Molecular Cloning, Expression, and Mapping of the High Affinity Actin-capping Domain of Chicken Cardiac Tensin

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Abstract. Tensin, an actin filament capping protein first purified from chicken gizzard, is localized to various types of adherens junctions in muscle and non-muscle cells. In this paper, we describe the isolation and sequencing of tensin cDNA from a chicken cardiac library. The 6.3-kb chicken cardiac tensin cDNA encodes an open reading frame of 1,792 amino acids. Mammalian cells transfected with the chicken tensin cDNA expressed a polypeptide of ~200 kD recognizable by antibodies to chicken gizzard tensin. The expressed protein was incorporated into focal adhesions and other actin-containing structures in the transfected cells. To map the domain associated with tensin's high affinity, barbed-end F-actin-capping activity, bacterially expressed recombinant fusion proteins containing various segments of tensin were prepared and assayed for activity. The results of these experiments show that the high affinity capping domain (KD = 1-3 nM) lies within amino acid residues R1037-V1169. Additional studies on a shorter construct, S1061-H1145, showed that these 85 residues were sufficient for producing complete inhibition of actin polymerization and depolymerization. While this active domain is located within that of the "insertin" sequence (Weigt, C., A. Gaertner, A. Wegner, H. Korte, and H. E. Meyer. 1992. J. Mol. Biol. 227:593-595), our data showing complete inhibition of polymerization and shift in critical concentration are consistent with a simple barbed-end capping mechanism rather than the "insertin model." Our results also differ from those of a recent report (Lo, S. H., P. A. Janmey, J. H. Hartwig, and L. B. Chen. 1994. J. Cell Biol. 125:1067-1075), which concluded that their recombinant tensin has an "insertin-like" inhibitory effect on barbed-end actin polymerization, and that this activity is attributed to residues T936-R1037 (residues 888-989 in their numbering system). In our study, a fusion construct (N790-K1060) encompassing T936-R1037 had no significant effect on actin polymerization and depolymerization, even at high concentrations.
Materials and Methods

Cell Culture

Human 293 embryonic kidney cells (Gorman et al., 1990), kindly provided by Dr. Jeremy Nathans (Department of Molecular Biology and Genetics, Johns Hopkins School of Medicine), were cultured in a 1:1 mixture of DME and Ham’s F12 medium supplemented with 10% fetal calf serum. NIH 3T3 mouse fibroblasts were cultured in DME supplemented with 10% calf serum. Chicken embryo fibroblasts (CEF) were grown as described by Vogt (1969). Cells were grown on glass coverslips for immunofluorescence studies.

Antibody Preparations

Affinity-purified polyclonal antibodies, designated as T(D), raised in a rabbit to a preparation enriched in chicken gizzard 165-kD tensin was used in the screening of the cDNA library. A mouse mAb, TL-1, was also prepared by using chicken gizzard tensin as antigen. This antibody is chicken specific, and it does not cross-react with anything in human or mouse cells. A preparation of polyclonal antibodies to actin homology domain-1 (anti-AH1), was raised in a New Zealand rabbit against a synthetic peptide (Peptide Synthesis Facility, Biology Department, Johns Hopkins University) containing the deduced amino acid sequence of M49-TT8 of tensin, a region with 50% sequence identity to amino acid residues 221-249 of aodn (see results and discussion for details). The anti-AH1 antibody preparation for immunoblotting was purified by affinity chromatography on a column of tensin fusion protein G52-$887 linked to Sepharose CL-4B. For localization studies.

Expression of Cardiac Tensin in Mammalian Cells

Transient transfection of mammalian cells was performed essentially as described by Gorman et al. (1990). Briefly, cells were seeded on culture plates of glass coverslips and cultured overnight. 3 h before transfection, the growth medium was changed. The cells were cotransfected with 5 μg of tensin expression plasmid and 0.5 μg of pRSVT+, using a calcium phosphate method, followed by a 15% glycerol shock step 3 h after transfection. The human 293 cells were shocked for 15 s, and the mouse 3T3 cells were shocked for 90 s. Transfected cells were incubated for 18 h before they were used for immunofluorescence studies.

Tensin Fusion Constructs

All fusion constructs encoding tensin deletion derivatives were cloned into pMAL (New England Biolabs, Beverly, MA) or pGEX (Pharmacia Fine Chemicals, Piscataway, NJ). In addition to pMAL-c2 and pMAL-cRI, a preparation of polyclonal antibodies to actin homology domain-1 (anti-AH1), was raised in a New Zealand rabbit against a synthetic peptide (Peptide Synthesis Facility, Biology Department, Johns Hopkins University) containing the deduced amino acid sequence of M49-TT8 of tensin, a region with 50% sequence identity to amino acid residues 221-249 of aodn (see results and discussion for details). The anti-AH1 antibody preparation for immunoblotting was purified by affinity chromatography on a column of tensin fusion protein G52-$887 linked to Sepharose CL-4B. For localization studies.

Northern Blots

Total RNA from various chicken tissues and cultured CEF was obtained by extracting in RNAxol according to the manufacturer’s directions (Biotec B Laboratories, Friendswood, TX). 30 μg of each RNA sample was separated by electrophoresis in formaldehyde-agarose gels in MOPS buffer (Lehrach et al., 1977) and transferred to an Immobilon-N membrane (Millipore Corp., Bedford, MA). Hybridization probes used for Northern blotting were labeled with ^32P by using the random primed DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Eukaryotic Expression Constructs

Two eukaryotic tensin expression constructs were prepared with the pCIS vector (Gorman et al., 1990; kindly provided by Dr. Gorman, Genetech, San Francisco, CA). The 3' Splh-Xhol of JC43 was replaced by the 3' Splh-Xhol fragment of JC42 to obtain JC97. The cDNA insert of JC97 was then released by double digestion with XbaI and Xhol, ligated with the XbaI- and Xhol-digested pCIS vector to yield JC101, which contains the full-length cDNA except nucleotides 1-54. The 5' XbaI-BamI region of JC97 was replaced with that of JC9 to yield JC98. The full-length cDNA was released from JC98 by digestion with XbaI and Xhol, and it was ligated into XbaI- and Xhol-digested pCIS to obtain JC100.
Immunoprecipitation and Immunoblotting

CEPs were grown on 100-mm plates, and the lysate was made by extraction with RIPA buffer (Davis et al., 1991). The 293 cells, also grown on 100-mm plates, were extracted with buffer containing 10 mM phosphate, pH 7.2, 0.25% Tween 20, 0.1 M NaCl, 10 mM dithiothreitol, 10 mM of EDTA and EGTA, 1 μM leupeptin, 20 μM E-64, and 1 μM pepstatin A. After clarification, the NaCl concentration in 293 cell lysate was brought up to 0.5 M. Tensin in CEF and tensin expressed in transiently transfected human 293 cells were immunoprecipitated from cell lysates with mAb TL-1 conjugated to Sepharose CL-4B beads. Immunoprecipitated proteins were separated by SDS-PAGE (10%) gel, transferred to Immobilon-P (Millipore Corp.), and probed with anti-AHI antibody.

Immunofluorescence Staining

Cells were fixed and stained 18 h after transfection. Concentration of antibodies used were: TL-1, 1:500 dilution of ascites fluid; anti-AHI, 1:200 dilution of antiserum; IgG from T(D), 5 μg/ml; and vin 11.5, 1:200 dilution of ascites fluid. FITC-conjugated anti-mouse and biotinylated donkey anti-rabbit were used at 20 μg/ml. Samples stained with biotinylated antibodies were washed in phosphate-buffered saline and further incubated with Texas red-streptavidin at 20 μg/ml. For actin staining, FITC-phalloidin was added along with the Texas red-streptavidin. Fluorescence microscopy was performed on an IM35 microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with 40× and 100× objectives. Photographs were taken with TMAX 400 film (ASA 800-1600) (Eastman Kodak Co., Rochester, NY).

Preparation of Tensin Fusion Proteins

MBP-tensin fusion proteins and GST-tensin fusion proteins were prepared and purified according to suppliers’ instructions (New England Biolabs and Pharmacia, respectively). A protease inhibitor mixture (1 μM leupeptin, 0.3 μM aprotinin, 1 μM pepstatin, 20 μM E-64, and 0.1 μM pefabloc SC) was included in all buffers used during purification. Protein concentrations were determined with the method of Bradford (1976), with the following exceptions. The concentration of fusion protein EI11-V1169 was estimated from the intensity of stained bands on immunoblots probed with mAb TL-1. In the case of fusion proteins R1037-E1149-P1162-V1169 and SI061-H1145, proteins eluted from affinity columns were further purified by ammonium sulfate precipitation and gel filtration chromatography (Superdex-75; Pharmacia, Uppsala, Sweden, in 20 mM Tris-HC1, pH 8, 0.1 M KCI, 1 mM EDTA, 10 mM dithiothreitol, 1 μM leupeptin, 0.3 μM aprotinin, 1 μM pepstatin, 20 μM E-64, 0.1 μM pefabloc SC, and 0.2 mM PMSF) to remove the major degradative fragments. Quantitation of the final products was based on UV absorbance at 280 nm, using extinction coefficients of 0.296 × 10^4 M^-1.cm^-1 for R1037-E1149-P1162-V1169 and 0.6 × 10^4 M^-1.cm^-1 for SI061-H1145, as calculated from the amino acid sequence of the fusion proteins according to the method of Rollin and Von Heijne (1989).

Actin Polymerization Assays

Actin was isolated from chicken breast muscle using established methods (Spudich and Watt, 1971), followed by chromatography on a Sepharacyl S-200 column in buffer A. Labeling of the actin with pyrene was carried out as described (Koyama and Mihashi, 1981). Samples of labeled or unlabeled G-actin were flash frozen in liquid nitrogen after column purification and stored at -80°C. The frozen actin samples were quickly defrosted and spun at 100,000 x g for 30 min immediately before use (Young et al., 1990).

In the actin polymerization assays, the concentration of G-actin (10% pyrene-labeled) was 0.5 or 2.0 μM in buffer A. G-actin, used as nuclei in the assays, was prepared by polymerizing 20–30 μM G-actin in buffer A containing 0.1 M KCl, 2 mM MgCl₂ (buffer F) for 30 min at room temperature or overnight at 4°C, and then diluting to 3 μM with buffer F and equilibrating for 2 h at room temperature before use. 1 min before the addition of G-actin nuclei, a 20× concentrated salt mixture was added to bring the salt concentration to 100 mM KCl, 2 mM MgCl₂ and 1 mM EGTA. Aliquots of G-actin were first mixed in a vortex mixer for 30 s, and then mixed with tensin fusion protein or an equivalent amount of buffer before adding to the G-actin to start polymerization. The initial rate of polymerization was measured by following the increase of fluorescence of the pyrene label at room temperature using a fluorescence spectrophotometer (650-105S; Perkin-Elmer Corp., Norwalk, CT).

The actin depolymerization assay was performed in the presence of G-actin (20% pyrene-labeled) was polymerized and further diluted to 3 μM and equilibrated as above. Samples of this pyrene-labeled G-actin were mixed in a vortex mixer for 30 s, and then mixed with tensin fusion protein for 10 s before adding to buffer F to initiate depolymerization.

To estimate the critical monomer concentration in the presence of tensin fusion proteins, 2 μM G- or F-actin was mixed with various concentrations of protein in buffer F. The amounts of G- and F-actins were calculated from pyrene fluorescence measured after 24 h.

Amino Acid Sequence Analysis

The amino acid composition, molecular weight, isoelectric point (pI), and the properties of tensin were analyzed with the use of the MacVector software package. For nucleotide and amino acid sequence searches and comparisons, the software program GENmenu was run on a VAX 8530 computer at The Johns Hopkins Medical School.

Results

Isolation and Sequencing of Chicken Cardiac Tensin cDNA

A LambdaZap II chicken cDNA library made from mRNA from a single adult chicken heart was initially screened with an affinity-purified polyclonal antibody preparation, T(D), raised to chicken gizzard 165-kD tensin. A single clone, Tn3.2, was isolated and shown to contain a 3.2-kb cDNA insert that produced a fusion protein recognizable by both T(D) and an mAb to chicken gizzard tensin (TL-1). Eight more clones were isolated from the same library with the use of three nucleotide probes derived from Tn3.2 (Fig. 1).

Figure 1. Tensin cDNA clones isolated from a chicken cardiac lambda ZapII library. A schematic diagram of tensin cDNA (nucleotides 1–6347) is shown at the top of the figure. The thick line marks the area of the open reading frame of tensin. Nucleotide 1 is the first nucleotide from the 5’ end of clone JC91, which is the clone that extends furthest at the 5’ end. Clone Tn3.2 was the first and only clone isolated with the use of affinity-purified polyclonal anti tensin T(D). 32P-labeled 5’ Apal-Apal fragment of Tn3.2 (probe 1) was used to isolate clones JC9, JC10, JC13, JC14, and JC16 from the same cDNA library. A mixture of 32P-labeled 5’ EcoRI-HaeII fragment of JC13 (probe 2) and 3’ Hinfl-Hinfl fragment of Tn3.2 (probe 3) were used for the isolation of clones JC42, JC43, and JC91. Alignment of the sequences of these overlapping clones resulted in the 6,347-nucleotide tensin cDNA as shown at the top of the figure.

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Figure 2. cDNA and deduced amino acid sequences of chicken cardiac tensin. The nucleotide (1–6347) and deduced amino acid sequences (the open reading frame, 1,792 amino acid residues) of chicken cardiac cDNA are shown. The coding sequence is indicated by uppercase letters and the 5' and 3' noncoding sequences represented by lowercase letters. Actin homology domains (AH/ and A/-/2), actin-binding protein homology domain (ABPH), as well as the src homology domain (SH2), are underlined. The area in which the high affinity actin filament capping domain is located (cap) is highlighted by dark shading.
Alignment of the sequences of the nine overlapping clones resulted in a composite tensin cDNA sequence of 6,347 nucleotides (Fig. 2). This sequence contains a single open reading frame (ORF) of 5,379 nucleotides, starting from nucleotide 3 and ending with the termination codon at nucleotide 5,381. Because cardiac tensin had not yet been isolated, and attempts to determine the NH2-terminal of purified gizzard 215-kD tensin did not yield any useful information (presumably because of a blocked NH2 terminus), the NH2-terminal amino acid of cardiac tensin cannot be assigned at this time. To establish a point of reference, the arginine at the very beginning of the ORF is tentatively designated as amino acid residue 1 for cardiac tensin. The complete sequence of chicken cardiac tensin has been deposited in GenBank (accession No. L06662).

Examination of the amino acid composition deduced from tensin cDNA sequence indicated higher contents of proline (10.7%) and serine (11.9%) as compared to those of average vertebrate proteins (Doolittle, 1986). These high values are consistent with the results of amino acid composition analysis performed on 215- and 165-kD tensin isolated from chicken gizzard (Butler, J. A., and S. Lin, unpublished results). These two amino acids are unevenly distributed in the cardiac tensin sequence. Short stretches of high concentrations of proline or serine (20-30% of each) are found between amino acid residues 800 and 1,500. Of the 17 cysteine residues in the tensin sequence, all but one (C819) are distributed near the NH2 or the COOH terminus of the molecule. The significance of these observations is not clear at this time.

The calculated overall pI of the entire ORF of cardiac tensin sequence was 7.94, whereas that of the sequence starting from M55 is 7.22. Analysis of pI by small sections (50 amino acids) of tensin sequence showed that the molecule can be divided into three domains on the basis of acidity. Both the NH2-terminal and COOH-terminal portions of the molecule are highly basic (pI > 9), while the middle portion of the molecule (from amino acid residues 350-1,300) is highly acidic (pI of ~5). 41% of the amino acids in tensin are nonpolar, which falls in the range normal for nonmembrane proteins.

Unlike other large proteins such as dystrophin and spectrin, the amino acid sequence of tensin does not appear to be composed of repeated domains, except for four short stretches between residues 1,305 and 1,383 (Fig. 3).

### Size and Distribution of Tensin mRNA in Chicken Tissues

Previous studies involving immunoblotting and immunofluorescence staining indicated that tensin is present at relatively high levels in fibroblasts, gizzard, intestine, and heart, and at lower levels in skeletal muscles, brain, and liver (Risinger, M. A., and S. Lin, unpublished results). For comparison at a different level, Northern blot analyses were performed on total RNA from various chicken cells and tissues, using a 3.2-kb probe corresponding to the cDNA insert of Tn3.2. As shown in Fig. 4, the probe hybridized to a 10-kb RNA species in samples from gizzard, heart, and CEF. In addition, an 8-kb RNA species was detected in the gizzard sample. All other tissues tested showed only a faint 10-kb band, suggesting lower levels of expression of tensin in these tissues. It is interesting that in all cases, the size of tensin RNA was much larger than needed to encode a protein of 215 kDa. Since the total length of cardiac tensin cDNA we have isolated so far is 6.3 kb, much of the noncoding sequences have apparently not been obtained.

### Expression of Chicken Cardiac Tensin in Transiently Transfected Mammalian Cells

The 5,379 nucleotides of the ORF of chicken cardiac tensin cDNA can encode a polypeptide with a calculated molecular weight of 193,000. This value is somewhat less than the molecular weight of 215,000 estimated from the electrophoretic mobility of tensin from various tissues on SDS-polyacrylamide gels (Butler, J. A., and S. Lin, unpublished results).
For a more direct comparison of the size of the polypeptide encoded by the cardiac tensin cDNA with that of CEF tensin, human embryonic kidney cells (293 cells) were transfected with tensin cDNA. Protein expressed in the transiently transfected cells and in CEFs (serving as a control) was immunoprecipitated with mAb TL-1 and analyzed by SDS-PAGE followed by immunoblotting with polyclonal antibodies raised to a synthetic peptide with the deduced sequence of the AH1 domain (see Discussion for detailed description) of cardiac tensin. The immunoblots show that reactive polypeptides with similar electrophoretic mobility as CEF tensin ($M_r \sim 215,000$) were present in the 293 cell lysates prepared from both a full-length and a truncated (initiated from M55) tensin constructs, but not in the null-transfected 293 cell lysate (Fig. 5). This result shows that the coding region of the cardiac tensin cDNA we have obtained is probably complete, and that the AH1 region encoded by the nucleotide sequence near the beginning of the ORF was translated.

To test for biological activity of the cardiac tensin expressed in the human 293 kidney cells, immunolocalization experiments were performed. Over a background of non-specific nuclear staining, filamentous structures in transfected 293 cells were brightly stained by the chicken-specific antitensin mAb TL-1 (an example is shown in Fig. 6a). Furthermore, under conditions where the nontransfected cells showed only low levels of F-actin staining, the filamentous structures stained by TL-1 in transfected 293 cells were also brightly stained by phalloidin (compare Fig. 6, a vs b), suggesting that the expressed tensin promotes the formation of these actin filament bundles. Similarly, anti-AH1 antibodies also stained the structures stained by TL-1 in the expressed chicken tensin in the cells (Fig. 6, c and d).

Because human 293 kidney cells do not have well-defined focal adhesions, structures that have been shown in fibroblasts to be enriched in tensin (Lin et al., 1989; Lin, 1993) we extended the immunolocalization study on expressed cardiac tensin to mouse 3T3 fibroblasts. As shown in Fig. 7, a and b, while all cells in the field showed typical phallodin-stained actin stress fibers, only the transfected cell in the center showed intense staining at the ends of stress fibers with mAb TL-1. Co-localization of tensin and vinculin in another double immunofluorescence staining experiment confirmed that the expressed chicken cardiac tensin was incorporated into the focal adhesions of the transfected cells (Fig. 7, c and d). These results indicate that certain functional domain(s) in tensin required for this type of cellular distribution is conserved across the different species and cell types studied here.

**Studies on the High Affinity F-Actin Capping Domain in Tensin**

Tensin isolated from chicken gizzard has previously been shown to have high affinity actin capping activity, reflecting a dissociation constant ($K_a$) for the barbed end of F-actin in the nanomolar range (Butler, J. A., and S. Lin, unpublished results). To locate the tensin domain responsible for this activity, a large number of fusion proteins from constructs containing different regions of tensin sequence were produced in bacteria and assayed for ability to inhibit barbed-end polymerization of pyrene-labeled actin (Fig. 8A). In this survey part of the study, the fusion proteins scored "+" were able to reduce the initial rate of actin polymerization at the barbed end by >95% as compared to the control, while those scored "-" inhibited polymerization by <5%. As indicated in Fig. 8A, fusion proteins containing the NH$_2$-terminal half of tensin up to residue K1060, which included regions of homology to actin and actin-binding protein sequences (see Discussion for details), had no significant activity. In contrast, all fusion proteins containing the COOH-terminal half (I311-R1792 and S1061-R1792) or the middle portion of tensin (I311-V1169 and N790-Y1521) were effective in inhibiting actin polymerization. Experiments on fusion proteins with further deletions showed that the high affinity actin capping domain definitely lies within the region of R1037-Y1169. Furthermore, those from shorter constructs including S1061-V1169 and S1061-H1145 were also capable of complete inhibition of actin polymerization and depolymerization (see also Fig. 10 for additional data on the latter construct). In contrast, the region immediately adjacent to the NH$_2$-terminal side of the actin capping domain, N790-K1060, did not exhibit any significant activity (see also Fig. 10).

Coomassie blue-stained gels of some of the fusion protein preparations purified by affinity chromatography or a combination of affinity and gel filtration chromatography were shown in Fig. 8, B and C. Most of the fusion proteins appeared to be proteolytically degraded, notably S1061-H1145, the shortest active fusion protein, while the inactive N790-K1060 appeared to be intact (Fig. 8 C).

Two of the fusion proteins from constructs that inhibited actin polymerization in the above experiments were studied in greater detail (Fig. 9). The first is from a long construct (I311-V1169), which also covers a region (L419-M443) homologous to the actin-binding domain found in a number of actin cross-linking proteins (see Fig. 2 and Discussion for details). The other fusion protein is from a short construct.
Figure 6. Immunolocalization of chicken tensin in transiently transfected human 293 cells. Cells transiently transfected with JCI101 were double stained with mAb TL-1 for chicken tensin (a and c) and phalloidin for F-actin (b), or with anti-AHI (d). In some cells, the expressed chicken tensin is localized to what appears to be adhesion plaques at the terminals of actin stress fibers (c), while in other cells, the expressed chicken tensin is colocalized with F-actin containing structures distinct from stress fibers (a). Bar, 150 μm.
Figure 7. Colocalization of tensin with vinculin in transiently transfected mouse 3T3 cells. Cells transiently transfected with JC101 were double stained with TL-I for the expressed chicken tensin (a) and phalloidin for F-actin (b), or they were double stained with polyclonal antibodies to tensin T(D) (c), and antivinculin (d). The micrographs showed that both polyclonal antibodies to tensin and mAb to vinculin-stained adhesion plaques in the transfected cells. Bar, 20 μm.

(R1037-El149*P1162-V1169), which lacks the 12-amino acid stretch absent in the insertin sequence previously reported by Weigt et al. (1992) (see Discussion for details). In both cases, nanomolar concentrations of the proteins effectively inhibited actin polymerization under conditions where monomer addition was limited to the barbed end (at 0.5 μM G-actin); inhibition was essentially complete at the higher concentrations of fusion proteins used (Fig. 9, A and D). Analysis of the kinetic data from these polymerization experiments gave K_d's of ~1 and 3 nM for the long and short fusion proteins, respectively. Similarly, nanomolar concentrations of the two fusion proteins inhibited depolymerization of actin (Fig. 9, B and E). Furthermore, both proteins were capable of shifting the critical actin concentration from 0.1 μM to 0.5–0.6 μM (Fig. 9, C and F). All of these results are quite comparable to the K_d of 2–5 nM obtained with tensin isolated from chicken gizzard (Butler, J. A., and S. Lin, unpublished results), and with another actin capping protein isolated from chicken skeletal muscle (Casella et al., 1986).

After the completion of the above experiments and while this manuscript was in preparation, Lo et al. (1994a) reported the identification of T936-R1037 (888-989 in their numbering system) as the domain responsible for tensin's ability to inhibit actin polymerization. This apparent discrepancy between their conclusion and ours prompted us to carry out a side-by-side comparison of fusion proteins from three of our constructs: R1037-El149*P1162-V1169, which had high affinity actin capping activity in the previous experiments (Fig. 9); a shorter construct, S1061-H1145; and N790-K1060, which encompasses the region of interest (T936-R1037) described by Lo et al. (1994a). In this set of experiments, activities were measured both by following polymerization of G-actin at concentrations below (0.5 μM) and above (2 μM) the critical concentration at the pointed end (0.8 μM under the salt conditions used in these experiments), as well as by following depolymerization of actin. As shown in Fig. 10 A, at 0.5 μM G-actin, R1037-El149*P1162-V1169 and S1061-H1145 completely inhibited actin polymerization, whereas N790-K1060 did not have any appreciable effect. Similar results were obtained with the three fusion proteins in polymerization assays at 2 μM G-actin (Fig. 10 B). The residual level of actin polymerization observed in the presence of the two active fusion proteins under these conditions was presumably from monomer addition at the pointed ends of filaments. The lack of significant inhibitory effect by
N790-K1060 was again demonstrated in an experiment in which R1037-E1149³P1162-V1169 and S1061-H1145 effectively blocked depolymerization of actin (Fig. 10 C). In conclusion, the high affinity actin capping domain of tensin is clearly within amino acid residues R1037-V1169 and not T936-R1037. Furthermore, although the presence of degradative products in S1061-H1145 (Fig. 8 C) precluded an accurate comparison of its specific activity with that of R1037-E1149³P1162-V1169, these experiments further narrowed down the location of the essential sequence for capping activity to the 85 amino acid residues in tensin.

Discussion

Several lines of evidence strongly support the authenticity of the cardiac tensin cDNA described here. First, the amino acid composition deduced from the cDNA is in good agreement with that of the 215-kD tensin purified from chicken gizzard (Butler, J. A., and S. Lin, unpublished results). Second, the cDNA was able to initiate translation of a polypeptide (M55-R1792) with similar electrophoretic mobility as that of CEF tensin on SDS-PAGE. Third, M55-R1792 and CEF tensin both reacted with polyclonal and monoclonal antibodies to gizzard tensin, as well as antibodies to the AH1 region (M49-T78) of the deduced sequence of cardiac tensin. Fourth, M55-R1792 was colocalized with vinculin to adhesion plaques of 3T3 cells. Finally, tensin fusion proteins exhibit all of the high affinity actin capping characteristics of purified gizzard tensin.

We do not yet know whether the total length of tensin cDNA we have isolated covers the complete sequence of the cardiac tensin molecule because the NH2 terminus of the
Figure 10. Direct comparison of the effects of three tensin fusion proteins on actin polymerization and depolymerization. The samples contained N790-K1060 (filled circles), R1037-E1149^P1162-V1169 (open square), S1061-H1145 (filled square), and the control (open circle). (A) Actin polymerization was measured at 0.5 μM of G-actin in the absence or presence of 0.5 μM N790-K1060, 90 nM R1037-E1149^P1162-V1169, or 1.8 μM S1061-H1145. (B) Actin polymerization was measured at 2 μM of G-actin in the absence or presence of 2.5 μM N790-K1060, 0.2 μM R1037-E1149^P1162-V1169, or 2.5 μM S1061-H1145. (C) Actin depolymerization in the absence or presence of the same concentrations of fusion proteins as in (B). Note that in these experiments, the concentrations given for S1060-H1145 are overestimates caused by the presence of substantial amounts of degradative products (see Fig. 8 C).

The sequence of chicken cardiac tensin (GenBank accession No. L06662) has been compared to all other entries deposited in GenBank and in a report published after our sequencing work has been completed (Lo et al., 1994b). In general, our sequence is in good agreement with all other available tensin sequences, except for some minor discrepancies that might be artifacts of cDNA synthesis. However, two significant sequence variations were also detected. The more notable one is a stretch of 12 amino acids (L1150-E1161) in cardiac tensin that is not found in the CEF tensin (M74165) or gizzard insertin (Weigt et al., 1992; see below for details), a presumptive degradation product of tensin. This short segment in the cardiac tensin sequence is unlikely to be a cloning artifact because it was present in two independent cDNA clones (Th3.2 and JC42), and it is present in the latest entry for chicken cardiac tensin from another laboratory (M96625; Lo et al., 1994b). The other notable variation is that the other tensin sequences (M96625 and M74165) lack the first 35 amino acids, including the first methionine, M30, in our ORF. This variation led Lo et al. (1994b) to designate M49 as the NH2-terminal of their sequence. According to Kozak (1989), the requirement for a strong initiation site is having either A or G at position −3, and less importantly, G at position +4. M49 has G at the +4 position but C at the −3 position, making it less likely an initiation site. In contrast, both M30 and M55 have A or G at position −3 and A at position +4, making them more likely to be initiation sites. A 5′-deleted construct, in which the coding sequence for M30 and M49 was missing, expressed a polypeptide recognized by tensin antibodies in transfected 293 cells, suggesting that M55 can serve as an initiation site.

A most significant advance stemming from the molecular cloning work is our finding that a short sequence of ~120 amino acids (R1037-E1149^P1162-V1169) can produce all of the typical effects of a high affinity F-actin capping protein—complete inhibition of barbed-end polymerization with a Ke in the nanomolar range, inhibition of depolymerization, and shifting of critical monomer concentration. To our knowledge, this is the first time a single, short domain with comparable F-actin-capping activity as the native protein has ever been identified. Furthermore, experiments with the fusion protein containing S1061-H1145 showed that the sequence essential for capping activity can be reduced to 85 residues, although the presence of degradative products precluded an accurate comparison of its specific activity with that of fusion proteins with longer sequences.

Wegner and co-workers (Ruhnau et al., 1989) described a polypeptide designated as "insertin," with properties similar to a group of proteolytic fragments of gizzard tensin (HA1 peptides) previously reported by our laboratory (Wil-
kins and Lin, 1986). To explain why their preparation inhibited barbed-end actin elongation at a maximal level of 80%, they proposed a model in which insertin binds to one of the actin subunits at the end of a filament, inhibiting monomer addition to this subunit while allowing monomer insertion between this protein and the other actin subunit. The amino acid sequence of insertin has since been determined (Weigt et al., 1992). It is almost identical to the amino acid sequence of the CEF tensin sequence in the GenBank (accession No. M74165), and it is also missing the 12-amino acid stretch present in the region containing the high affinity capping domain of our cardiac tensin sequence. To test whether the absence of these 12 amino acids causes a change in mechanism from capping to "inserting," we made a construct (R1037-E1149) that lacks these residues. As stated above, we found that the fusion protein from this construct has all of the properties of a typical actin filament capping factor, completely inhibiting barbed-end elongation and shifting critical concentration at substoichiometric concentrations. Our data, therefore, do not support the insertin model. It is possible that previous experiments on insertin were affected by such limiting factors as the extreme instability of the samples used (Ruhnau et al., 1989).

In the recent study of Lo et al. (1994a), fusion proteins from two short constructs (p27 and p11) containing sequences within the "insertin" sequence of Weigt et al. (1992) were prepared, and their effects on actin polymerization were studied. They concluded that the retardation of actin polymerization at the barbed-end, or "inserting," activity of tensin was associated with residues T936-R1037 (888-989 in their numbering system). In direct contrast to their conclusion, we found that the fusion protein from this construct has all of the properties of a typical actin filament capping factor, completely inhibiting barbed-end elongation and shifting critical concentration at substoichiometric concentrations. Our data, therefore, do not support the insertin model. It is possible that previous experiments on insertin were affected by such limiting factors as the extreme instability of the samples used (Ruhnau et al., 1989).

Figure 11. Structural features and sequence homology in the vicinity of the tensin F-actin capping domain. Indicated in the diagram are the PEST regions (dotted bars), as well as the 12 residues missing in CEF tensin and gizzard insertin (striped bars). Also indicated are annotation of some of the amino acid residues (1050, 1075, etc.) and consensus phosphorylation sites for cdc-28 (KSP and KTP; Langan, 1978), tyrosine kinase (RpsDshY; Cooper et al., 1984), and protein kinase C (SpR and HSyK; Kishimoto et al., 1985). The lower half of the figure shows sequence alignment of the repeated sequence in the tensin capping domain. Identical residues in the repeated sequence are connected by bars and conservative substitutions are connected by double dots. A Gap (−) was inserted for best alignment.
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Table 1. PEST Sequences of Cardiac Tensin

<table>
<thead>
<tr>
<th>Residues</th>
<th>PEST sequence</th>
<th>PEST score</th>
</tr>
</thead>
<tbody>
<tr>
<td>448-464</td>
<td>REDSTEGTWAEPALPGK</td>
<td>6.9</td>
</tr>
<tr>
<td>533-546</td>
<td>RTDEPGAPGAPGTH</td>
<td>5.0</td>
</tr>
<tr>
<td>610-625</td>
<td>RETDILODELPQDGH</td>
<td>5.6</td>
</tr>
<tr>
<td>974-982</td>
<td>RETYTPSYQTVPPEPR</td>
<td>4.9</td>
</tr>
<tr>
<td>1039-1049</td>
<td>KSPEESTVPAR</td>
<td>7.0</td>
</tr>
<tr>
<td>1099-1119</td>
<td>KEAEEMESASPSSLTSGGVR</td>
<td>3.3</td>
</tr>
<tr>
<td>1145-1163</td>
<td>HPVGGEAGADEESEPR</td>
<td>12.2</td>
</tr>
<tr>
<td>1258-1287</td>
<td>HSSYQTSPSSFQATGLGPSYPDPYGDPR</td>
<td>5.4</td>
</tr>
<tr>
<td>1433-1446</td>
<td>RGGQSTQPALPEK</td>
<td>1.4</td>
</tr>
</tbody>
</table>

PEST sequences are identified by using the PEST-FIND computer program developed by Rogers, and PEST scores are calculated as described (Rogers et al., 1986). To qualify as a PEST sequence, the PEST score value has to be $\geq 5.0$, and a PEST score $>0$ indicates a strong PEST region.

We thank W. L. Carter and M. E. Cunningham for assisting in the initial phase of cloning and sequencing, J. Allendorf for expressing some of the truncated tensin fusion proteins in Escherichia coli, J. A. Butler for analysis for PEST sequences, and D. Haynie for making the initial observation of the similarity of the AH1 region of tensin with actin. This work was supported by research grant GM22289 from the National Institutes of Health and a grant-in-aid from the American Heart Association.

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References


