Tropomyosin-Binding Site(s) on the *Dictyostelium* Actin Surface As Identified by Site-Directed Mutagenesis†

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ABSTRACT: To identify tropomyosin-binding site(s) on the surface of actin molecule, we examined the effect of mutagenesis introduced to subdomain 4 of actin. Because the sequence of Gln228−Ser232 of *Dictyostelium* actin differs from that of *Tetrahymena* actin that does not bind tropomyosin, the *Dictyostelium/Tetrahymena* chimeric actin was produced. Also, Lys238 and Glu241 were replaced with alanine (mutant 645) to study the role of charged residues which are located at both ends of a β-sheet. As a control experiment, a negative charge was introduced near to the N-terminus (mutant 663). To facilitate the separation of mutant actins without affecting the normal function, Glu360 was replaced with histidine. As a control mutant to such mutants, the mutant 647 (E360H) was produced. Mutant actins were expressed in *Dictyostelium* cells. All mutant actins were functional: they (i) polymerize and (ii) activate ATPase activity of rabbit skeletal myosin subfragment-1 (S1). The mutant 663 (G2E) showed tropomyosin binding and activated myosin ATPase almost as well as rabbit skeletal actin. However, the tropomyosin binding of the mutant 645 (K238A/E241A/E360H) became magnesium dependent. The chimeric actin (mutant 646: QTAAS-to-KAYKE replacement and E360H) showed decreased tropomyosin binding even in the presence of magnesium ions. These results indicate that the tropomyosin-binding sites of "on"-state actin are on subdomain 4. Surprisingly, the chimeric actin showed more cooperative calcium regulation than rabbit skeletal actin in the presence of tropomyosin−troponin. The mutant actin 645 can hardly activate S1 ATPase irrespective of calcium concentration in the presence of tropomyosin−troponin, even though this actin by itself can activate S1 ATPase. The steric blocking or cooperative/allosteric mechanism of thin filament regulation is discussed.

Muscle contraction is regulated by the intracellular concentration of calcium ions. The major proteins involved in the regulation of mammalian skeletal muscle are actin, tropomyosin, and troponin. When the free calcium concentration is lower than micromolar, the interaction between actin and myosin in the presence of Mg-ATP is inhibited by the tropomyosin−troponin system, provided that the ionic strength and Mg-ATP concentration are in a physiological range. The binding of calcium ions to troponin suppresses the inhibition and triggers the actin−myosin interaction (Ebashi & Endo, 1968). It is important, therefore, to determine the location of tropomyosin and troponin on actin filaments to elucidate the regulatory mechanism at a molecular level. The position of tropomyosin and its movement induced by calcium ions have been studied by various methods including three-dimensional electron microscopy (Spudich et al., 1972; Wakabayashi et al., 1975; Wakabayashi & Toyoshima, 1981; O'Brien et al., 1983; Toyoshima & Wakabayashi, 1985; Milligan & Flicker, 1987; Milligan et al., 1990; Lehman et al., 1994; Ishikawa & Wakabayashi, 1994). Recently, Lorenz et al. (1995) presented a model of the actin−tropomyosin complex that shows strong electrostatic interactions between charged side chains of tropomyosin residues and actin residues in the inner domain. All of these studies suggest that tropomyosin binds to the inner domain of actin in the presence of calcium ions ("on" state), which corresponds to subdomains 3 and 4 (Kabsch et al., 1990). However, a definite conclusion has not yet been reached, because clear three-dimensional images with high enough resolution were not yet obtained.

Chemical cross-linking studies showed that residues 1−11 of actin are near the region responsible for the binding of myosin (Sutoh, 1982) and troponin I (Grabareck & Gergely, 1988), but no such residues are found for tropomyosin binding. Chemical modification studies, however, suggested that Arg95 (Johnson et al., 1978), Lys238 (El-Saleh et al., 1984), and Lys336 (Szilagyi & Lu, 1982) are involved in tropomyosin binding and that Lys61 (Miki, 1989) is important for the calcium-mediated regulation of the actin−myosin interaction.

Recent progress in recombinant DNA techniques enables one to replace amino acid residues in a site-directed manner instead of chemical modification, which may not be perfectly site specific. Recombinant DNA techniques were used to study the interaction between actin and mutant tropomyosins (Hitchcock-DeGregori & Varnell, 1990; Hitchcock-DeGregori & An, 1996). Mutagenesis of the actin gene has been described in yeast (Aspenstrom & Karlsson, 1991; Aspenstrom et al., 1992, 1993; Cook et al., 1991, 1992, 1993; Chen et al., 1993) and in *Dictyostelium* (Sutoh et al., 1991; Hirono

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et al., 1992; Johara et al., 1993). Actin gene expression in *Escherichia coli* was reported (Frankel et al., 1990), but treatment with detergent was necessary to prepare actin as a functional protein. We chose to use *Dictyostelium* cells for expressing recombinant actins, because purification without detergent is possible and myosin expression is also reported (Manstein et al., 1989; Itakura et al., 1993; Ritchie et al., 1993; Uyeda et al., 1993). The mutant actins (E316K and G368E) from *Drosophila* were characterized previously (Drummond et al., 1990; Anson et al., 1995), but the effect on tropomyosin binding is not yet reported.

We focused our attention to subdomain 4, because cryoelectron microscopy showed that the density of subdomain 4 of actin−tropomyosin−troponin increased following the addition of calcium ions (Ishikawa & Wakabayashi, 1994). Electron microscopic studies showed that one tropomyosin molecule covers seven actin monomers on the thin filaments (Ohtsuki & Wakabayashi, 1972), and Fourier analysis of the distribution of charged amino acid residues along a tropomyosin molecule showed 14 repeats (Parry, 1974; McLachlan & Stewart, 1976). These results suggest that the charged groups may be important for tropomyosin-binding sites on actin. Therefore, we changed the actin sequence (Wertman et al., 1992) so that charged amino acids at both ends of the sole β-sheet in subdomain 4 were replaced with noncharged ones (K238A/E241A) or noncharged amino acids were changed to charged ones. As an example of the latter case the sequence Gln228−Ser232 was replaced by the corresponding sequence of *Tetrahymena* (*Dictyostelium/Tetrahymena* chimeric mutant that involves Q228K/A231K/S232E replacement), because *Tetrahymena* actin does not bind tropomyosin (Hirono et al., 1987). There are other regions where the sequence of *Tetrahymena* actin differs from "conventional" actins such as rabbit skeletal actin and *Dictyostelium* actin. However, the difference is most conspicuous in the region containing the sequence Gln228−Ser232 except for the N-terminal region where there is no consensus sequence.

As a control experiment, an extra negative charge was introduced at the N-terminal region (G2E mutant), which is located far from subdomain 4. This mutation makes the sequence of *Dictyostelium* actin similar to that of rabbit skeletal actin in the N-terminal region. The G2E mutant showed normal tropomyosin binding and activated myosin ATPase almost as well as rabbit skeletal actin. Another control mutant E360H was almost indistinguishable from the wild-type actin as far as tropomyosin binding and the activation of myosin ATPase are concerned.

MATERIALS AND METHODS

Plasmid Construction. Figure 1 shows the mutants produced in the present work. Mutations were introduced into the actin 15 gene by oligonucleotide-directed mutagenesis (Kunkel, 1985) so that Lys238 and Glu241 were replaced with alanine (p645: K238A/E241A/E360H), Glu360 was replaced with histidine (p647: E360H), or Gly2 was replaced with glutamic acid (p663: G2E). The *Dictyostelium/Tetrahymena* chimeric actin mutant (p646) was produced by replacing the Gln228−Ser232 sequence (QTAAS) with the *Tetrahymena* sequence KAYKE. In mutants 645 and 646, Glu360 was changed to histidine to facilitate separation of mutant actins from the wild-type actin by HPLC using DEAE-5PW. It is already known that the mutant E360H

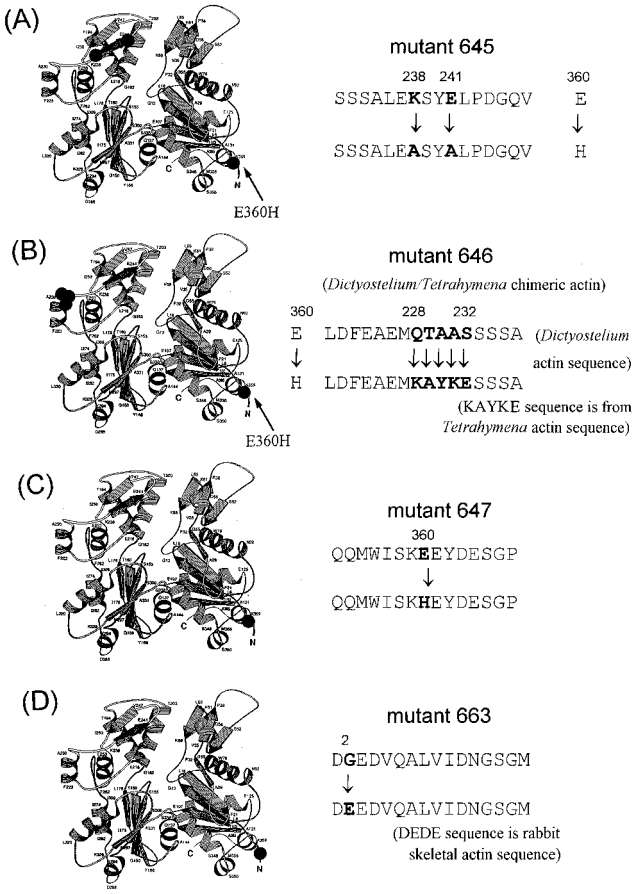

FIGURE 1: Ribbon model representation (Kabsch et al., 1990) of the positions of site-directed mutagenesis (left panels) and single letter representation of the changes in the sequence of *Dictyostelium* actin (right panels): (A) mutant 645 (K238A/E241A/E360H), (B) mutant 646 (*Dictyostelium/Tetrahymena* chimeric actin), (C) control mutant 647 (E360H), and (D) mutant 663 (G2E). The changed residues are shown with filled circles in the ribbon models and in bold letters in the sequences.

produces actin that polymerizes and activates ATPase activity of myosin S1 as well as the wild-type actin (Johara et al., 1993).

Transformation vectors were constructed by inserting the mutant actin 15 gene (Knecht et al., 1986) into an integration vector B10Tp2′ (Early & Williams, 1987) as described by Sambrook et al. (1989).

Transformation of Dictyostelium Cells. Transformation vectors were introduced into *Dictyostelium* cells by electroporation (Howard et al., 1988). Transformed cells were selected by culturing them in the presence of G418 (neomycin analogue) as described (Sutoh et al., 1991). Cells were cloned twice by picking them from a colony visible on a culture dish and plating them on a new dish. The expression level of mutant actin in these cloned cells was estimated by two-dimensional gel electrophoresis (O'Farrel, 1975; Mikawa et al., 1981), because all types of mutations described in this paper altered the isoelectric point of actin.

Preparation of Proteins. Transformed *Dictyostelium* cells were used to purify both mutant and wild-type actins as described (Sutoh et al., 1991). Rabbit skeletal actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (1971). Myosin was prepared from rabbit skeletal muscle (Szent-Gyorgyi, 1951). S1 (myosin subfragment-1) was obtained by digesting myosin with chymotrypsin following the procedure described previously (Weeds & Taylor, 1975; Okamoto & Sekine, 1985).

Tropomyosin and tropomyosin−troponin were prepared from rabbit skeletal muscle as described by Ebashi et al. (1968) and Ebashi and Ebashi (1964), respectively.

Assay of Tropomyosin-Binding Ability. The tropomyosin-binding ability was examined by the method described by Hitchcock-DeGregori and Varnell (1990) with slight modification so that no radioactive isotope is required. The various concentrations of tropomyosin (1, 2, 3, or 4 μM, final) were mixed with a fixed amount (5 μM, final) of *Dictyostelium* wild-type actin, rabbit skeletal muscle actin, or mutant actins. The mixtures were incubated for 12 h 25 °C in 150 mM NaCl, 10 mM imidazole hydrochloride (pH 7.0), 0.01% NaN_3, and 5 mM $MgCl_2$ or 0.2 mM EDTA. The mixtures were then centrifuged for 30 min at 360000*g* at 25 °C in a Beckman TL-100 ultracentrifuge.

In the presence of tropomyosin−troponin, the various concentrations of tropomyosin−troponin (1, 2, 3, or 4 μM, final) were mixed with a fixed amount (5 μM, final) of *Dictyostelium* wild-type actin or mutant 646 actin. The mixtures were incubated for 5 h at 25 °C in 150 mM NaCl, 10 mM imidazole hydrochloride (pH 7.0), 0.01% NaN_3, 5 mM $MgCl_2$, and 0.1 mM $CaCl_2$ or 0.2 mM EGTA. The mixtures were then centrifuged for 7 min at 360000*g* at 25 °C in a Beckman TL-100 ultracentrifuge. The shorter ultracentrifugation time was chosen so that unbound tropomyosin−troponin stays in the supernatant.

The resulting pellets were resuspended in the same buffer in volumes equal to those of the supernatants, and then aliquots of both supernatants and suspended pellets were subjected to SDS−PAGE. The amount of tropomyosin bound to actin was quantified by densitometry of the stained bands. The density per weight of actin and that of tropomyosin were different. This difference was corrected when the molar ratio was calculated assuming that the molecular weights of actin and tropomyosin are 42 000 and 70 000, respectively. The saturation level of tropomyosin binding was about 0.2 mol/mol of actin. This value is higher than 0.14 which is calculated from the 1:7 molar ratio of tropomyosin to actin determined by electron microscopy (Ohtsuki & Wakabayashi, 1972). The higher values may be due to the free tropomyosin in the pellet, which we did not subtract.

Other Procedures. SDS gel electrophoresis was done on 12.5% polyacrylamide slab gels (Laemmli, 1970), and gels were stained with Coomassie Brilliant Blue. Two-dimensional electrophoresis was carried out by the method of O'Farrel (1975) and Mikawa et al. (1981). The first dimension (isoelectric focusing) was performed in the presence of 9 M urea. A 10% (w/v) polyacrylamide gel was used for the second dimension. Gels were stained with Coomassie Brilliant Blue.

Actin activation of S1 ATPase activity was measured in the reaction mixture containing 50 mM KCl, 10 mM imidazole hydrochloride (pH 7.0), 5 mM $MgCl_2$, 1 mM ATP, 50 μM $CaCl_2$ or 0.2 mM EGTA, 0.1 mg/mL S1, 0.6 mg/mL actin, 0.6 mg/mL tropomyosin−troponin, and 5 μg/mL phalloidin. In the absence of tropomyosin−troponin, actin activation of S1-ATPase activities was measured in 50 mM KCl, 10 mM imidazole hydrochloride (pH 7.0), 5 mM $MgCl_2$, 1 mM ATP, 0.1 mg/mL S1, 0.6 mg/mL actin, and 5 μg/mL phalloidin. All reactions were carried out at 25 °C. Phosphate assays were done by the malachite green method (Kodama et al., 1986).

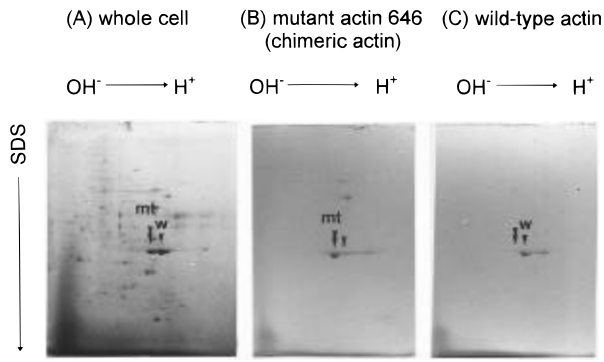

FIGURE 2: Two-dimensional gel electrophoretic patterns of (A) whole cell lysate of the mutant 646, (B) HPLC-purified mutant actin 646 (chimeric mutant), and (C) HPLC-purified wild-type actin. Single and double arrowheads indicate wild-type actin and mutant actin, respectively. The mutant actin 646 is more basic than wild-type actin.

Protein concentration was measured by determining absorbance at 280 nm or by the Bradford method (Bradford, 1976) according to Read and Northcote (1981), calibrated with ultraviolet absorbance unless otherwise stated. The value of absorbance of actin and tropomyosin was assumed to be 11.1 cm^{-1} (1%) and 2.9 cm^{-1} (1% at 278 nm), respectively (Houk & Ue, 1974; Eisenberg & Kielley, 1974).

RESULTS

Expression and Separation of Mutant Actins from Wild-Type Actin. Because a *Dictyostelium* cell contains multiple sets of actin genes, the separation of mutant actin from wild-type actin is required. It is known that the mutants with aspartate/glutamate-to-histidine replacement can be separated from wild-type actin by HPLC using DEAE-5PW (Sutoh et al., 1991; Johara et al., 1993) probably due to the decrease in negative charges of mutant actin. However, we could not separate the mutant actin (D311A/E316A) from wild-type actin by this method (data not shown), even though the extent of decrease in negative charges is more than some of the previous cases. It was reported that E360H actin can be separated from wild-type actin and that this mutant actin is functionally normal (Johara et al., 1993). Therefore, we introduced E360H mutation on top of the main mutation and could achieve good separation from wild-type actin.

The two-dimensional electrophoretic pattern of the whole cell lysate of mutant 646 (QTAAS to KAYKE and E360H, *Dictyostelium/Tetrahymena* chimeric actin) reveals the two major actin spots, which correspond to mutant and wild-type actin (Figure 2A). The wild-type cell lysate showed only one major spot in the actin area (data not shown). By comparison with this pattern, it was found that the mutant actin was more basic than the wild-type actin, as expected. After the HPLC using DEAE-5PW, the mutant or wild-type actin showed only one major spot (Figure 2B,C). There were some minor spots, which may be due to variations of posttranslational modification. The amount of such minor spots was very small. Thus, the introduction of E360H mutation was effective for the separation of the mutant actin from the wild-type actin. The mutant actins 645 (K238A/E241A/E360H), 647 (E360H), and 663 (G2E) were also expressed and purified in the same way as the mutant actin 646.

When the incubation time of 30 min was selected, the extent of polymerization of any of the *Dictyostelium* actins

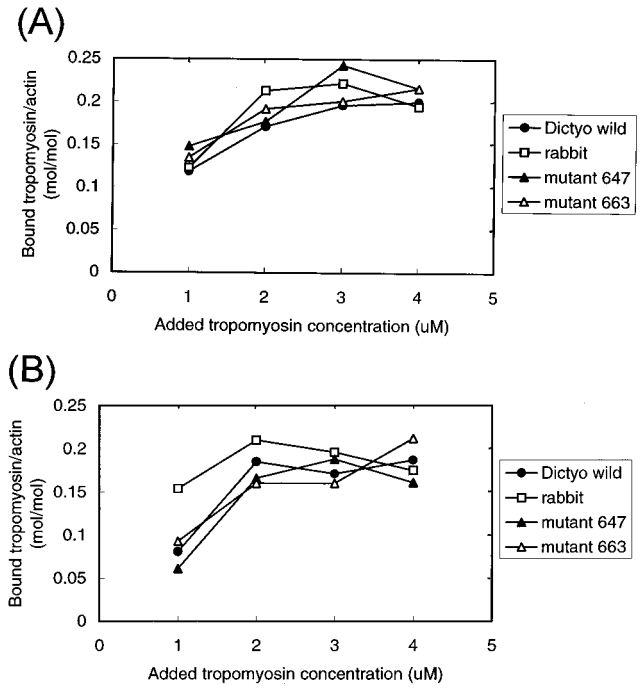

FIGURE 3: Tropomyosin-binding ability of the *Dictyostelium* actins compared with that of rabbit skeletal actin by cosedimentation experiments (A) in the presence of magnesium ions and (B) in their absence. Five micromolar (final) wild-type actin (filled circle), rabbit skeletal actin (open square), mutant actin 647 (filled triangle), or mutant actin 663 (open triangle) was mixed with various concentrations of tropomyosin (1, 2, 3, or 4 μM, final) and incubated for 12 h at 25 °C in 5 mM $MgCl_2$ or 0.2 mM EDTA, 10 mM imidazole hydrochloride (pH 7.0), 150 mM NaCl, and 0.01% NaN_3. The mixture was ultracentrifuged. The supernatant and the pellet were analyzed by SDS−PAGE. *Dictyostelium* actins bound tropomyosin well even in the absence of magnesium ions.

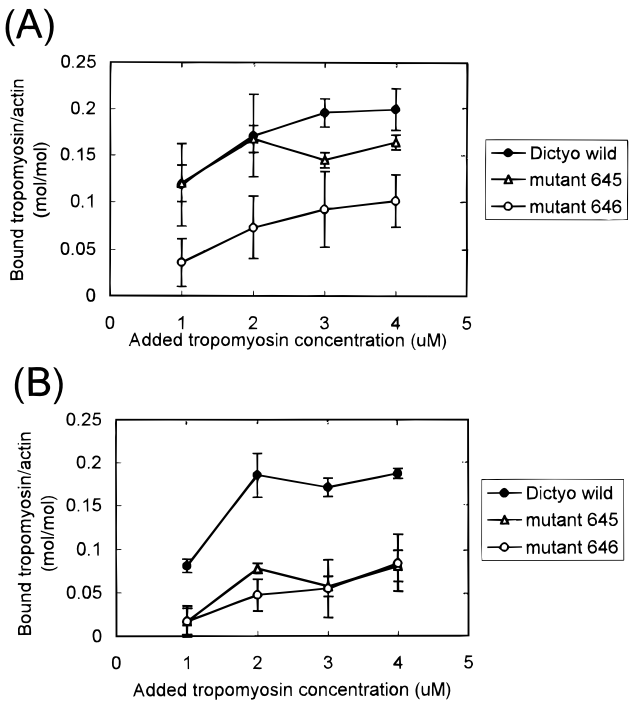

FIGURE 4: Tropomyosin-binding ability of the mutant actins compared with that of the wild-type actin by cosedimentation experiments. Five micromolar (final) wild-type actin (filled circles), mutant actin 645 (open triangle), or mutant actin 646 (open circle) was mixed with various concentrations of tropomyosin (1, 2, 3, or 4 μM, final) and incubated for 12 h at 25 °C in 5 mM $MgCl_2$ or 0.2 mM EDTA, 10 mM imidazole hydrochloride (pH 7.0), 150 mM NaCl, and 0.01% NaN_3. The mixture was ultracentrifuged, and the supernatant and the pellet were analyzed by SDS−PAGE. Each plotted point represents the mean ± SD (n = 4) from the independent experiments (A) in the presence of magnesium ions or (B) in their absence. The latter condition is believed to be less favorable for tropomyosin binding to actin. Much less tropomyosin cosedimented with the mutant actins 645 and 646 than with wild-type actin in the absence of magnesium ions, but binding of tropomyosin to the mutant actin 645 was partially restored by magnesium ions.

(wild type and mutants) monitored by the pelleting experiment was lower than that of rabbit skeletal actin, even though the rate of polymerization monitored by pyrene fluorescence was comparable (data not shown). Yet, we could recover most of the actin in the pellet under the conventional conditions of ultracentrifugation when the incubation time was 5 or 12 h. We, therefore, incubated the actin solution for 5 or 12 h at 25 °C to ascertain full polymerization and used actin that was polymerized in this manner to characterize mutant actins.

Binding of Rabbit Skeletal Tropomyosin to Dictyostelium Actin. To examine binding ability, we measured the binding of tropomyosin purified from rabbit skeletal muscle to *Dictyostelium* actins by cosedimentation experiments (Hitchcock-DeGregori & Varnell, 1990). Wild-type or one of the mutant actins (5 μM, final) was mixed with various concentrations of tropomyosin in the presence of 5 mM magnesium ions and ultracentrifuged. The resulting supernatant and suspended pellet were subjected to SDS−PAGE analysis.

Figure 3 shows that the *Dictyostelium* wild-type actin binds rabbit skeletal tropomyosin well. The binding to rabbit skeletal actin was also measured as a control experiment. The mutagenesis introduced to subdomain 1 [mutant 663 (G2E) or mutant 647 (E360H)] did not affect tropomyosin binding both in the presence (Figure 3A) and in the absence (Figure 3B) of magnesium ions.

Figure 4A shows that tropomyosin does not bind well to the mutant actin 646 (*Dictyostelium/Tetrahymena* chimeric actin) even in the presence of magnesium ions that facilitate the actin−tropomyosin interaction (Laki et al., 1962). Tropomyosin binds to the mutant actin 645 (K238A/E241A/E360H) more firmly in the presence of magnesium ions (Figure 4A) than in their absence (Figure 4B): tropomyosin binding became magnesium dependent. These results indicate that the C-terminal half of the Phe223−Ala230 α-helix and the following loop (Gln228−Ser232) are important for tropomyosin binding and intact Lys238 or Glu241 is indispensable for the stable binding of tropomyosin in the *absence* of magnesium ions.

Figure 5 shows the effect of troponin on the tropomyosin binding of the chimeric mutant actin 646. The tropomyosin binding of the chimeric actin is restored by the addition of troponin. This may be consistent with the analysis that troponin increases the apparent tropomyosin-binding constant of actin by 1000 times (Hill et al., 1992; Dahiya et al., 1994): the decreased tropomyosin binding ability may be compensated by such an effect of troponin. The other possibility is the indirect binding of tropomyosin: tropomyosin binds to troponin, which may be able to bind even to the chimeric actin.

Activation of S1 ATPase Activity. Figure 6 shows the ability of various actins to activate S1 ATPase either in the presence of the tropomyosin−troponin complex or in its absence. It should be noted that the background S1 ATPase was not subtracted.

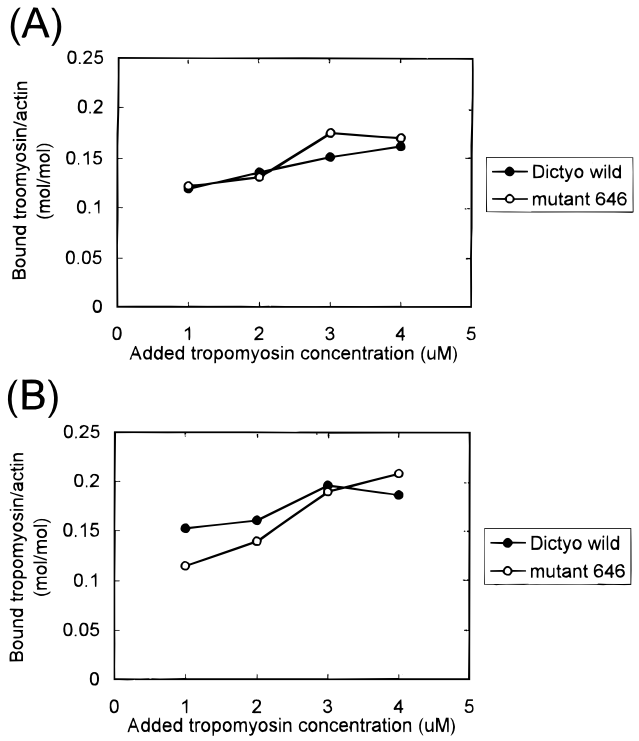

FIGURE 5: Effect of troponin on binding of tropomyosin to actin (A) in the presence of calcium ions or (B) in their absence. The latter condition is believed to be more favorable for troponin binding to actin. The various concentrations of tropomyosin−troponin (1, 2, 3, or 4 μM, final) were mixed with a fixed amount (5 μM, final) of *Dictyostelium* wild-type actin (filled circle) or chimeric actin 646 (open circle). The mixtures were incubated for 5 h at 25 °C in 150 mM NaCl, 10 mM imidazole hydrochloride (pH 7.0), 0.01% NaN_3, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, or 0.2 mM EGTA. The mixtures were then centrifuged for 7 min at 360000*g* at 25 °C in a Beckman TL-100 ultracentrifuge. The chimeric actin 646 binds tropomyosin well in the presence of troponin irrespective of calcium concentration, even though the chimeric actin can bind tropomyosin very weakly in the absence of troponin as shown in Figure 4.

In the absence of tropomyosin−troponin, all actins activated S1 ATPase. In the presence of tropomyosin−troponin and in the absence of calcium ions, the activation by all actins was suppressed. Thus, all kinds of *Dictyostelium* actins preserved the ability to be regulated. The suppression by the regulatory proteins could be released by calcium ions except for the mutant 645 (K238A/E241A/E360H), which showed almost negligible activation of S1 ATPase: tropomyosin−troponin suppressed the S1 ATPase activation by the mutant actin 645 irrespective of calcium concentration.

Interestingly, the chimeric actin (mutant 646), which can bind tropomyosin very weakly by itself, showed the best calcium regulation when rabbit skeletal tropomyosin−troponin was added: it showed the highest activation in the presence of calcium ions but almost no activation in their absence, though the standard deviation of the data in the presence of calcium ions was rather high. Also, this chimeric actin is the only actin to which tropomyosin−troponin showed no inhibitory effect in the presence of calcium ions.

It was confirmed that the control mutant 647 (E360H) showed almost the same ATPase activation as the wild-type actin. After the replacement of Gly2 with glutamate to mimic the rabbit actin sequence at the N-terminus (mutant 663: G2E), S1 ATPase activation by *Dictyostelium* actin became almost as high as that by rabbit skeletal actin. This is consistent with previous work (Sutoh et al., 1991; Aspenstrom & Karlsson, 1991; Cook et al., 1992), which pointed out the importance of the negative charges in the N-terminal region of actin for activation of myosin ATPase.

DISCUSSION

Separation of Mutant Actins from Wild-Type Actin. Though it has been reported that the replacement of aspartate/glutamate with histidine enables the separation of the mutant actins from the wild-type actin by HPLC using DEAE-5PW (Sutoh et al., 1991; Johara et al., 1993), the mutant actin (D311A/E316A) could not be separated from the wild-type actin. The mutant actins that could be successfully separated from wild-type actin have mutations in subdomain 1, but the mutant actin (D311A/E316A) has mutations on subdo-

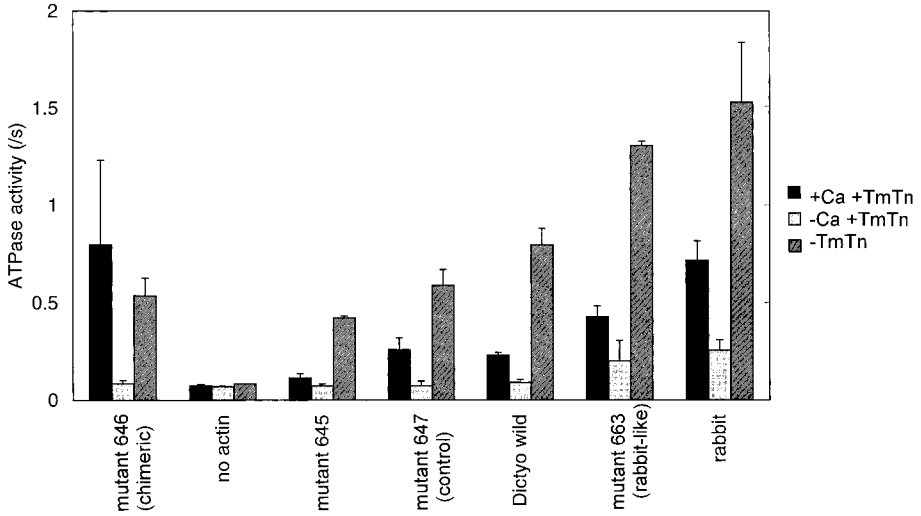

FIGURE 6: Effect of tropomyosin−troponin on the activation of S1 ATPase activity by various actins. Actin activation of S1 ATPase activity was measured in the reaction mixture containing 50 mM KCl, 10 mM imidazole hydrochloride (pH 7.0), 5 mM $MgCl_2$, 1 mM ATP, 50 μM $CaCl_2$ or 0.2 mM EGTA, 0.1 mg/mL S1, 0.6 mg/mL actin, 0.6 mg/mL tropomyosin−troponin, and 5 μg/mL phalloidin. In the absence of tropomyosin−troponin, actin activation of S1 ATPase activity was measured in 50 mM KCl, 10 mM imidazole hydrochloride (pH 7.0), 5 mM $MgCl_2$, 1 mM ATP, 0.1 mg/mL S1, 0.6 mg/mL actin, and 5 μg/mL phalloidin. All reactions were carried out at 25 °C. In the absence of the tropomyosin−troponin complex, all actins except the chimeric mutant 646 showed higher activation than in its presence. Except for mutant 645, all actins activated ATPase in a calcium-sensitive manner in the presence of tropomyosin−troponin. In the presence of tropomyosin−troponin the mutant actin 645 can hardly activate S1 ATPase irrespective of calcium concentration. Interestingly, the chimeric actin showed more cooperative calcium regulation (less activation in the absence of calcium ions and comparable activation in the presence of calcium ions) than rabbit skeletal actin.

main 3. Thus, the important factor for the separation with anion-exchange HPLC is not just the net charge but the amount of the negative charge of subdomain 1. This is consistent with the results obtained by Nakamura et al. (1992, 1994), who calculated the electrostatic potential and field around the surface of F-actin: the electrostatic potential of subdomain 1 is the lowest and the field around subdomain 1 is the strongest. To facilitate the separation of mutant actins, therefore, we additionally introduced E360H mutation in subdomain 1. Indeed, the mutant actins 645 (K238A/E241A/E360H) and 646 (the chimeric mutant: QTAAS to KAYKE and E360H) could be separated from the wild-type actin as expected.

Activation of Myosin ATPase by Mutant Actins and Its Calcium-Mediated Regulation. In the absence of tropomyosin−troponin, all the *Dictyostelium* actins including wild-type and mutant actins activated the S1 ATPase activity. This finding excludes the possibility that the decreased tropomyosin-binding ability may be due to the abnormal folding of the mutant actins.

All the *Dictyostelium* actins examined could activate S1 ATPase, and this activation was suppressed by rabbit skeletal tropomyosin−troponin in the absence of calcium ions. This indicates that the regulatory mechanism of muscle contraction tolerates the species-specific differences between rabbit and *Dictyostelium* and suggests that the regulatory mechanism may not depend on the precise interactions between particular amino acid residues. This may be consistent with the steric block mechanism (Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973; Wakabayashi et al., 1975), in which the position of tropomyosin on actin is of primary importance. However, the steric blocking mechanism cannot explain the very low ability of the mutant 645 (K238A/E241A/E360H) to activate S1 ATPase irrespective of calcium concentration when tropomyosin−troponin is added (Figure 6). To interpret the mutant 645 data according to the steric blocking, one must postulate that tropomyosin is trapped in the "off"-state position in this mutant actin. The mutagenesis, however, was introduced in subdomain 4, which is far from the outer domain that is the proposed position of off-state tropomyosin (Kabsch & Vandekerckhove, 1992; Lehman et al., 1994; Ishikawa & Wakabayashi, 1994). Thus, some kind of transmission of the effect of mutagenesis in subdomain 4 to other regions has to be assumed. This is outside the framework of the steric blocking model.

It was suprising that the chimeric actin 646, which binds tropomyosin very weakly, can be regulated better than the wild-type actin, the control mutant (E360H), and rabbit skeletal actin when tropomyosin−troponin is added. To account for the effect of the two mutations (mutant 646 and mutant 645) in subdomain 4, a combination of the steric block model and the cooperative/allosteric model may be required. According to the latter model (Greene & Eisenberg, 1980; Hill et al., 1980; Nagashima & Asakura, 1982; Lehrer & Morris, 1982; Geeves & Halsall, 1987), regulated thin filaments are inhibited from binding myosin when the myosin concentration is low even in the presence of calcium ions, but a further increase in myosin concentration removes this inhibition in a manner analogous to the transition from the T state to R state of hemoglobin (Perutz, 1989). If this T-to-R transition does not take place easily in the mutant actin 645 (K238A/E241A/E360H) and occurs more cooperatively in the chimeric actin 646, our results can be explained.

The assay condition was chosen so that tropomyosin would inhibit actin-activated ATPase of S1. In this condition, activation of S1 ATPase by the wild-type actin decreased by a factor of 3 when tropomyosin−troponin and calcium ions were added. This kind of inhibition was hardly observed in the chimeric actin. This result indicates that the inhibitory activity of tropomyosin depends on the sequence of Gln228−Ser232.

Tropomyosin-Binding Site on Actin. As mentioned previously, the sequence of the α-helix (Phe223−Ala230) is not conserved in *Tetrahymena* (Hirono et al., 1987, 1990). Interestingly, this α-helix is missing in the ATPase fragment of a 70-kDa heat shock cognate protein, of which the three-dimensional structure is very similar to that of actin (Flaherty et al., 1991). Flaherty et al. (1991) suggested the possible involvement of the region Cys217−Leu236 in the interaction of actin with tropomyosin. Our results provide the experimental evidence for such a suggestion. It is also known that the temperature factor of the α-helix (Phe223−Ala230) is high (Kabsch et al., 1990); this might indicate that flexibility of this α-helix is important for the regulatory mechanism.

We showed that Lys238 or Glu241 is indispensable for the stable binding of tropomyosin to actin in the absence of magnesium ions. The alanine-scanning experiment also shows the importance of these two charged residues for the survival of yeast (Wertman et al., 1992). The chemical modification of Lys238 by pentanedione decreases tropomyosin binding in the absence of divalent cations (El-Saleh et al., 1984). This observation is similar to ours. El-Saleh et al. (1984) reported also that actin−tropomyosin−troponin can activate myosin ATPase even in the absence of calcium ions after the modification of Lys238. This observation is different from ours: almost no activation of ATPase was observed irrespective of calcium concentration after Lys238 and Glu241 were replaced with alanine (mutant 645). The observed difference may be related to the difference between the bulkiness of modified Lys238 and that of alanine, of which the side chain is smaller. Another possibility for the difference may be related to the coexistence of the E241A replacement in the mutant 645.

By comparison of the atomic model of actin (Kabsch et al., 1990; Holmes et al., 1990) and the three-dimensional image of actin−tropomyosin−troponin (Tomioka et al., 1991), it can be seen that Gln228−Ser232, Lys238, and Glu241 are on the top part of the inner domain (Toyoshima & Wakabayashi, 1985; Milligan & Flicker, 1987), which corresponds to subdomain 4 of actin. Lys336 that has been shown to be involved in tropomyosin binding (Szilagyi & Lu, 1982) is also located in the inner domain.

Figure 7 shows the model of F-actin (Lorenz et al., 1995), in which the mutated residues are displayed in color. We showed that the α-helix (Phe223−Ala230) flanked by flexible loops in both ends is important for tropomyosin binding. The interaction between this α-helix and α-helices of tropomyosin−troponin may be important, because tropomyosin consists of two α-helices and troponin-T (tropomyosin-binding subunit of troponin) is believed to contain an α-helix.

In conclusion, our results indicate (i) that the sequence Gln228−Ser232 is important for tropomyosin binding and (ii) that Lys238 or Glu241 that flanks the sole β-sheet in subdomain 4 is important for tropomyosin binding in the absence of magnesium ions. The mutations introduced to subdomain 4 of actin also change the mode of calcium

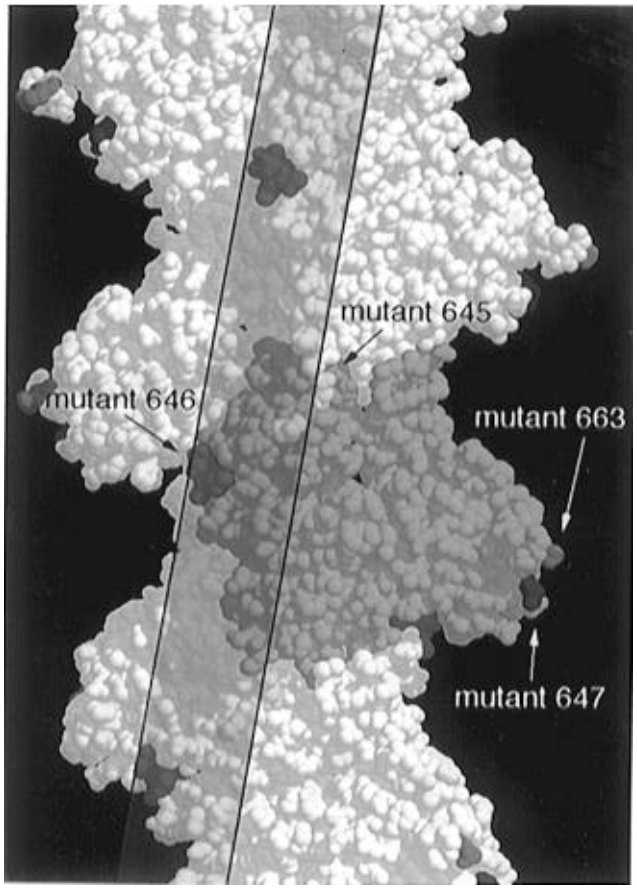

FIGURE 7: Atomic model of F-actin (Lorenz et al., 1995) showing the mutated residues (graphic display by RasMol, Roger Sayle). The residues are identified as follows: mutated Gln228−Ser232 (mutant 646) in red, mutated Lys238 and Glu241 (mutant 645) in yellow, mutated Gly2 (mutant 663) in green, and mutated Glu360 (mutant 647) in blue. Atomic coordinates kindly supplied by Drs. Ken Holmes and Michael Lorenz (Max Planck Institute, Heidelberg) were used. The orientation of the molecules along the helix axis is the same as that in the ribbon model of G-actin (Kabsch et al., 1990). The proposed position of tropomyosin in the presence of calcium ions is shaded.

regulation. Our results also indicate that the tropomyosin-binding sites are on subdomain 4 of actin at least in the presence of calcium ions. This is consistent with the results of cryoelectron microscopy, which showed that the densities in the inner domain of actin are significantly higher in the presence of calcium ions than those in their absence (Ishikawa & Wakabayashi, 1994).

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