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G- to F-actin modulation by a single amino acid substitution in the actin binding site of actobindin and thymosin β4

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The actin binding sites of actobindin and thymosin β4, two small polypeptides that inhibit actin polymerization by interacting with monomeric actin, have been localized using peptide mimetics. Both sites are functionally similar and extend over 20 residues and are located in the NH_2-terminus of the polypeptides. They can be dissected into two functional entities: a conserved hexapeptide motif (LKHAET or LKKTET), which forms the major contact site through electrostatic interactions with actin, and a non-conserved NH_2-terminal segment preceeding the motif, which exerts the inhibitory activity on actin polymerization probably by steric hindrance. The introduction of a glutamic acid at the third position in the motif, creating LKEAET or LKETET sequences, which are similar to those found in some F-actin binding proteins, converts the peptide's inhibitory phenotype into an F-actin stimulatory property. These results allow the proposal of a simple model for G- to F-actin modulation.

Key words: actin-binding motif/actin polymerization/microfilaments/peptide mimetics

Introduction

Eukaryotic cells contain proteins that specifically interact with monomeric actin and inhibit its polymerization or promote its depolymerization (for reviews see Hartwig and Kwiatkowski, 1991; Vandekerckhove and Vancompernolle, 1992). For a long time, profilin was considered to be one of the most important representatives of this class of proteins (Carlsson *et al.*, 1977; Reichstein and Korn, 1979). More recent studies have identified a number of other small monomeric actin binding proteins in various organisms. This includes actobindin in *Acanthamoeba castellanii* (Lambooy and Korn, 1986), actophorin in *Acanthamoeba* (Cooper *et al.*, 1986), depactin in sea urchin eggs (Mabuchi, 1983) and cofilin, destrin and ADF in mammalian cells (Nishida *et al.*, 1987; Nishida *et al.*, 1985; Giuliano *et al.*, 1988). Thymosin β4 has recently been added to this group. It is a 43mer polypeptide for which thymic hormone activity, responsible for some immunomodulatory and endocrine effects, has been described (Low *et al.*, 1981; Rebar *et al.*, 1981). Thymosin β4 is now found in most mammalian tissues as an abundant polypeptide, probably responsible for sequestering the major part of the unpolymerized pool of actin (Safer *et al.*, 1991).

The smallest proteins of this group, actobindin (88 residues) (Vandekerckhove *et al.*, 1990) and Thymosin β4 (43 residues) (Low *et al.*, 1981) are convenient targets for detailed protein and chemical analysis of their interactions with actin. The interfaces between actobindin and actin have been studied by chemical cross-linking, combined with amino acid sequencing. These studies revealed the formation of an isopeptide linkage between Glu100 of actin and Lys16 of actobindin. In addition, connections between one of the first three acidic residues of actin and Lys16 and Lys52 of actobindin were found (Vancompernolle *et al.*, 1991). Both lysine residues are located at the beginning of the 33 residue repeats present in the actobindin sequence. They are also part of an LKHAET motif, which is found in an almost conserved form in several other actin-binding proteins, including *Dictyostelium* α-actinin, fimbrin, plastin, myosin heavy chain, tropomyosin and thymosin β4 (Vancompernolle *et al.*, 1991). In the latter, the LKKTET hexamotif is located, like in actobindin, at about 16 residues away from the NH_2-terminus (Low *et al.*, 1981). These observations gave rise to the idea that this sequence could function as a general actin-binding motif.

We have verified this hypothesis by studying the actin-binding properties of synthetic peptides that have been derived from the NH_2-terminal region of actobindin and thymosin β4 and that contained the LKHAET or LKKTET hexamotifs or variants thereof. These studies suggest a binding principle in which not only the hexamotif but also the non-conserved NH_2-terminally located sequences play an important role. Furthermore, we detected G- or F-actin modulatory properties, depending on the nature and order of charged residues in the hexapeptide motif. These findings provide new ideas on the underlying mechanisms controlling actin polymerization.

Results

Synthetic peptides corresponding to an actin binding site in actobindin

A peptide, which contained the LKHAET hexapeptide motif, extending towards the NH_2-terminal side and covering residues 1-29 of actobindin (AB1-29), was synthesized. As shown by fluorescence studies (Figure 1A) and F-actin sedimentation analysis (Figure 1B), this peptide displayed an activity that was qualitatively indistinguishable from that previously reported for intact actobindin (Lambooy and Korn, 1988). The peptide prolonged the lag phase of actin polymerization, but had little effect on the extent of polymerization of already existing filaments. The K_d of this peptide for binding to skeletal muscle actin, as measured by the method of Lal and Korn (1985), is 200 μM, a value that is ~30 times higher than that of actobindin for rabbit skeletal muscle actin (Lambooy and Korn, 1986). In order to delineate the shortest segment that was still able to display

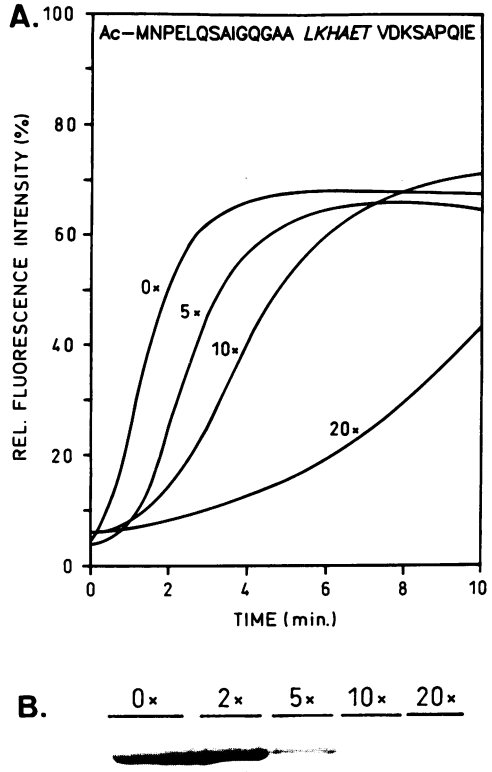

Fig. 1. Inhibition of actin polymerization by the actobindin peptide AB1−29. **A.** Actin polymerization measured by fluorescence enhancement of pyrenyl actin (10%). The amino acid sequence at the top of the figure is that of AB1−29, Ac refers to the acetylated NH_2-terminus. Numbers indicate molar excess of peptide over actin. **B.** Actin polymerization measured by sedimentation of F-actin. The formed actin filaments were airfuge precipitated after 8 min of polymerization. The pellets were analysed by SDS−PAGE on mini slab gels (only the actin bands are shown).

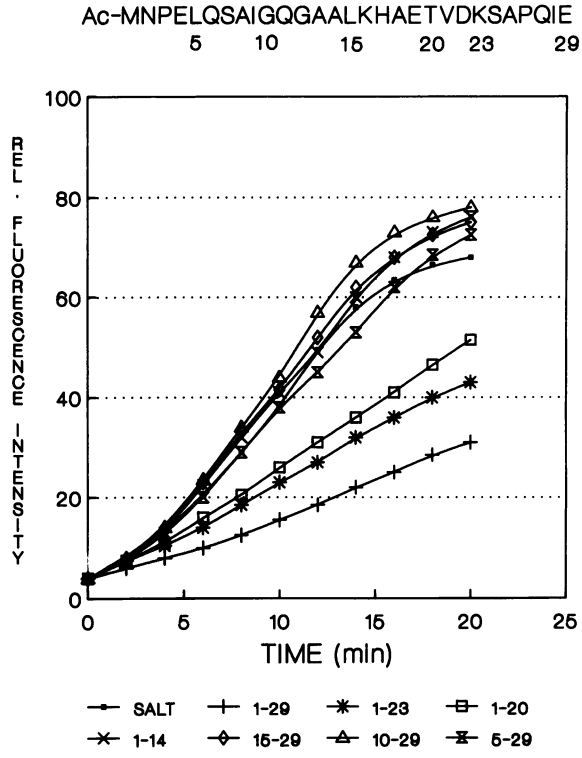

Fig. 2. Effect of NH_2- and COOH-terminal deletions on the inhibitory activity of AB1−29. The amino acid sequence at the top is that of AB1−29, the numbers below the sequence indicate the position of the deletions. A 20 molar excess of each peptide was mixed with pyrenyl-G-actin (10%). Polymerization was initiated after 10 min of incubation (time 0) and followed by fluorescence enhancement. Salt, polymerization curve of actin alone; 1−29 and 1−23 etc, curves of actin polymerization in the presence of AB1−29, AB1−23, etc.

full biological activity, which we define here as the capacity to inhibit salt-induced actin polymerization, we analysed several NH_2- and COOH-terminal deletion variants of AB1−29 (Figure 2). Deletion of the first 4, 9 or 14 NH_2-terminal residues (peptides AB5−29, AB10−29 and AB15−29) resulted in a total loss of the inhibitory activity. In contrast, deletion of sequences located COOH-terminal to the LKHAET motif (peptides AB1−20 and AB1−23) had only a reduced effect. Further removal of the LKHAET motif, leaving only residues 1−14 of actobindin (peptide AB1−14), resulted in a abrupt loss of activity. These results were confirmed by F-actin sedimentation (not shown). Therefore, all further experiments were carried out with variants of peptide AB1−29.

Amino acids in the LKHAET motif that are important for interaction with actin

To obtain more information on the contribution of the individual amino acids in the LKHAET motif to the biological activity of the peptide, we synthesized several substitution variants (Figure 3A and B). Replacing Glu19 by Lys (AB1−29 E19K) or Thr20 by Ile (AB1−29 T20I) had no or little effect on the activity. However, when Lys16 (the lysine that was cross-linked to Glu100 in the actobindin-actin complex; Vancompernolle *et al.*, 1991) was substituted for Glu (AB1−29 K16E), the peptide had completely lost its inhibitory activity. This was also the case when the bulky and hydrophobic Leu was replaced by the small Gly (AB1−29 L15G) and when His17 was substituted by Glu (AB1−29 H17E). With the latter, an increase in fluorescence was already observed even before the polymerization was initiated by salt (see Figure 3A). This observation incited us to study actin polymerization in G-buffer. As expected, peptide AB1−29 H17E was now able to induce actin polymerization in low salt buffer (Figure 4). In contrast with salt-induced actin polymerization, no lag phase was observed. Also the double mutant AB1−29 KH16/17EE, but not the single mutant AB1−29 K16E, was able to induce actin polymerization under these conditions (data not shown). Falling ball viscometry and F-actin sedimentation studies on the actin−peptide mixtures (data not shown) confirmed that the increased fluorescence was indeed the result of polymer formation, and electron microscopic analysis of the actin filaments induced by these peptides revealed no differences with filaments formed in the presence of high salt (Figure 4, insert). To exclude the possibility that the induction of actin polymerization was caused by salt present in the peptide solutions, we measured the conductivity of the samples. This increased only very slightly compared with a G-actin solution alone. By contrast, the conductivity of an actin filament solution, formed by the addition of salt (KCl + $MgCl_2$), was ~30 times higher than of a G-actin solution.

Thus, although no inhibition of salt-induced actin polymerization was observed, the peptides displaying a glutamic acid at position 17 still interacted with actin and were even able to induce actin polymerization. Therefore

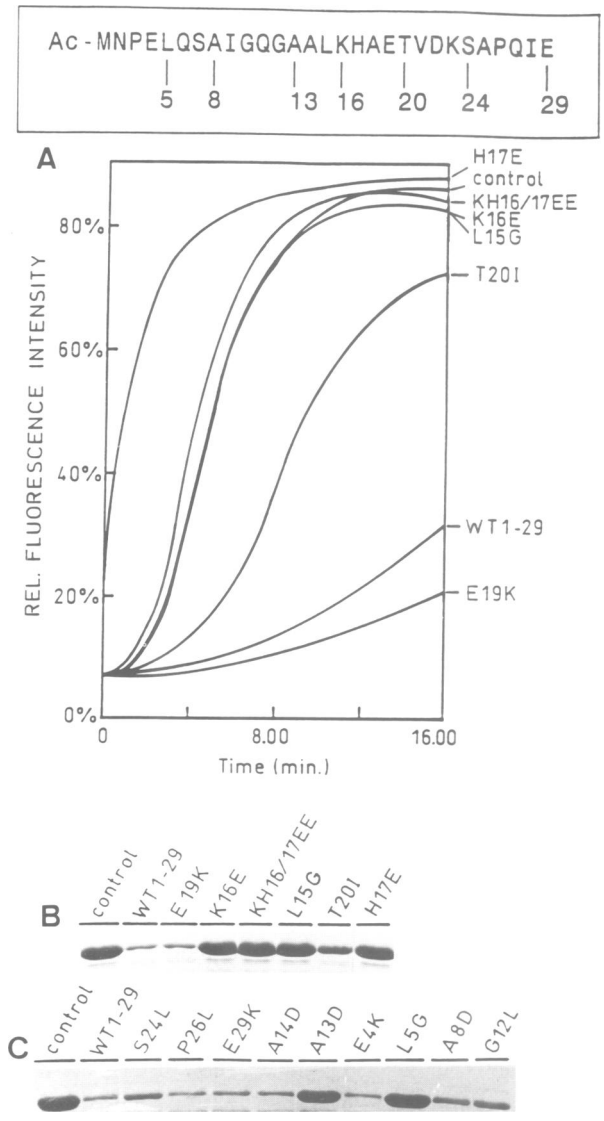

Fig. 3. Effect of point mutations in AB1−29 on the inhibitory activity of the peptide. The sequence of AB1−29 is shown and the positions of the substitutions are indicated. The substitution variants are indicated as follows: the left amino acid(s) are the originals, on the right side is the substituted residue(s) and the number in between indicates the position. WT1-29 refers to AB1−29; control, actin alone. All peptides were tested at a 30 molar excess over actin. **A** and **B**. Effect of substitutions in the hexamotif measured by fluorescence (A) and F-actin sedimentation (B). **C.** Effect of point mutations outside the motif measured by F-actin sedimentation (only the actin bands are shown).

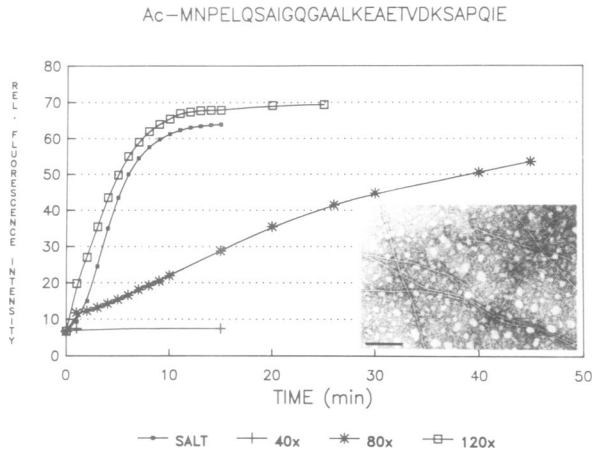

Fig. 4. Induction of actin polymerization by peptide AB1−29 H17E in low salt buffer. The sequence at the top is that of peptide AB1−29 H17E. The peptide was added at time 0 and the polymerization was followed continuously as a function of time. The numbers refer to the molar excess over actin. Salt refers to the polymerization curve of actin obtained by the addition of salt. Note the absence of lag phase in the peptide-induced actin polymerization. The insert in the figure shows the negative stain analysis of actin filaments formed by this peptide. Bar, 0.02 μm.

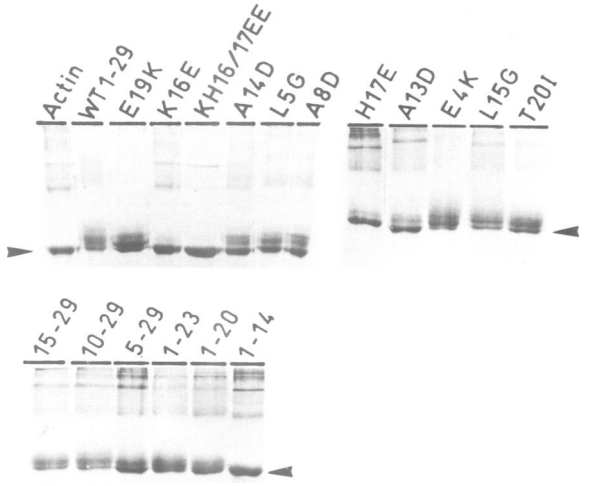

Fig. 5. EDC cross-linking of actobindin variant peptides to actin. All peptides were cross-linked in 30 molar excess over actin. The arrow indicates the position of actin. With the exception of AB1−14, which is missing the hexamotif and the variants in which Lys 16 was replaced by a glutamic acid (K16E and KH16/17EE), all peptides could be cross-linked to actin. For peptide nomenclature, see legend Figure 3.

these interactions must induce a conformational change favourable for filament formation.

In the light of these results, it is possible that a missing phenotype is not necessarily associated with absence of actin binding. Therefore, we used EDC-catalysed actin peptide cross-linking as a criterion for specific binding. EDC is a zero length cross-linker connecting COOH-groups with NH_2-groups into an isopeptide linkage only when the participating residues are in close contact with each other. All peptide variants were able to cross-link, except those in which Lys16 was replaced by Glu (AB1−29 K16E, AB1−29 KH16/17EE) and the peptide in which the LKHAET motif was missing (AB1−14) (Figure 5). Since it is not possible to show by EDC cross-linking if peptide AB1−14 still binds to actin (because it has no lysin), we tested if this peptide was able to compete with AB1−29. Since cross-linking of AB1−29 was not hindered even by a 10 times molar excess of AB1−14 over AB1−29, we concluded that AB1−14 did not bind to actin or that binding was very weak. Here it is important to recall that the EDC cross-linking must have been specific, since Lys16 is the only cross-linkable residue, which is similar to results obtained with intact actobindin (Vancompernolle *et al.*, 1991).

The region located NH_2-terminally of the LKHAET motif is important for inhibition of actin polymerization

Results obtained with the NH_2-terminal deletion variants of AB1−29 already indicated the importance of the

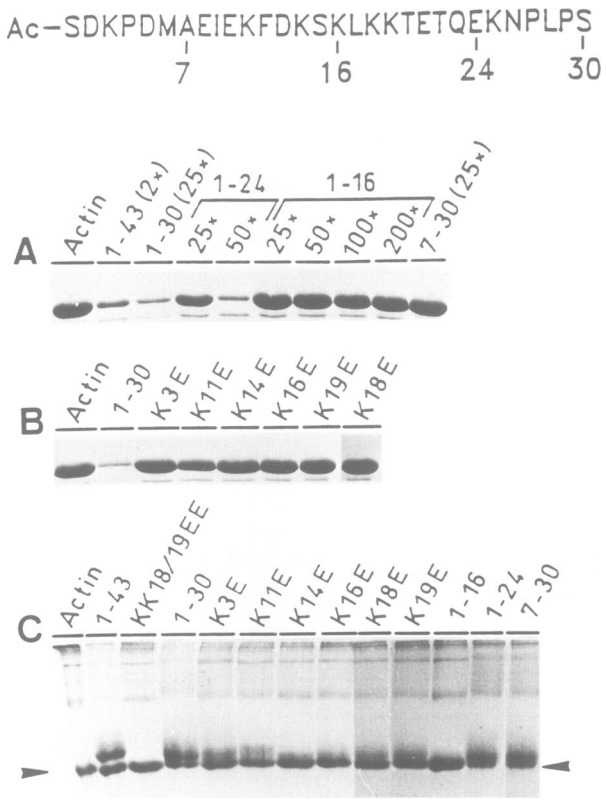

Fig. 6. Analysis of the actin binding site of thymosin β4. The sequence shown at the top is that of the thymosin β4 fragment 1−30. Numbers under the sequence refer to the borders of the deletion fragments. 1−43, the intact thymosin β4 molecule; Actin, actin alone (in the absence of peptide). **A.** Effect of NH_2- and COOH-terminal deletions on the inhibitory activity of thymosin β4 measured by sedimentation. The deletion variants are named according to their respective start and end residue. The numbers indicate the molar excess over actin. **B.** Effect of Lys−Glu exchanges in fragment TH1-30 on the inbibitory activity measured by sedimentation. All peptides were tested in a 20 molar excess over actin (only the actin bands are shown). **C.** EDC cross-linking of thymosin β4 peptide variants to actin. The arrow indicates the position of actin. The double mutant KK 18/19 EE is made in the complete thymosin β4 molecule. All other substitution variants are made in fragment TH1-30.

NH_2-terminus of actobindin. This was further confirmed by studies using single amino acid substitutions located in the region preceding the hexamotif (Figure 3C). For instance, substituting Leu5 and Ala13 by Gly and Asp respectively, resulted in a total loss of inhibitory activity. Replacing Ala8 and Gly12 by Asp and Leu respectively resulted in a reduction of the peptide's activity, as documented by a small increase in the F-actin pellet formation (Figure 3C) and by an increased rate in fluorescence enhancement relative to the wild type peptide (results not shown). In contrast, Glu/Lys and Ala/Asp exchanges at positions 4 and 14 respectively had no effect on the inhibitory activity of the peptide. On the other hand, all amino acid substitutions introduced in the COOH-terminal part of peptide AB1−29 had very little effect. Thus it was possible to replace Ser24, Pro26 and Glu29 by Leu, Leu and Lys respectively, without measuring a considerable change in the peptide's activity.

In summary, the region in actobindin necessary for inhibition of actin polymerization extends over ~20 residues, comprising the hexapeptide motif and an NH_2-terminally located segment. Residues located COOH-terminally of the hexamotif seem to be of less importance. Amino acid substitutions resulting in loss of activity do not necessarily cause the loss of specific binding. Indeed, all peptide variants with substitutions outside the hexamotif showed nearly identical actin cross-linking efficiencies (Figure 5).

Thymosin β4 variants exert similar effects on actin as actobindin variants

Thymosin β4 is another small actin-binding polypeptide of which the chemically synthesized form is equally as active as the biological counterpart (Sanders *et al.*, 1992). As previously mentioned, thymosin β4 contains an LKKTET motif, similar to the actobindin LKHAET motif and located at a similar distance from the NH_2-terminus. We hypothesize therefore that thymosin β4 could display actin-binding properties similar to actobindin.

First, we identified the shortest fragment that still displayed actin binding properties similar to intact thymosin β4. Fragment TH1−30 was able to inhibit completely salt-induced actin polymerization when present in a 25-fold molar excess, which is ~10 times higher than intact thymosin β4 (Figure 6A). Further deletion yielded peptides with even more reduced activities. Peptide TH1−24 was only fully active when present in a 50 molar excess and TH1−16 (now missing the LKKTET motif) completely lost its inhibitory activity. Similar studies with the NH_2-terminally truncated thymosin β4 variant TH7−30 also emphasized the importance of the NH_2-terminal part of the molecule, since no activity was measured not even at a 100 molar excess (Figure 6A).

These studies show that the 20 COOH-terminal residues of thymosin β4 are not essential for inhibiting actin polymerization and that the active segment extends over a minimal distance comprising the 24 NH_2-terminal residues. Since segment TH1−30 had a higher activity than TH1−24, we decided to perform further studies with the former. As for the actobindin derived peptides, these studies consisted of measuring the effect of several point exchanges on the inhibitory activity of salt-induced actin polymerization by fluorescence enhancement (data not shown) and sedimentation, the induction of actin filament formation in low salt and the capacity to form specific EDC-catalysed conjugates with actin.

Most interactions between actin and actin binding proteins characterized so far (Vandekerckhove and Vancompernolle, 1992) involve salt bridges in which actin is the donor of the acidic residue and the actin-binding protein is the donor of the amino group. Since the NH_2-terminus of thymosin β4 is very rich in lysine residues, we tried to identify which of these were involved in interaction with actin. Therefore, we individually substituted each of the lysine residues (positions 3, 11, 14, 16, 18 and 19) in TH1−30 for glutamic acid.

Each of the substitutions resulted in a drastic decrease or total loss of inhibitory activity (Figure 6B), again emphasizing the relative importance of amino acids located in the NH_2-terminal part as well as of the lysine residues in the LKKTET motif. EDC cross-linking was found with all of the variants, but was in general less pronounced than that of the WT peptide TH1−30 (Figure 6C). Crosslinking with the deletion variants revealed that the residues involved in specific binding were located exclusively in the LKKTET motif (Figure 6C) since no conjugate with TH1−16 was found. Thereupon we made a double exchange in the

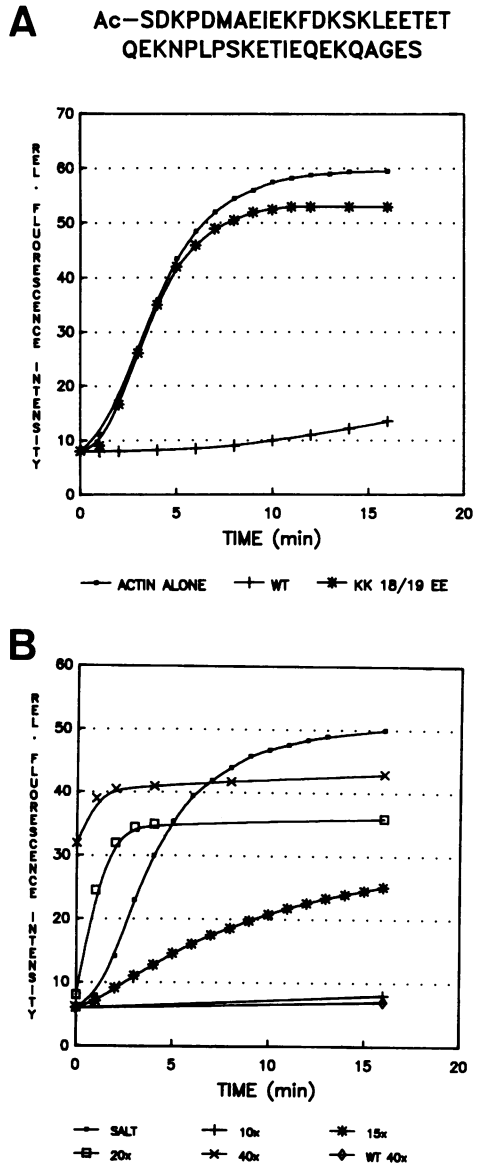

Fig. 7. Activity of peptide TH1-43 KK18/19EE. The sequence shown at the top is that of TH1−43 KK18/19EE. **A.** Inhibition of salt-induced actin polymerization measured by fluorescence. Thymosin β4 (WT) was used in a 2 molar excess over actin. The variant peptide KK18/19EE was tested in a 10 molar excess. **B.** Induction of actin polymerization by TH1-43 KK18/19EE in low salt buffer measured by fluorescence. The peptide was added at time 0, the numbers indicate the molar excess over actin. In comparison to salt-induced polymerization, no lag phase was observed in the peptide-induced actin polymerization. Note also that WT thymosin β4 was assayed at a 40 molar excess and that no increase in fluorescence was observed.

complete thymosin β4 molecule by replacing the lysine residues at positions 18 and 19 by glutamic acid (TH KK18/19EE). The thymosin β4 variant now showed no inhibitory activity (Figure 7A) and could also not be cross-linked to actin even when present in a 40 molar excess (Figure 6C). This demonstrates that Lys18 and 19 form important salt bridge interactions with actin, similar to Lys16 and His17 in the actobindin NH_2-terminus.

Since the essential lysin residues for EDC cross-linking are missing in the fragment TH1−16, we tested whether it could compete with intact thymosin β4. As cross-linking of the latter was only hindered for 50% at a 100 molar excess of TH1−16 over thymosin β4, we concluded that the

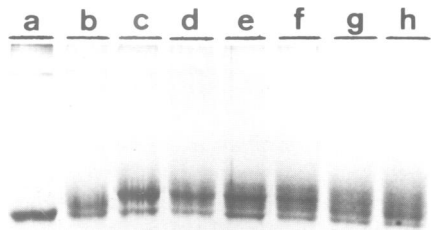

Fig. 8. Competition between AB1−29 and thymosin β4 for the same site on actin, studied by EDC-cross-linking. **a**, actin alone; **b**, actin-AB1−29 conjugate (AB1−29 was used at a 20 molar excess over actin); **c**, actin−thymosin β4 conjugate (thymosin β4 was used at a 2 molar excess over actin); **d**, **e**, **f**, **g** and **h**, equal amounts of actin were first incubated with a 2 molar excess of thymosin β4, then increasing amounts of AB1−29 were added (d, 5 ×; e, 10 ×; f, 20 ×; g, 40 ×; h, 100×molar excess over actin) and cross-linking was started. Note the complete disappearance of the actin−thymosin β4 conjugate in lanes g and h and the appearance of the actin−AB1−29 conjugate.

interaction of this fragment with actin must be very weak (data not shown).

In analogy with the results obtained with AB1−29 H17E, we also tested all single substitution variants and TH KK18/19EE for their ability to induce actin filament formation in low salt. This was measured by fluorescence enhancement and falling ball viscometry. Only the double mutant TH KK18/19EE (Figure 7B) and the single point mutant TH1−30 K19E (those peptides that contained a glutamic acid on the third position of the motif) were able to induce actin polymerization in low salt buffer.

In conclusion, our studies revealed common features between the actin binding sites of actobindin and thymosin β4. First, both sites extend over ~20 amino acids and are located in the NH_2-terminal part of the respective proteins. Secondly, they contain a similar hexapeptide motif in which the charged residues are at identical positions and which both start with the Leu-Lys sequence. Thirdly, amino acid substitutions outside the motif do not affect actin binding, but mostly affect the F-actin inhibitory phenotype. Fourthly, substitutions in the motif mostly affect both the peptide's activity and actin binding. Fifthly, peptides with a glutamic acid at the third position in the motif induce actin polymerization in low salt conditions.

Actobindin and thymosin β4 compete for the same site on actin

Since both peptides and their variants exhibit very similar actin binding properties, we expected that AB1−29 and thymosin 4 could compete with each other for the same site on actin. Such competition is difficult to measure by the polymerization assay. However, EDC cross-linking and subsequent analysis of the differently sized conjugates by SDS−polyacrylamide gels could be used here.

Equal amounts of actin were first incubated for 10 min with a 2×molar excess of thymosin β4. Then, increasing amounts of AB1−29 were added and after 10 min, EDC cross-linking was started. At low concentrations of AB1−29, mainly the thymosin β4−actin conjugate is formed, while at higher concentrations (40−100 molar excess over actin) the thymosin β4−actin conjugate disappears and is replaced by the AB1−29−actin conjugate (Figure 8). We never observed covalently cross-linked products with a size larger than that of the actin−thymosin β4 complex, which is in agreement with the absence of the triple conjugate consisting

of thymosin β4, AB1−29 and actin. Thus both peptides compete for the same binding site on actin in concentration ranges in agreement with their measured K_d values.

The hexamotif determines actin conformation

From the competition experiments and the detailed analysis described above, a picture emerges in which the amino acids present in the motif are the first line determinants for induction of monomeric or polymeric actin and that the NH_2-terminal extension is only a secondary element in determing the phenotype. This is supported by the finding that the hexapeptide alone can also bind to actin, although it does not inhibit polymerization. In case of the LKHAET and LKKTET peptides, we could demonstrate binding by EDC cross-linking and NH_2-terminal sequence analysis of the conjugates (data not shown). For the LKEAET and LKETET peptides, we found induction of actin polymerization in low salt (data not shown).

Thus, although the short motifs alone all bind to actin, the LKHAET- or LKKTET-containing sequences need a specific NH_2-terminal extension for the inhibitory phenotype, while for the LKEAET- or LKETET-containing peptides, such an additional sequence does not seem to be necessary for induction of F-actin formation.

Discussion

We have analysed in detail the potential actin binding capacities of a conserved hexapeptide motif found in a number of actin-binding proteins (Safer *et al.*, 1991; Vancompernolle *et al.*, 1991). Its consensus sequence is LbbxEy, with b representing a basic amino acid, x a small residue and y an -OH- or -SH-containing side chain (L and E represent the one letter notation for Leu and Glu respectively). This motif was chemically synthesized in a number of peptides derived from the NH_2-terminus of either actobindin or thymosin β4. In general, these peptides contained 29 or 30 residues and in some experiments we used intact thymosin β4 or synthetic variants thereof. The 'wild type' peptides displayed a biological activity similar to actobindin or thymosin β4, an activity defined as the capacity to inhibit or to retard KCl- and $MgCl_2$-induced actin filament formation when added to G-actin solutions prior to addition of salt. For the actobindin-derived peptides, which covered the NH_2-terminal 29 residues, full actin inhibitory activity was noticed when a 30-fold molar excess over actin was present, which is in agreement with its higher K_d value. Similar concentrations were also necessary for the thymosin β4-derived fragments. These are low concentrations, excluding the possibility that the effects measured on actin polymerization could be due to an increase in ionic strength, either produced by working with incompletely desalted peptides or by the charges of the peptides themselves. It should be further noted that all peptides are very soluble in water, making effects due to unspecific hydrophobic interactions very unlikely.

In the first place, we studied the participation of segments of amino acids in actin-binding by deleting sequences either at the COOH-terminal or the NH_2-terminal side of the peptide. For both the actobindin and thymosin β4 derived peptides we observed that deletion of the hexamotif resulted in complete loss of actin binding activity, as measured by all criteria mentioned above. We further noticed that an intact NH_2-terminus (but not an intact COOH-terminus) of the peptide was essential for the inhibition of actin polymerization, but not for binding to actin. Hence, we concluded that the hexapeptide motif contained residues essential for binding (and EDC cross-linking), but that this only resulted in an inhibitory phenotype when the motif was located COOH-terminal of an intact actobindin or thymosin β4 NH_2-terminal segment. Since substitutions in the NH_2-terminus could destroy actin inhibitory activity, we concluded that not only any NH_2-terminal sequence sufficed for activity, but that such segments should have specific sequences and structures. Secondary structure predictions postulate a high preference for α-helix formation for this sequence, in line with NMR-studies of thymosin 4 (Zarbock *et al.*, 1990). It is thus possible that some of the substitutions could have altered the structural requirements necessary to obtain the inhibitory phenotype either by weakening the α-helix or by disturbing the charge distribution in this area.

The actin binding sites of actobindin or thymosin β4 mimicked by these peptides are probably functionally very similar and consist of at least two functional entities: first, an NH_2-terminal segment, with a variable sequence likely to fold into a helix configuration and secondly, a COOH-terminally located hexapeptide motif with a specific conserved sequence. The latter is responsible for actin binding, while the former determines the inhibitory phenotype. The actin-binding hexamotifs contain an LK sequence followed by a short cluster of either basic or acidic residues. The first lysine residue is directly involved in contact with actin, probably via Glu100 or the acidic NH_2-terminus, as shown for the actobindin-actin complex (Vancompernolle *et al.*, 1991). The third residue is also important in actin-binding. When this position is occupied by lysine or histidine, the peptide binding induces the formation of an unpolymerizable form of actin or actin−peptide complex. When glutamic acid is present at this site, then binding to actin promotes a configuration favourable for filament formation in low salt. A similar effect was also observed with peptides having glutamic acid in positions 2 and 3 of the motif. The common properties among the actobindin and thymosin β4 peptides argued for similarity in actin-binding. This is confirmed by competition of the two peptides for the same site on actin.

Since it is still possible that peptides bind to actin without exerting measurable effect on actin polymerization, we assayed this so called 'neutral binding' by establishing EDC cross-linking between the peptide and actin. The conjugates could be easily visualized by their higher molecular weights on SDS gels or by NH_2-terminal sequence analysis of the electroblotted conjugates using the non-acetylated forms of the peptides. Since the peptide concentrations were low (20−30 molar excess over actin), we could expect that random cross-linking would be minimal. This was indeed confirmed in control experiments where a double mutant (TH KK18/19EE) showed no cross-linking at a concentration that was 20 times higher than that for wild-type thymosin β4 for which 50% cross-linking was observed (notice that the non-cross-linkable thymosin β4 variant still contained seven lysin residues). Thus EDC cross-linking can be considered a true measure of specific interaction between the peptides and actin.

An unexpected feature of some of the variant peptides was their capacity to induce the formation of actin filaments in low salt buffer. These filaments could not be distinguished

from those formed in salt. Neither aggregates nor other precipitable forms of actin, often seen in the presence of polycations (Brown and Spudich, 1979) or highly charged peptides (Friederich *et al.*, 1992), could be detected by electron microscopy.

It is unlikely that these findings are based on artifactual effects associated with the use of peptides rather than the entire polypeptides. First, the observations made for the TH1−30 fragments have been confirmed with the corresponding mutants of complete thymosin β4. Secondly, the lysine residues involved in the peptide cross-linking are the same as those present in the intact molecules, pointing to identical interactions. Thirdly, the peptide mimetics are not derived from an internal segment of the native molecules but represent their NH_2-terminal parts. Since NH_2- or COOH- extremities of proteins often fold independent of the rest of the molecule, the peptides used here could easily reflect true folding in the native molecule.

Similar LKK- and/or LKE-containing sequences have been observed in the presumptive actin-binding site of caldesmon 498-LKEKQQ-503 (Wang *et al.*, 1991). An LKEAET-sequence is observed in the COOH-terminal end of the tropomyosins (Lewis *et al.*, 1983). The latter motif is also present in one of our variant peptides and induces actin polymerization. This sequence, which is also in an α-helix configuration, could therefore constitute one of the F-actin binding sites in tropomyosin.

How can binding of very similar peptides result in either the inhibition or the induction of actin polymerization? And what can we learn from this about the actin regulatory activities of actobindin, thymosin β4 and other actin-binding proteins? The most likely mechanism following from our studies starts from the assumption that there are two functional entities in the actin binding site: a conserved hexapeptide motif forming the major contact with actin by salt bridge formation and an NH_2-terminal segment exerting a secondary effect. Peptides containing Lys at the third position of the motif (typical of actobindin and thymosin β4) bind to actin via the motif, while the NH_2-terminal segment hinders the association of a second actin protomer. Deletions or substitutions in this segment could increase its local mobility and therefore reduce the hindering effect. In the case of the binding of peptide with Glu at the third position of the motif (typical of caldesmon and tropomyosin), a conformational change is induced in actin and possibly also in the peptide, so that a polymerization competent actin is formed. Due to this change the NH_2-terminal segment has moved away from the actin−actin contact site, leaving a free contact with the annealing actin protomer.

Further details of this model remain to be analysed. For instance, we cannot exclude that the NH_2-terminal segment participates either in the stabilization of the motif or in interaction with actin. However, these latter effects should be minor, as the synthesized NH_2-terminal segments are only slightly competitive for the motif containing peptides when present at high molar excess.

The mechanism described here can also explain the properties of some actin binding proteins. LKE-containing sequences, as they are present in caldesmon (Bryan *et al.*, 1989) and in tropomyosin, could stabilize F-actin filaments by reinforcing the conformation favourable for the filament protomer. The use of a hindering α-helical rod to reduce or to inhibit actin polymerization could be an effective mechanism to fine tune the rate of actin polymerization. Indeed, such a system would allow fast binding to actin via the motif and at the same time would leave a mechanism that could temper the rate of actin polymerization proportional to its segmental motility.

Materials and methods

Proteins

Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (1971). Immediately before use actin was centrifuged in a TL100 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) for 2 h at 55 000 r.p.m. (rotor type TLA 100.2) to remove remaining F-actin. Actin was labelled with *N*-pyrenyliodoacetamide according to the procedure of Brenner and Korn (1983) and stored in the dark on ice in G-buffer (2 mM Tris, 0.2 mM $CaCl_2$, 0.5 mM mercaptoethanol and 0.2 mM ATP pH 7.6).

Peptides

Peptides were synthesized with a model 431 A peptide synthesizer (Applied Biosystems Inc., Foster City, CA) on *p*-hydroxymethyl resin using the Fmoc chemistry procedure and following the manufacturer's instructions. After cleaving the peptide−resin conjugate with trifluoroacetic acid (TFA), the peptide was precipitated with *t*-methyl-butylether and collected by centrifugation at 2000 *g*. The residue was dried for 2 h in a Speedvac concentrator (Savant Instruments, Farmingdale, NY) and then redissolved in H_2O and adjusted to pH 8 with 4 M NaOH. The peptides were desalted over a Sephadex G25 column (2×25 cm) in H_2O. The eluate was monitored by UV absorbance at 254 nm. Peptide fractions were collected, lyophilized and further purified by preparative HPLC on a C4 reversed phase column (1×25 cm, Vydac Separations Group, Hesperia, CA) as described in Friederich *et al.* (1992). Peptide concentrations were measured by alkaline hydrolysis (Hirs, 1967) followed by ninhydrin reaction (Moore and Stein, 1948) using bovine serum albumin solutions as standard.

Actin polymerization assays

Inhibition of actin polymerization. Actin polymerization in the presence of peptides was measured by the increase in fluorescence of pyrenyl-labelled actin (Brenner and Korn, 1983) and by F-actin sedimentation. Peptides were incubated at 20°C with a constant amount of G-actin (12 μM, of which 10% was pyrene labelled actin) in G-buffer and the polymerization was initiated 10 min later by addition of salt (final concentrations 150 mM KCl and 1 mM $MgCl_2$). The molar ratios of actin and peptide are indicated for each case individually. The increase in fluorescence was monitored as a function of time at 20°C with an SFM25 fluorimeter (Kontron Instruments, Zürich) using excitation and emission wavelengths of 365 nm and 388 nm respectively. The fluorescence could be measured continously without noticeable bleaching in the first 20 min.

The sedimentation assays were carried out with solutions of 24 μM G-actin. Peptides (molar excess indicated in the figures) were incubated for 10 min with G-actin at 20°C. The polymerization was started by addition of salt and after 8 min the formed actin filaments were sedimented for 15 min at 30 psi in an Airfuge (Beckman Instruments Inc., Palo Alto, CA). The supernatant was discarded and the pellets were washed twice with G-buffer and redissolved in 50 μl Laemmli sample buffer. The protein composition of the pellets was analysed by SDS−PAGE on mini slab gels.

Induction of actin polymerization. The conditions for the fluorescence assay were identical as described above, except that now the peptides were added at time zero and no salt was added.

Peptide-induced actin polymerization was measured by incubating G-actin solutions (24 μM) in G-buffer with various amounts of peptide. After 30 min the formed F-actin filaments were sedimented by Airfuge centrifugation and analysed as above.

Electron microscopy

G-actin was used at a final concentration of 1 mg/ml. Polymerization was induced by the addition of KCl and $MgCl_2$ to a final concentration of 150 and 1 mM respectively and allowed to continue for 30 min at 20°C. Induction of actin polymerization by the addition of peptides was performed at the same G-actin concentration in the absence of salt. The molar ratio of peptide to G-actin used in the experiments varied from 40−60. Samples were immediately applied to carbon coated grids and stained with 1% aqueous phosphotungstic acid.

Crosslinking of the synthetic peptides to actin

Actin was dialysed at 4°C for 16 h against a buffer containing 5 mM potassium phosphate, 0.2 mM $CaCl_2$, 0.5 mM mercaptoethanol and 0.2 mM ATP pH 8. Peptides (a 20–30 molar excess for most of the peptides was used; only a 2 molar excess for thymosin β4) were first incubated with G-actin for 10 min at room temperature, then 1-ethyl-3 (3 dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), both freshly prepared as 100 mM stock solutions in water, were added to a final concentration of 4 mM (Staros *et al.*, 1986). The reaction was stopped after 4 min by the addition of glycine to a final concentration of 5 mM. Aliquots of the samples were immediately analysed by SDS–PAGE.

Falling ball viscometry

Changes in the viscosity of actin solutions were measured with falling ball viscosimetry according to MacLean-Fletcher and Pollard (1980).

Amino acid sequencing

Protein bands from SDS–polyacrylamide gels were electroblotted onto PVDF membranes (Bauw *et al.*, 1987) and subjected to automated amino acid sequence analysis using a model 477A sequenator and a 120A phenylthiohydantoin amino acid analyser (Applied Biosystems Inc., Foster City, CA). The instrument was run according to the manufacturer's instructions.

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