichiometry ranged between 0.80 and 1.00 probe/actin. 1,5-IAEDANS modified actin was prepared by a procedure identical to that described for pyrene iodoacetamide except that F-actin was incubated with a 20-fold molar excess of 1,5-IAEDANS and the reaction time was 3 h. The molar extinction coefficient used for the protein-dye complex was $E_{337} = 6300\ M^{-1}\ cm^{-1}$. The modified protein contained between 0.75 and 1.00 dye/actin. The protein concentrations of modified actin were determined by using the Bradford assay (1976). All actins tested for in vitro motility were labeled with phalloidin-tetramethyl rhodamine isothiocyanate according to the method of Kron and Spudich (1986).

Nucleotide exchange experiments

Actin containing bound ϵ-ATP was prepared as described by Root and Reisler (1992). Fluorescence measurements were made in a Spex Fluorolog Spectrofluorimeter (Spex Industries, Inc., Edison, NJ). The excitation and emission wavelengths were 340 and 410 nm, respectively. The exchange of bound ϵ-ATP for unlabeled ATP was monitored by the decrease in fluorescence that occurs when ϵ-ATP is released into the surrounding medium. The buffer consisted of 2 mM Tris, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, pH 7.6. Fluorescence contributions of the fluorescent probes were subtracted as background. The rates of nucleotide exchange were determined by fitting the data to a single exponential expression with Sigma Plot 4.1 software program.

Tryptic digestion of actin and DNaseI

G-actin (24 μM) with or without 24 μM DNaseI was cleaved by 0.87 μM trypsin at 25°C. The reactions were stopped with an excess of soybean trypsin inhibitor after 1, 3, 5, 10, 20, and 30 min. The samples were denatured and electrophoresed on 10% polyacrylamide gels (Laemmli, 1970). Duplicate gels were either stained with Coomassie Blue R or electrotransferred to nitrocellulose and immunoblotted. Western blots of SDS gels were performed according to Bulinski et al. (1983). An N-terminal antiserum (recognizing actin residues 1–7) and a C-terminal antiserum (recognizing actin residues 359–367) were used as the primary antibodies in Western blots. The antibodies were gifts from Dr. G. DasGupta. Coomassie Blue R-stained protein bands were scanned with a Biomed Instruments Densitometer (Fullerton, CA). The apparent molecular mass of digestion products was determined by comparing their mobilities in SDS-PAGE to those of molecular weight standards.

Actin-activated myosin S-1 ATPase

The actin-activated MgATPase activities were measured for control, pyrene-modified, and 1,5-IAEDANS-modified actin as previously described (Reisler, 1980; DasGupta and Reisler, 1989). Actin (5–60 μM) was incubated with 1 μM S-1 in a buffer consisting of 4.8 mM KCl, 2 mM $MgCl_2$, 5.5 mM Tris, pH 8.0. The ATPase measurements were performed at 25°C. V_{max} values are reported as turnover rates, i.e., micromoles of phosphate liberated per micromole of S-1 per second.

In vitro motility assay

The in vitro motility experiments were performed as described by Homsher et al. (1992). A fluorescent microscope (Orthoplan, E. Leitz Inc., Rockleigh, NJ) with a 200 Watt mercury bulb, built-in neutral density filter, and a 63X oil immersion objective (N.A. = 1.3) was used to image the rhodamine phalloidin-labeled actin filaments. Phalloidin does not affect the K_m or V_{max} of actin-activated ATPase activities (Uyeda et al., 1990). The images were recorded onto videocassette (recorder model SLV-RS; Sony, Park Ridge, NJ) from a camera (model SIT68; Page-MTI Inc., Michigan City, IN) after digital image processing (processor model ARGUS10, Hamamatsu Corp., Bridgewater, NJ). Temperature was maintained at 25°C with a brass microscope stage and a sleeve for the objective. 0.1–0.3 mg/ml HMM was applied to nitrocellulose coated glass coverslips (Kron et al., 1991). Special precautions were taken before and after applying HMM to the coverslips to remove ATP-insensitive myosin heads. These precautions are detailed by Homsher et al. (1992). Rhodamine-labeled control actin or modified actin was added to the HMM coated coverslip. 1 mM ATP in 0.5% methylcellulose was added to initiate filament movement. More than 95% of fluorescent actin filaments moved after removing the rigor-like heads. An Expert-Vision motion analysis system was used (Motion Analysis, Santa Rosa, CA) to track and analyze the velocities of the moving centroids. Only filaments with an SD of one-third of the mean velocity were included in the data analysis. The distribution of over 75 filament velocities was plotted in each histogram.

RESULTS

Conformational coupling of distant regions within the actin monomer

Actin was modified at Cys-374 with pyrene iodoacetamide and 1,5-IAEDANS to analyze the effects of C-terminal modifications on other regions within actin. Because C-terminal probes detect changes in the actin's nucleotide cleft, we have examined the signal transmission in the reverse direction, from the C-terminus to the nucleotide site. Nucleotide exchange of modified and control actin was employed as an indicator of conformational changes in the cleft. The exchange of bound ϵ-ATP for unlabeled ATP was monitored by the decrease in fluorescence that accompanies the release of ϵ-ATP into the surrounding medium. Fig. 1 shows that the ϵ-ATP exchange rate (off-rate) of pyrenyl actin is substantially reduced ($1.9 \times 10^{-3} \pm 1.2 \times 10^{-5}\ s^{-1}$) compared with values obtained for control actin ($3.2 \times 10^{-3} \pm 1.2 \times 10^{-5}\ s^{-1}$). The nucleotide exchange rate of 1,5-IAEDANS actin was also decreased, by about 20% compared with control values (data not shown).

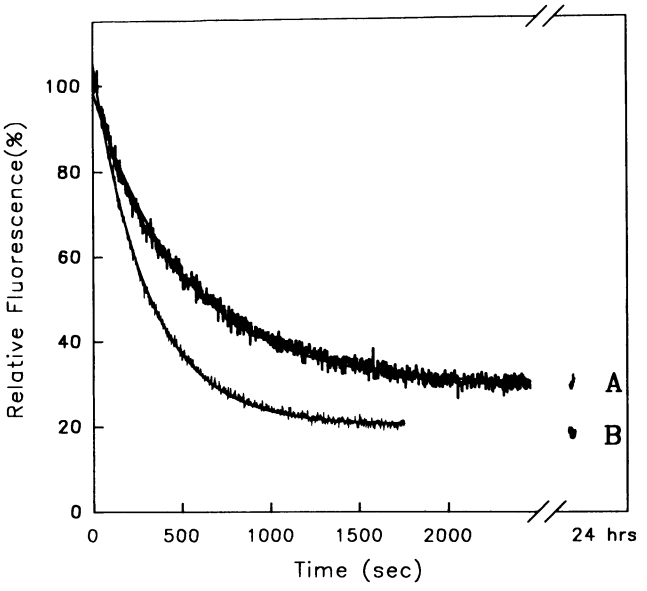

FIGURE 1 Effect of Cys-374 modification on nucleotide exchange. Nucleotide exchange experiments were performed with pyrene-modified (0.8 pyrene/actin) (*A*) and -unmodified actin (*B*). The nucleotide exchange was monitored by the fluorescence decrease as ϵ-ATP was replaced by ATP. G-actin (3 μM) containing bound ϵ-ATP was mixed with 0.5 mM ATP as described in Materials and Methods. The buffer conditions were 2 mM Tris, 0.2 mM $CaCl_2$, and 0.5 mM β-mercaptoethanol, pH 7.6. Control actin in each case was subjected to the same modification procedures but for the addition of fluorescent reagent. The observed first-order off-rates of ϵ-ATP exchange were $3.2 \times 10^{-3} \pm 1.2 \times 10^{-5}$ s^{-1} for control actin and $1.9 \times 10^{-3} \pm 1.2 \times 10^{-5}$ s^{-1} for pyrenyl actin ($n = 7$).

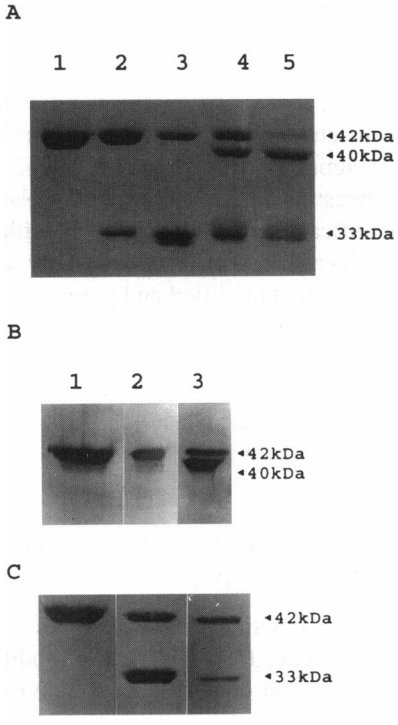

FIGURE 2 Effect of DNaseI binding on the tryptic cleavage of actin's C-terminus. 24 μM G-actin in the presence and absence of 24 μM DNaseI was digested at 25°C with trypsin as described in Materials and Methods. (*A*) Coomassie-blue stained protein bands of actin and DNaseI. (*lane* 1) Control actin; (*lanes* 2 and 3) actin digested for 10 and 30 min, respectively; (*lanes* 4 and 5) actin and DNaseI digested for 10 and 30 min, respectively. (*B*) Immunoblots of electrotransferred protein bands blotted with the N-terminal antiserum specific for actin residues 1–7. (*lane* 1) Control actin; (*lane* 2), actin digested for 30 min; (*lane* 3) actin and DNaseI digested for 30 min. (*C*) Immunoblot of electrotransferred protein bands blotted with the C-terminal antiserum specific for actin residues 359–367. (*lane* 1) Control actin; (*lane* 2) actin digested for 30 min; (*lane* 3) actin and DNaseI digested for 30 min.

Protease digestions were used to probe other regions of actin that could be affected by C-terminal modifications. Tryptic digestion cleaves G-actin at residues Arg-62, Lys-68, Arg-372, and Lys-373 (Jacobson and Rosenbusch, 1976; de Couet, 1983; Mornet and Ue, 1984). Previous studies indicated that cleavage of the last three C-terminal residues is not affected by the presence of a probe at Cys-374 (Mossakowska et al., 1993). Our results show that cleavages at Arg-62 and Lys-68 are also not affected by Cys-374 modification (data not shown). Digestions at Met-47/Gly-48 in the DNaseI loop (subdomain II, residues 39–50) by subtilisin were also identical for unmodified, pyrene-modified, and 1,5-IAEDANS-modified actins (data not shown).

Although subtilisin digestion within the DNaseI loop of actin was not affected by the presence of pyrene at Cys-374, the binding of DNaseI to this loop changes the digestion of actin at the C-terminus. Tryptic cleavage of the actin C-terminus is dramatically altered by the binding of DNaseI in subdomain II. In the absence of DNaseI, G-actin is cleaved to produce a 33-kDa fragment (Fig. 2 *A*) as shown previously (Jacobson and Rosenbusch, 1976; de Couet, 1983; Mornet and Ue, 1984). However, in the presence of DNaseI a new cleavage site results, as seen by the appearance of the 40 kDa digestion product (Fig. 2 *A*). This 40 kDa actin fragment was not detected in an earlier study that reported on the protection of actin sites 62/63 and 68/69 from tryptic cleavage in the presence of DNaseI (Burtnick and Chan, 1980). To determine the location of the new digestion site, we immunoblotted the digestion products to nitrocellulose and probed the proteins with N-terminal (recognizing residues 1–7)- and C-terminal (recognizing residues 359–367)-specific antisera. As seen in Fig. 2 *B*, the 40-kDa fragment reacts with the N-terminal antiserum, indicating that the N-terminus remains intact. However, reactivity with the C-terminal antibody is lost in the 40 kDa fragment (Fig. 2 *C*), indicating that either the entire 359–367 sequence or its major part on actin is no longer present. The 33 kDa actin fragment (Fig. 2 *A*) generated by trypsin digestion in the absence of DNaseI results from cleavage at residues Arg-62 and Lys-68 (Mornet and Ue, 1984). Accordingly, this peptide reacts with the C-terminal antiserum and not the N-terminal antiserum (Fig. 2, *B* and *C*). Approximately a 2-kDa fragment (about 20 amino acids) is cleaved from the C-terminus of actin in the presence of DNaseI to yield the 40 kDa actin fragment. This suggests, based on actin's amino acid sequence, that the cleavage site occurs at Lys-359. Such a cleavage would reduce actin's mass by approximately 2 kDa and would also remove the C-terminal antibody epitope from the 40-kDa

fragment. It is unlikely that C-terminal digestion in the presence of DNaseI would occur at Lys-336; such a reaction should produce a smaller, 37- or 38-kDa, product.

The coupling between the actin C-terminus and the DNaseI loop in subdomain II was analyzed further with the use of pyrenyl actin. The protection of Arg-62 and Lys-68 from tryptic digestion in the presence of DNaseI was not altered by modification of the C-terminus with pyrene iodoacetamide (data not shown). DNaseI activity was inhibited to the same extent in unmodified and pyrene modified actin (data not shown). Also, pyrenyl actin titrated with DNaseI does not show any perturbation of C-terminal fluorescence. These results suggest that subdomain II is not as sensitive to Cys-374 modification as the site around Lys-359 to DNaseI binding in the subdomain II region.

Functional changes in the actin polymer induced by C-terminal modifications

To determine the functional consequences of structural perturbations stemming from C-terminal modification, we examined the activation of S-1 ATPase activity by the labeled actins and their in vitro motilities. The myosin S-1 ATPase activated by unmodified and 1,5-IAEDANS-labeled actin was measured over a range of actin concentrations, as shown in Fig. 3. The V_{max} and K_m values of modified and unmodified actin were determined by a Michaelis-Menton analysis of the ATPase data (see legend). The V_{max} of modified actin (13.8 ± 1.1 s^{-1}) was increased by 25% over that of control (10.8 ± 0.8 s^{-1}), whereas the K_m value (23.7 ± 4.5 and 27.5 ± 4.4 μM, respectively) was not significantly different.

Velocities of filament sliding for unmodified and 1,5-IAEDANS-modified actins were determined by using an in vitro motility assay as described in Materials and Methods. Fig. 4 *A* shows the velocity distribution of sliding filaments for control and modified actins. 1,5-IAEDANS (75% modified) actin moved with an increased mean velocity (4.5 ± 0.5 μm/s) compared with that of control actin filaments (3.3 ± 0.6 μm/s). Fig. 4 *B* presents the mean velocities of actin as a function of Cys-374 modification with 1,5-IAEDANS (100% modification corresponds to a labeling stoichiometry of one IAEDANS molecule per actin). The sliding velocity of actin varies with the extent of labeling, reaching a maximum at about 75% modification. Modification at sites other than Cys-374 could be a cause for the decrease in sliding velocities observed at 85% and higher levels of modification.

Pyrene iodoacetamide modification of Cys-374 also affects acto-S-1 ATPase and the in vitro motility of actin filaments but in the opposite direction to the 1,5-IAEDANS modification. The activation of S-1 ATPase by pyrenyl and control actins is plotted as a function of actin concentration (Fig. 5). The V_{max} of S-1 ATPase activation by pyrenyl actin was 7.5 ± 1.0 s^{-1}, and that of unmodified actin was 13.0 ± 0.6 s^{-1}. The K_m value was not significantly altered by pyrenyl modification of the C-terminus (19.4 ± 2.3 and 23.8 ± 6.5

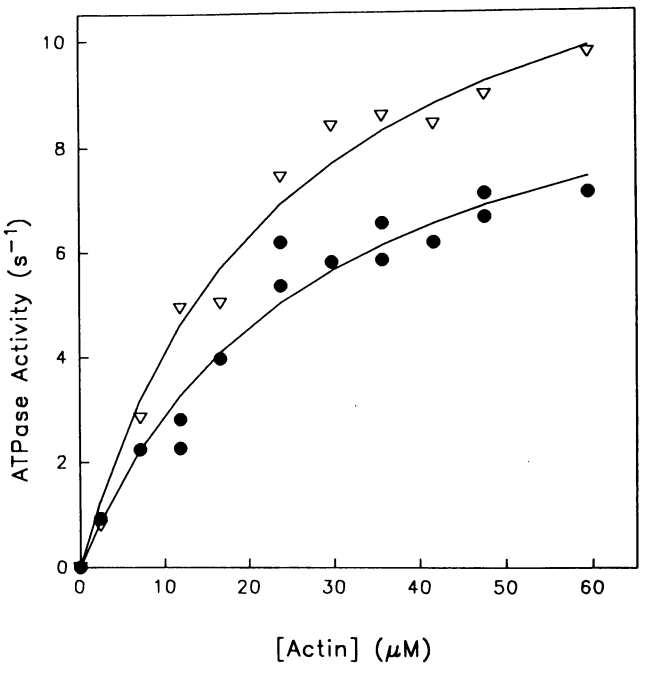

FIGURE 3 Activation of S-1 ATPase activity by 1,5-IAEDANS-modified actin. Actin-activated ATPase activities were measured as described in Materials and Methods. Actin (5–60 μM) was incubated with 1 μM S-1 in a buffer consisting of 4.8 mM KCl, 2 mM $MgCl_2$, 5.5 mM Tris, pH 8.0. The V_{max} and K_m values for unmodified actin (●) determined from this plot are 10.8 ± 0.8 s^{-1} and 27.5 ± 4.4 μM, respectively. The V_{max} and K_m values for 1,5-IAEDANS actin (▽) are 13.8 ± 1.1 s^{-1} and 23.7 ± 4.5 μM, respectively. The 1,5-IAEDANS actin was 75% modified in this preparation. S-1 ATPase activity in the absence of actin has been subtracted from all values. The lines are computer-generated fits of data points to the Michaelis-Menton equation (n = 2).

μM for unmodified and modified actins, respectively). The in vitro sliding velocity of pyrene-labeled actin was reduced to 2.8 ± 0.5 μm/s compared with a mean value of 3.9 ± 0.4 μm/s for control actin, as shown in the distributions given in Fig. 6. Thus, modification of the actin penultimate residue affects polymer structure and function, as evidenced by its effects on actomyosin ATPase and sliding velocities of actin filaments. The opposing effects of 1,5-IAEDANS and pyrene iodoacetamide on these functions reflect probe-dominated conformational changes at the C-terminus, which in turn determine the changes at other sites on actin. Other probe dependent effects of Cys-374 modification have been observed by Duong and Reisler (1994), Prochniewicz et al. (1993) and Drewes and Faulstich (1993).

DISCUSSION

The contribution of the C-terminus to actin's structure and function is important for several reasons. First, the C-terminus is involved in intermonomer contacts and contributes to polymer stability. Second, myosin S-1 indirectly affects the C-terminus of actin. This is suggested by studies demonstrating that although S-1 binding changes the fluorescence of probes attached to actin Cys-374 (Kouyama and

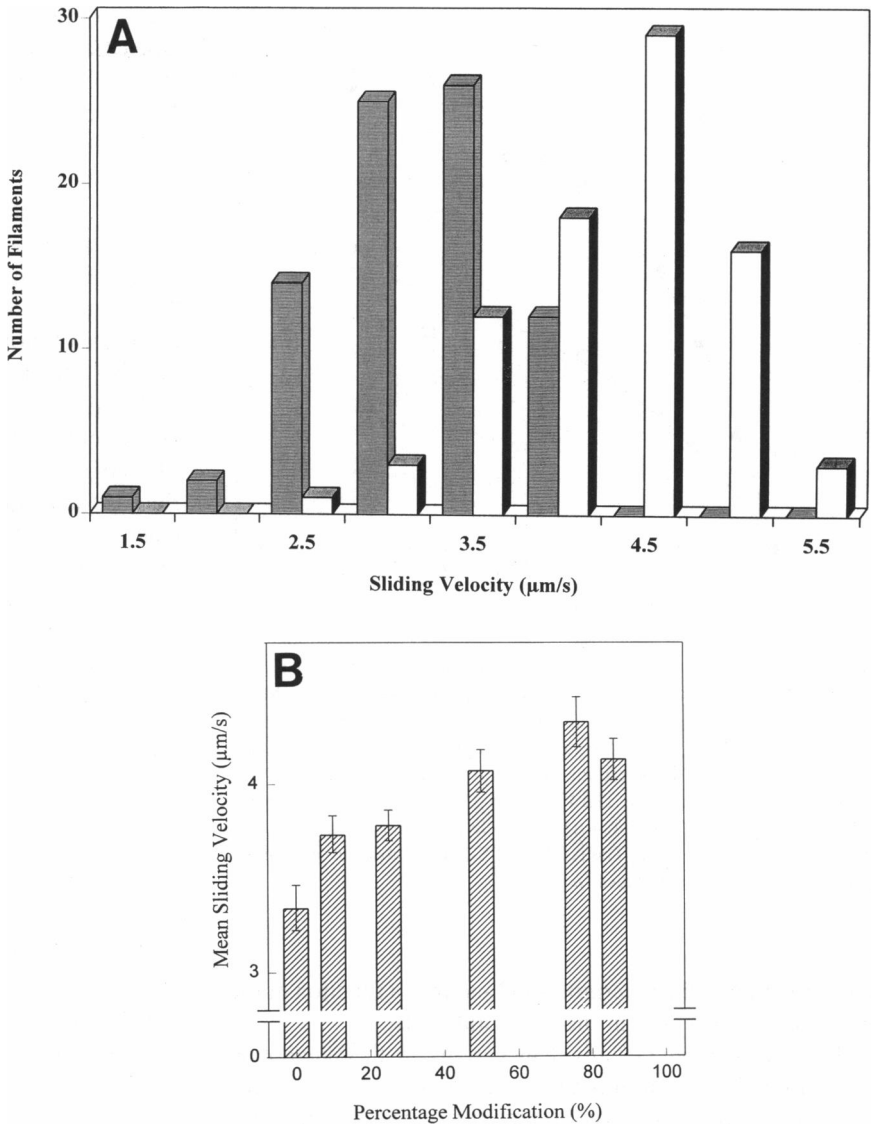

FIGURE 4 The in vitro motility of 1,5-IAEDANS actin. Actin sliding velocities over HMM-coated coverslips were measured using the in vitro motility assay, as described in Materials and Methods. Buffer conditions used were 25 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, 25 mM imidazole, pH 7.4. (*A*) Distribution of sliding velocities of 1,5-IAEDANS actin (*open bars*) and control actin (*filled bars*). In this preparation, 1,5-IAEDANS actin was 75% modified. Mean sliding velocities for control and 1,5-IAEDANS actins are 3.3 ± 0.6 and 4.5 ± 0.5 μm/s respectively. (*B*) The mean sliding velocities of 1,5-IAEDANS actin plotted as a function of the percentage of actin modified at Cys-374. Error bars indicate the 99% confidence limits of the mean velocity of actin filaments. ($n > 75$ filaments for each sample of actin).

Mihashi, 1981), it is not altered significantly by truncation of the C-terminal residues or their chemical modification (Crosbie et al., 1992; Makuch et al., 1992, Duong and Reisler, 1994). Third, the C-terminus is a common binding region for several regulatory proteins, including gelsolin (Doi et al., 1991), profilin (Malm et al., 1983; Vandekerckhove et al., 1989; Schutt et al., 1993) and, to some degree, caldesmon (Graceffa and Jancso, 1991). Finally, the C-terminus seems to be conformationally coupled to other regions within actin. A recent study (Drummond et al., 1992) and this work indicate that structural changes at the C-terminus are transmitted to distant regions within the actin monomer. These long range communications may have functional significance.

To define further the conformational coupling of distant regions in the actin structure, we modified actin at Cys-374 with two different fluorescent reagents: 1,5-IAEDANS and pyrene iodoacetamide. Such modifications of actin provide a convenient method to alter the local structure of the C-terminus and examine its effects on the rest of the molecule. Our results show that modification at the C-terminus reduces the rates of nucleotide exchange by 40 and 20% for pyrene- and 1,5-IAEDANS-modified actins, respectively (Fig. 1). Thus, C-terminal modifications affect the structure of the nucleotide cleft, reducing its ability to exchange ATP. The converse signal transmission, from the nucleotide site on actin to its C-terminus, has been observed in several studies. The conversion of ATP to ADP in Mg^{+2}-G-actin has been recently shown to enhance the susceptibility of C-terminal residues Arg-372 and Lys-373 on actin to trypsin (Strzelecka-Golaszewska et al., 1993). Also, the alkylation at Cys-374 is reduced in F-actin in the presence of BeFx, a phosphate analog that binds to F-actin (Muhlrad et al., 1994). The sensitivity of the C-terminus to the subtle differences between ATP and ATPγS states of the nucleotide cleft, as noted in x-ray diffraction studies on profilin/actin crystals (Schutt et al., 1989), provides additional evidence for conformational coupling between the nucleotide cleft and the C-terminus. Most recently, Orlova and Egelman (1994) used three-dimensional reconstructions to show that the

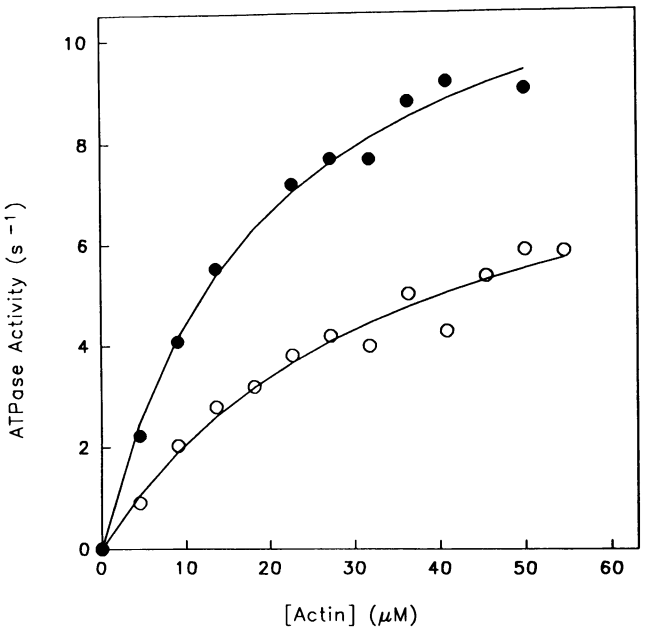

FIGURE 5 Pyrenyl actin-activated ATPase of S-1. Actin-activated ATPase activities of S-1 were measured as described in Materials and Methods. Actin (5–60 μM) was incubated with 1 μM S-1 in a buffer consisting of 4.8 mM KCl, 2 mM $MgCl_2$, 5.5 mM Tris, pH 8.0. The V_{max} and K_m values for unmodified actin (●) determined from this plot are 13.0 ± 0.6 s^{-1} and 19.4 ± 2.3 μM, respectively. The V_{max} and K_m values for pyrenyl actin (○) are 7.5 ± 1.0 s^{-1} and 23.8 ± 6.4 μM, respectively. Pyrenyl actin was 85% modified in this experiment. The ATPase of S-1 in the absence of actin has been subtracted from all values (n = 6).

conformation of the C-terminus in F-actin is a function of the divalent cation bound in the nucleotide cleft.

The observation that divalent cations in the nucleotide cleft affect the C-terminus also strengthens the evidence for conformational transmissions between these two regions. Actin strongly binds a divalent cation that interacts with the β and γ phosphates of ATP in the nucleotide cleft. A protection of the C-terminal tryptic cleavage sites was observed when Ca^{2+} in ATP-Ca^{2+} G-actin was replaced with Mg^{2+} (Strzelecka-Golaszewska et al., 1993). Earlier work of Carlier et al. (1986) showed the effect of divalent cations in the cleft on the C-terminus via fluorescence changes in 1,5-IAEDANS attached to Cys-374.

The mechanism of communication between the C-terminus and the nucleotide cleft may be understood in terms of the molecular hinge model of Tirion and ben-Avraham (1993). Their normal mode analysis predicts the existence of molecular hinges within actin that allow for the opening and closing movements of the nucleotide cleft. One of the predicted hinges (at residues 137 and 339) is fewer than 20 Å from the C-terminal residue 372 (Kabsch et al., 1990; McLaughlin et al., 1993) and might be easily perturbed by fluorescent reagents such as pyrene and IAEDANS. The distance between Cys-374 and the molecular hinge is uncertain given that the crystal structure of actin is lacking the last three amino acid residues (Kabsch et al., 1990).

The communication between actin's C-terminal region and the DNaseI loop in subdomain II is evidenced by tryptic digestion of the actin-DNaseI complex. The binding of DNaseI exposes the actin site 359/360 to cleavage by trypsin (Fig. 2). This does not necessarily imply an extensive change in the affected site. The fact that Cys-374 probes do not alter subtilisin digestion in the DNaseI binding loop and, vice versa, that DNaseI does not change pyrene actin fluorescence is pertinent. The conformational coupling between the C-terminus and the DNaseI loop may not be as strong and extensive as that between the C-terminus and the nucleotide cleft. This may mean that the DNaseI-induced perturbation in the C-terminal region is localized to the Lys-359 site and does not spread to Cys-374.

Alternatively, it is conceivable that the very strong binding of DNaseI to actin precludes the detection of any changes transmitted from the C-terminus to the DNaseI site. In support of the former interpretation, it should be noted that

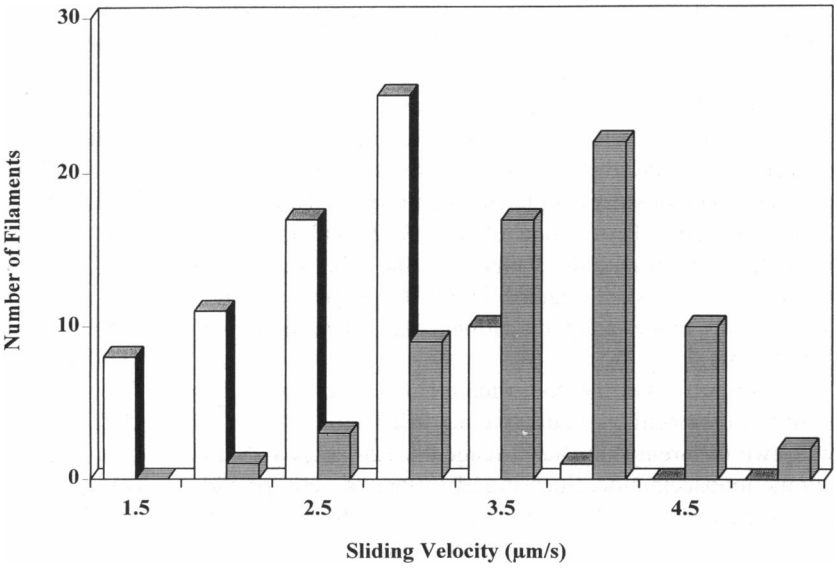

FIGURE 6 The in vitro motility of pyrenyl actin. Actin sliding velocities over HMM-coated coverslips were determined in the in vitro motility assays, as described in Materials and Methods. Buffer conditions are given in the legend to Fig. 4. Distributions of sliding velocities of pyrene modified (*open bars*) and control actin filaments (*filled bars*) are in a histogram form. In this preparation, pyrene actin was 85% modified. The mean velocities for control and pyrenyl actin are 3.9 ± 0.4 and 2.8 ± 0.5 μm/s. (n > 75 filaments).

the crystal structures of actin complexed with DNaseI (Kabsch et al., 1990) and fragment I of gelsolin (McLaughlin et al., 1993) are in excellent agreement with each other.

The polymer form of actin is also altered by C-terminal modifications. Both the in vitro motility and actin activation of S-1 ATPase were affected by modifications at the C-terminus of actin. The V_{max} of S-1 ATPase and the mean velocity of in vitro sliding were both enhanced by 25% with 1,5-IAEDANS (Figs. 3 and 4) and reduced with pyrene iodoacetamide modifications of actin (Figs. 5 and 6). The pyrene modification effect is surprising because this probe appears to have only limited impact on actin polymerization (Cooper et al., 1983) and does not alter much actomyosin binding and dissociation rates (Criddle et al., 1985). Other Cys-374 probes have also been noted to alter acto-S-1 interactions. A report by Lin (1978) showed a 25% decrease in acto-S-1 ATPase activity upon Cys-374 modification with dansyl aziridine.

The structural coupling between the C-terminus and distant regions of actin, as documented by the probes, has important biological implications. Clearly, attachment of small probes to actin's C-terminus results in long range structural alterations and functional modulation of actin, as revealed in ATPase and motility assays. This point is especially relevant considering the interactions of the actin C-terminus with other proteins including filamin (Mejean et al., 1992), caldesmon (Graceffa and Jansco, 1991), and α-actinin (Lebart et al., 1990), etc. These proteins modulate actomyosin interactions by influencing actomyosin ATPase activity and motility. As in the case of IAEDANS and pyrene modifications of actin, actin-binding proteins can potentiate (Sosinski et al., 1984) or inhibit (Dabrowska et al., 1985) actomyosin ATPase activity. The fact that small probes can do the same, at least to some extent, indicates that the proper positioning of the C-terminus, which in our case depends on the nature of the probe, may be an important component of the regulatory protein mechanism of action. Thus, by probing the structural alterations resulting from C-terminal modifications, we may be able to learn more about the molecular mechanisms of regulation by these proteins.

In conclusion, we have documented the conformational coupling between the actin C-terminus and two distant regions in the monomer: the nucleotide cleft and the DNaseI binding loop. Our results also show that modifications of the C-terminus affect the filament function, as observed in ATPase and motility experiments. Probe-dominated effects on the ATPase activity and motility demonstrate that the nature of the probe determines the conformation of the C-terminus, and the resulting changes are transmitted throughout the molecule. Alternatively, local effects of the probe may alter packing within the filament such that the polymer structure is affected. These probe-dependent changes indicate that the C-terminus is an important region for determining actin structure and modulating its function by actin-binding proteins.

Our results also emphasize that caution is needed when using modified actins as structural and functional probes of this protein. The interpretation of any mutation, modification, and antibody-based experiments is more complicated given the conformational coupling of distant regions in actin.

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