Differential Localization and Sequence Analysis of Capping Protein β-Subunit Isoforms of Vertebrates

Dorothy A. Schafer, Yulia O. Korshunova, Trina A. Schroer,* and John A. Cooper

Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110; and *Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218

Abstract. Capping protein nucleates the assembly of actin filaments and stabilizes actin filaments by binding to their barbed ends. We describe here a novel isoform of the β subunit of chicken capping protein, the β2 isoform, which arises by alternative splicing. The chicken β1 isoform and the β2 isoform are identical in their amino acid sequence except for a short region at the COOH terminus; this region of the β subunit has been implicated in binding actin. Human and mouse cDNAs of the β1 and β2 isoforms also were isolated and among these vertebrates, the COOH-terminal region of each isoform is highly conserved. In contrast, comparison of the sequences of the vertebrate β subunit COOH-termini to those of lower eukaryotes shows no similarities.

The β2 isoform is the predominant isoform of non-muscle tissues and the β1 isoform, which was first characterized in studies of capping protein from chicken muscle, is the predominant isoform of muscle tissues, as shown by immunoblots probed with isoform-specific antibodies and by RNAse protection analysis of mRNAs. The β2 isoform also is a component of dynactin complex from brain, which contains the actin-related protein Arp1. Both β-subunit isoforms are expressed in cardiac muscle but they have non-overlapping subcellular distributions. The β1 isoform is at Z-discs of myofibrils, and the β2 isoform is enriched at intercalated discs; in cardiac myocytes grown in culture, the β2 isoform also is a component of cell-cell junctions and at sites where myofibrils contact the sarcolemma. The biochemical basis for the differential distribution of capping protein isoforms is likely due to interaction with specific proteins at Z-discs and cell-cell junctions, or to preferential association with different actin isoforms. Thus, vertebrates have developed isoforms of capping protein that associate with distinct actin-filament arrays.

FUNDAMENTAL processes of biological systems include generation of asymmetric shapes and movement. The actin cytoskeleton is an essential component of these processes, which are often dynamic and take on diverse forms in different cell types and tissues. Within a single cell, distinct populations of actin filaments can exist, some of which may be formed by a distinct actin isoform. The different actin filament arrays contain several different actin-binding proteins, which likely interact with actin to regulate its assembly. Many of the actin-binding proteins also are expressed as isoforms which may contribute to generate diversity in the organization of actin filaments in cells and tissues.

Capping protein is a candidate for regulating actin filament assembly in vivo. In vitro, capping protein nucleates filament assembly and binds to the barbed ends of filaments, preventing actin monomer addition and loss (9, 12, 13). Evidence supporting these functions in vivo include the colocalization of capping protein with actin filaments in skeletal muscle (13, 45), epithelia (47), and yeast (4). In addition, genetic studies in yeast indicate an interaction of capping protein with actin-binding proteins (1, 29). Furthermore, inhibition of capping protein's ability to bind actin dramatically alters the actin organization in muscle cells undergoing myofibrillogenesis (Schafer, D. A., C. Hug, and J. A. Cooper, manuscript submitted for publication).

Chicken capping protein has been purified from skeletal muscle and is a heterodimeric protein composed of an α subunit of $M_r \sim 36$ kD and a β subunit of $M_r \sim 32$ kD. Two-dimensional (2D)[1] gels of purified capping protein from skeletal muscle, which is also known as CapZ, resolve each subunit into two electrophoretically distinct components (9). The capping protein α subunits, α1 and α2, are the products of different genes; mRNAs encoding the α1 and α2 proteins are expressed in all chicken tissues (14). Even though multiple β-subunit proteins are resolved on 2D gels of purified skeletal muscle capping protein (9) and of extracts of chicken

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Please address all correspondence to Dr. Dorothy A. Schafer, Department of Cell Biology and Physiology, Washington University School of Medicine, Campus Box 8228, 660 S. Euclid Avenue, St. Louis, MO 63110. Telephone: (314) 362-4606; FAX: (314) 362-7463.

1. *Abbreviations used in this paper:* 2D, two dimensional; GST, glutathione-S-transferase; MBP, maltose-binding protein; SAP, sub-sarcolemmal adhesion plaques.

tissues (6), to date, only one cDNA for a capping protein β subunit has been identified in chickens (10) and other species (3, 5, 25, 52). Southern blots of genomic DNA indicated that the β subunit of chickens is encoded by a single gene. Northern blot analysis indicated that two mRNA transcripts were expressed in all chicken tissues (10). Interestingly, the sizes of the two RNA transcripts expressed in chicken muscle and non-muscle tissues differed slightly: in non-muscle tissues, the transcripts each were ~100 nucleotides smaller than those expressed in muscle tissues (10).

To understand the biological function of capping protein, we have now cloned a novel isoform of the β subunit, called β2. The sequence of the β2 isoform differs from the β1 isoform, which was characterized from skeletal muscle, only in a region of the molecule implicated in binding actin (28). The β1 isoform is the predominant isoform expressed in muscle tissues, whereas the β2 isoform is expressed in most tissues examined. In cardiac muscle, which express both β1 and β2, the β1 isoform is located at Z-discs of sarcomeres while the β2 isoform is enriched at intercalated discs. The different subcellular distributions for these two capping protein isoforms suggests that the two isoforms have unique functions that are essential for the organization of actin filaments at different locations within a single cell.

Materials and Methods

Reagents were purchased from Sigma Chem. Co. (St. Louis, MO) or Fisher Chemical Co. (Pittsburgh, PA) unless stated otherwise. Dynactin complex was purified from chick embryo brain as described (48). Actin was purified from chicken pectoral muscle as described (49).

Antibodies

Affinity-purified goat polyclonal antibodies to skeletal muscle capping protein were purified as described (45). Goat antibodies specific for the β subunit that were used to screen the cDNA libraries, were purified on columns containing a fusion protein of glutathione-S-transferase (GST) and the β subunit of skeletal muscle capping protein (47). Rabbit antibodies specific for the β2 isoform were elicited in rabbits by Cocalico Biological, Inc. (Reamstown, PA). The immunogen was a bacterially expressed fusion protein containing GST and the 27 amino acids that are unique to the COOH terminus of the β2 isoform. These antibodies were affinity-purified using an Affigel affinity column containing maltose-binding protein (MBP) fused to the same 27 COOH terminus amino acids of the β2 isoform. Mouse monoclonal antibody (mAb) 1B11 and mAb 1E5, which are specific for the α subunit and β subunit, respectively, of chicken skeletal muscle capping protein were purified on protein A-agarose as described (28). Monoclonal anti-vinculin and anti-A-CAM were purchased from Sigma Chem. Co. Fluorophore-conjugated antibodies used for indirect immunofluorescence experiments were purchased from Chemicon (Temecula, CA).

Electrophoresis and Western Blotting

One-dimension SDS gels were run as described by Laemmli (30). Two-dimensional electrophoresis was performed using a mini-isoelectric focusing apparatus according to the method of O'Farrell (37); ampholytes (Pharmacia LKB Biotechnology, Piscataway, NJ) were used in a combination of pH 3–10 at 0.4% (wt/vol) and pH 5–8 at 1.6% (wt/vol). The second dimension SDS gels were 10% acrylamide gels as described (30). Proteins were transferred to nitrocellulose as described (50) using 25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol. Blots were blocked in 2% fish gelatin, 5% heat-inactivated calf serum in TTBS (0.3 M NaCl, 20 mM TrisCl, pH 8.0 and 0.05% (vol/vol) Tween-20 and 0.01% NaN$_3$), and incubated overnight at 4°C with primary antibodies diluted in the blocking solution. Blots were washed in TTBS and bound antibodies were detected using the appropriate species-specific alkaline phosphatase–conjugated secondary antibody (Tago, Burlingame, CA). Blots were developed to yield the alkaline phosphatase reaction product (20).

Isolation of Chicken Capping Protein β-Subunit cDNAs

A λgt11 cDNA library (15) prepared from poly(A⁺) RNA from 10-d chicken embryos was kindly provided by Dr. Susan W. Craig of Johns Hopkins University. Approximately 2 × 10⁶ phage clones were screened using goat polyclonal anti-β subunit specific antibodies and swine anti-goat immunoglobulin conjugated with alkaline phosphatase (Tago) according to the method of Davis et al. (18). The cDNAs in λgt11 were isolated from the phage after EcoR1 digestion and ligated into pBluescript (Stratagene, La Jolla, CA). A set of cDNA clones that were originally isolated from a chicken muscle cDNA library by screening with a β subunit cDNA as described (10) also were used in these studies, including eight β1 cDNAs (β16, β25, β27, β29, β33, β53, β72, and β74d), and one β2 cDNA (β78).

Isolation of Mouse Capping Protein β-Subunit cDNAs

A fragment of chicken β2 cDNA was used to screen a λgt11 cDNA library of newborn mouse (P$_0$) skeletal muscle (kindly provided by Drs. Maria Donoghue, Joshua Sanes, and John Merlie, Washington University). Twenty-four cDNA clones were purified; the sequences at the ends of 22 clones indicated that they were similar to chicken and human β-subunit cDNAs. Additional internal sequence showed that 14 clones were analogous to the chicken β1 cDNA and eight clones were analogous to the chicken β2 cDNA. Two of the longest clones in each group were sequenced on both strands using a shotgun strategy. The sequences are in Genbank; accession numbers for the mouse β-subunit cDNAs are #U10406 for β1, and #U10407 for β2.

Isolation of Human Capping Protein β Subunit cDNAs

Two cDNAs with homology to the β subunit of capping protein were identified in searches of the non-redundant nucleic acid data bases at NCBI using BLASTX (2) and Client v. 1.7.5. This search identified two partial overlapping cDNAs, one from a HepG2 library (HUM0005409, accession number D12250, [38]) and one from a testis library (HUMTACEB, accession number M26658, [19]). The cDNAs are identical in their region of overlap, which includes the COOH-terminal portion of the coding region of the capping protein β subunit. Using this sequence, we constructed primers and performed PCR on a HepG2 cDNA library kindly provided by Dr. Mike Mueckler of Washington University (36). A clone matching the data base entries was recovered. Several sequential RACE PCR reactions were performed to extend the cDNA clones to full-length (22). The predicted amino acid sequence is very similar to that of the chicken β2 isoform reported here. This analysis agrees with a more complete characterization of a human β cDNA by others (Barron-Casella, E. A., M. A. Torres, S. W. Scherer, H. H. Q. Heng, L.-C. Tsui, and J. F. Casella, manuscript submitted for publication).

Nucleotide Sequencing of cDNAs

DNA sequencing was performed by the dideoxy termination method (44) using Sequenase as described by the manufacturer (U.S. Biochemical Corp., Cleveland, OH) or by PCR cycle-sequencing using Taq polymerase (17).

Plasmid Constructions

pBJ-378 contains the 533 nt KpnI-EcoR1 fragment corresponding to the 3' half of the β1 cDNA inserted into pBS. pBJ-380 contains the 419 nt Kpn-EcoR1 fragment corresponding to the 3' half of the β2 cDNA inserted into pBS. Plasmids encoding fusion proteins containing the 27 amino acids that are unique to the COOH terminus of the β2 isoform of capping protein β subunit (amino acids 246-272) and either GST or MBP were prepared by ligation of a PCR-derived DNA fragment encoding the 27 unique COOH-terminal amino acids into the EcoR1 site of either pGEX (Pharmacia LKB Biotechnology) or pMAL-c2 (New England Biolabs, Beverly, MA), respectively. The resulting plasmids are designated pBJ-538 for expression of GST-β2-COOH terminus fusion protein and pBJ-458 for expression of MBP-β2-COOH terminus fusion protein.

RNAse Protection Analysis

Ribonuclease protection analyses were performed using the RPA II assay kit as described by the manufacturer (Ambion, Austin, TX). Poly(A⁺) RNA was isolated from chicken tissues as described (8). RNA probes were

prepared by in vitro transcription in a reaction that contained 0.25 μg linearized DNA, 0.5 mM ATP, 0.5 mM CTP, and 0.5 mM UTP, 10 mM DTT, 20 U RNAsin (Promega, Madison, WI), 40 μCi ^{32}P-labeled GTP (Amersham Corp., Arlington Heights, IL), 0.025 U T3 RNA polymerase (Stratagene), 50 mM Tris-HCl, pH 7.5, 6 mM MgCl$_2$, 2 mM spermidine, and 10 mM NaCl. The DNA templates for preparation of the RNA probes were KpnI-digested pBJ-378 and pBJ-380 (described in the preceding section), for probe 533 and probe 419, respectively.

Cardiac Myocyte Cultures

Hearts from 6-d chick embryos were incubated in 2–3 ml 0.05% trypsin in calcium- and magnesium-free HBSS for 5 min in a 37°C shaking incubator. The suspension was vortexed at low speed for 10 s and the non-dissociated tissue was allowed to settle. The supernatant containing dissociated cells was removed and fresh trypsin solution was added. The trypsin digestion was repeated as described above six times. The first two supernatants were discarded; subsequent supernatants were added to 20 ml plating medium composed of MEM-Earle's salts, 5% "selected" FBS, 2 mM glutamine, penicillin, and streptomycin. Dispersed cells were preplated in a 100-mm culture dish for 1 h at 37°C to deplete fibroblasts. Myocytes were plated at 2 × 10^5 cells/35-mm dish containing a 22-mm × 22-mm coverslip in plating medium. Two days after plating, the medium was replaced with glutamine-free medium; this medium was replaced every other day thereafter.

Immunostaining

Cryosections (5 μm thick) of adult chicken heart tissue were applied to gelatin-coated glass slides and fixed in 2% paraformaldehyde in PBS (0.137 M NaCl, 2.6 mM KCl, 1.5 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, 0.01% NaN$_3$, pH 7.4) for 15 min at room temperature. Excess aldehyde was quenched by 10 min incubation in 1 mg/ml NaBH$_4$ in PBS. Slides to be labeled with mAb 1E5 also were treated for 10 min with methanol at −20°C followed by 5 min in PBS. Sections were blocked in 10% heat-inactivated newborn calf serum and 3% BSA prepared in TTBS for 30 min. For double labeling with the β2-specific antibodies and anti-vinculin, a mixture of the primary antibodies diluted in blocking buffer was applied to the sections for 4 h at room temperature. Slides were washed three times in TTBS and incubated with a mixture of fluorescein-conjugated donkey anti–rabbit IgG and rhodamine-conjugated donkey anti–mouse IgG for 2 h at room temperature. Slides were washed three times in TTBS and coverslips were applied using a mounting medium composed of 0.1% n-propylgallate in 10 mM TrisCl, pH 8.0, and 50% glycerol. Micrographs were obtained using an MRC-1000 (Bio-Rad Laboratories, Hercules, CA) scanning laser confocal microscope equipped with a krypton-argon mixed gas laser.

Cardiac myocyte cultures were rinsed in 30 mM Hepes, pH 7.0 containing 70 mM KCl, 5 mM MgCl$_2$, and 3 mM EGTA and fixed for 15 min at room temperature in freshly prepared 2% paraformaldehyde in the same Hepes buffer. Cells were permeabilized by incubation in 0.1% Triton X-100 in the Hepes buffer for 15 min and blocked in 10% heat-inactivated newborn calf serum and 3% BSA prepared in TTBS. For double labeling with anti-β2-specific antibodies and mAb 1E5, a mixture of the primary antibodies diluted in blocking buffer was applied to the cells overnight at 4°C. Cells were washed three times in TTBS and incubated with a mixture of fluorescein-conjugated donkey anti–rabbit IgG and rhodamine-conjugated donkey anti–mouse IgG for 2 h at room temperature. Cells were washed three times in TTBS and coverslips were mounted on slides using the mounting medium described above. Micrographs were obtained using a Zeiss Axioplan microscope equipped with a 63× planapochromat objective lens (1.4 NA). T-max 400 ASA film (Eastman Kodak, Rochester, NY) was used for photography.

Results

Three observations suggested that the capping protein β subunit of chicken non-muscle tissues was different from the β subunit of capping protein isolated from skeletal muscle. First, polyclonal antibodies prepared against skeletal muscle capping protein reacted well with the α-subunit proteins on Western blots of extracts of chicken epithelial tissues, but poorly with the β-subunit proteins (47). Second, the capping protein β subunit of epithelial cell extracts has a slightly faster mobility on SDS gels than the β subunit of capping

protein from skeletal muscle (47). Third, reports on a protein called β-actinin (33), which has since been found to be capping protein (34), indicated that some chicken tissues contained a capping protein subunit that was most likely a β subunit, but which was electrophoretically distinct from the β subunits of skeletal muscle (6).

To further characterize capping protein β subunit isoforms, we investigated the distribution of the capping protein subunits in chicken tissues using immunoblots of 2D gels probed with polyclonal antibodies (45, 47) and monoclonal antibodies (28) raised against capping protein of skeletal muscle. To facilitate this discussion, we have named the major polypeptides detected on the Western blots of the 2D gels according to the diagram in Fig. 1 a: the major α-subunits with M_r~36 kD are A1 (pI~5.7) and A2 (pI~5.9) and the major β subunits with M~32 kD are B1 (pI~5.5), B1' (pI~5.3), and B2 (pI~6.0). These roman letter designations are heuristic and temporary. Once a specific protein spot has been determined as the product of a specific cDNA, the protein is henceforth referred to using a Greek letter and a number that corresponds to the name of its encoding cDNA.
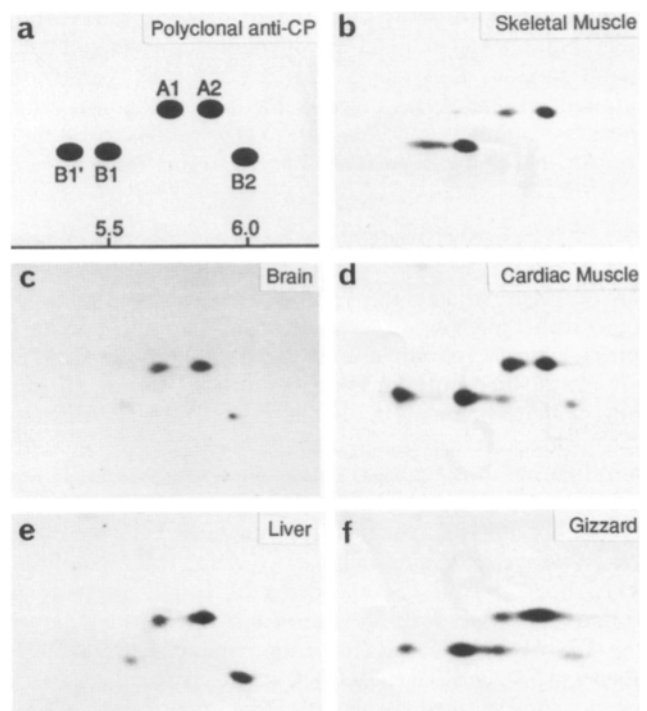
Figure 1. Western blots of two-dimensional gels of chicken tissue extracts probed with goat polyclonal anti-capping protein. This antibody detects all subunits of capping protein; however, because the antigen was skeletal muscle capping protein, the antibodies recognize the B1 and B1' proteins better than the B2 protein. Panel *a* is a drawing indicating the mobilities and names of the major capping protein subunits detected by the antibody; the scale at the bottom indicates the approximate pH range of the first dimension gel. Tissue extract samples are: *b*, Skeletal (pectoralis) Muscle; *c*, Brain; *d*, Cardiac Muscle; *e*, Liver; *f*, Gizzard. Only the portion of each blot containing capping protein is shown.

A Novel Capping Protein β Subunit Is Expressed in Chicken Brain, Liver, Cardiac Muscle, and Gizzard

Western blots of 2D gels containing whole tissue extracts of chicken brain, liver, pectoral muscle, cardiac muscle, and gizzard were probed with either the polyclonal antibodies (Fig. 1) or with a mixture of mAbs 1E5 and 1B11 (Fig. 2), which detect the β subunit and the α subunit (preferentially the A2 protein) of capping protein, respectively (28). mAb 1E5 also reacts with the protein product of the previously characterized β-subunit cDNA (10, 28). The distribution of the α-subunit proteins, A1 and A2, was nearly identical in the tissues examined. In contrast, the distribution of the β-subunit isoforms varied among different tissues. Three major isoforms of the β subunit were detected using the polyclonal anti-capping protein preparation (Fig. 1). In pectoral muscle, cardiac muscle and gizzard, the B1 and B1′ proteins were the major β subunits (Fig. 1, b, d, and f). These proteins had similar mobilities on 2D gels as the β-subunit isoforms of capping protein purified from chicken pectoral muscle (9). The B1 and B1′ proteins also reacted with mAb 1E5 (Fig. 2, b, d, and f). In brain, liver, cardiac muscle, and gizzard, proteins having a similar M_r in the SDS gels as the B1 and B1′ proteins, but having more alkaline isoelectric points also were detected with the polyclonal anti-capping protein. Brain and liver were particularly enriched for a β-subunit isoform with a pI∼6.0, designated here the B2 protein (Fig. 1, c and e). mAb 1E5 did not react with the B2 protein (Fig. 2, c, d, e, and f). The lack of reactivity of the B2 protein with mAb 1E5 suggests that this β subunit is either posttranslationally modified at the epitope bound by mAb 1E5 or that the epitope is missing from this isoform.

A Novel mRNA Encodes the β2 Isoform of Capping Protein β Subunit

To investigate whether the B2 protein results from expression of a distinct mRNA, we screened a chick embryo cDNA expression library for clones expressing protein recognized by affinity-purified polyclonal antibodies specific for all capping protein β subunits but not by mAb 1E5. Approximately thirty clones were obtained in the initial screen. Four clones also hybridized at high stringency with a full-length β-subunit cDNA probe previously isolated from a chicken muscle cDNA library (10). Sequence analysis showed that these four clones were overlapping and that two were identical siblings. The longest clone (EM7) encoded a full-length open reading frame; the consensus sequence of this cDNA, now named the β2 cDNA (Genbank accession number U07826), is shown in Fig. 3 A. The β2 cDNA was nearly identical to the previously identified β-subunit cDNA, now named the β1 cDNA (Genbank accession number J04959) (10), except that nucleotides 758-870 of the β1 cDNA were deleted. The sequence of the 5′ untranslated region of the β2 cDNA exactly matched that of one β1 cDNA (β33) clone previously described (10). Fig. 3 B shows the relationship between the β1 cDNA and the β2 cDNAs.
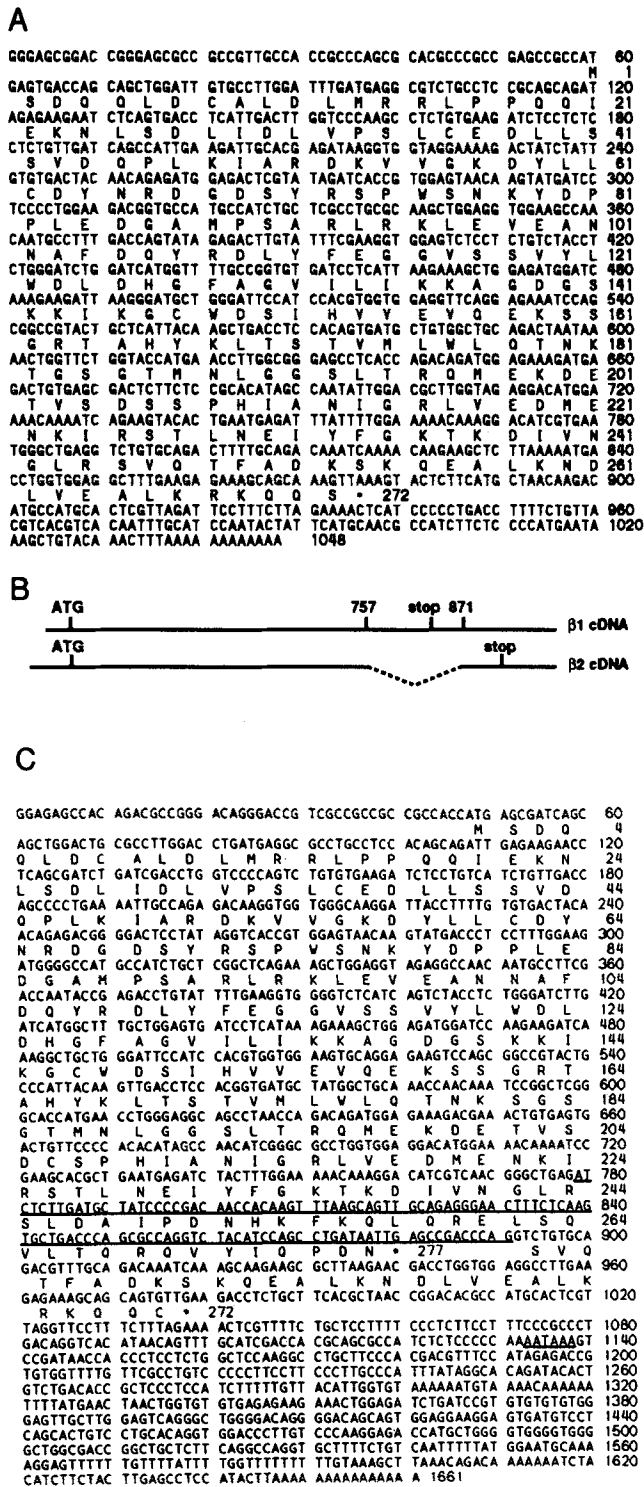
The 113 nucleotides missing from the β2 cDNA include those encoding the 32 COOH-terminal amino acids and the stop codon for translation of the β1 protein. Thus, the protein predicted from the β2 cDNA sequence is identical at its NH₂ terminus, including amino acids 1 through 245, to the NH₂ terminus of the β1 protein. The amino acids compris-
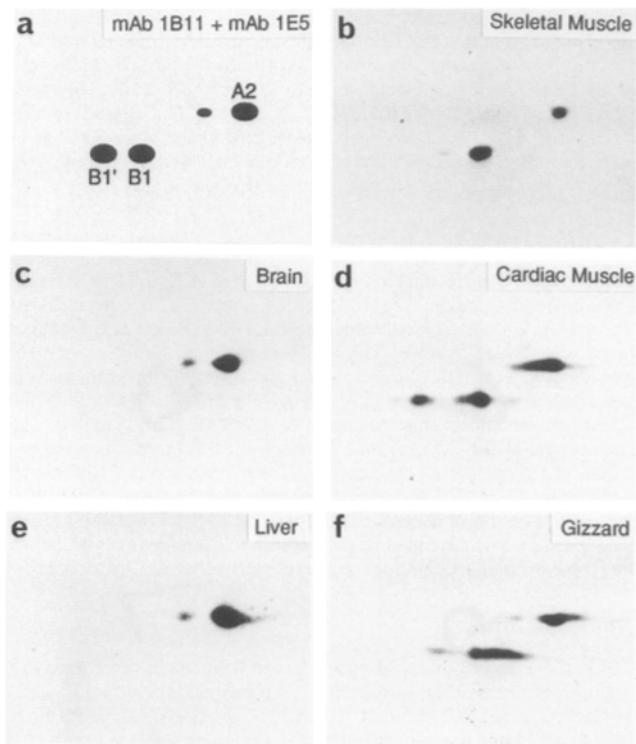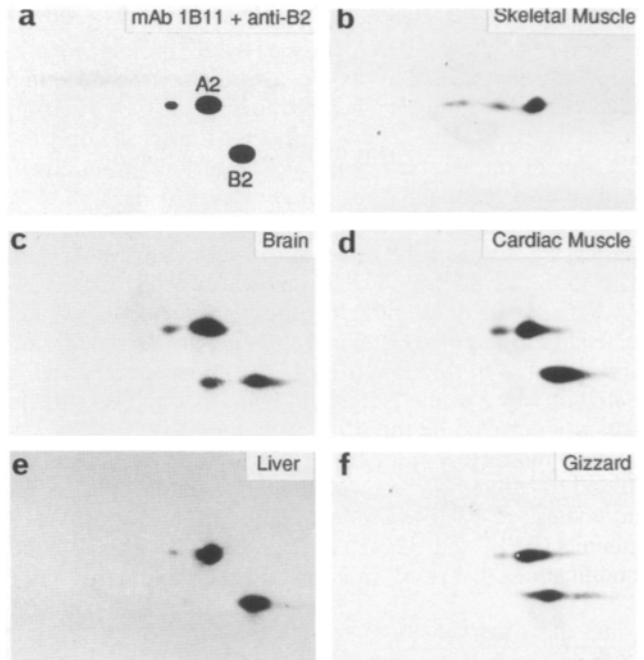

Figure 2. Western blots of two-dimensional gels of chicken tissue extracts probed with a mixture of mAb 1B11, which recognizes the A2 protein strongly and the A1 protein weakly, and mAb 1E5, which detects the B1 and B1′ proteins. Panel *a* shows a drawing indicating the capping protein protein subunits that react with this mixture of antibodies. Tissue extract samples are: *b*, Skeletal Muscle; *c*, Brain; *d*, Cardiac Muscle; *e*, Liver; *f*, Gizzard. These blots were reacted with a mixture of mAb 1E5 and mAb 1B11 in order to observe the A2 α-subunit as point of reference for the mobilities of the β-subunit isoforms. Only the portion of each blot containing capping protein is shown; the pH range of the first dimension gel is as indicated in Fig. 1 *a*.

ing the COOH terminus of the putatitve β2 protein are encoded by nucleotides that are present in the 3′ untranslated region of the β1 cDNA, which contains a second stop codon (Fig. 3 B). The organization of the β1 and β2 cDNAs suggests that the two β-subunit mRNAs arise from a primary RNA transcript that is alternatively spliced, with the β1 mRNA containing an exon corresponding to nucleotides 758-870 of the β1 cDNA that is absent from the β2 mRNA. This notion is supported by the nucleotide sequences at the sites where presumptive splice junctions formed in both cDNAs, which fit the consensus sequence for exon–exon boundaries (35). For the presumptive splice junction in the β2 mRNA and at one site in the β1 mRNA, this sequence is AGG, where the underline indicates the first nucleotide of the acceptor exon; the sequence at the other presumed splice junction in the β1 mRNA is AGA. In addition, the existence of alternatively spliced β-subunit mRNAs is consistent with the facts that only one β-subunit gene was detected by Southern blot analysis of genomic DNA and that the mRNA transcripts detected in muscle and non-muscle tissues differ in size by ∼100 nucleotides (10).

Several features can be deduced from the predicted peptide sequence of the β2 protein. First, the new COOH termi-

A

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GGGAGCGGAC CGGGAGCGCC GCCGTTGCCA CCGCCCAGCG CACGCCCGCC GAGCCGCCAT 60
                                                           M   1
GAGTGACCAG CAGCTGGATT GTGCCTTGGA TTTGATGAGG CGTCTGCCTC CGCAGCAGAT 120
 S  D  Q   Q  L  D   C  A  L  D   L  M  R   R  L  P   P  Q  Q  I  21
AGAGAAGAAT CTCAGTGACC TCATTGACTT GGTCCCAAGC CTCTGTGAAG ATCTCCTCTC 180
 E  K  N   L  S  D   L  I  D  L   V  P  S   L  C  E   D  L  L  S  41
CTCTGTTGAT CAGCCATTGA AGATTGCACG AGATAAGGTG GTAGGAAAAG ACTATCTATT 240
 S  V  D   Q  P  L   K  I  A  R   D  K  V   V  G  K   D  Y  L  L  61
GTGTGACTAC AACAGAGATG GAGACTCGTA TAGATCACCG TGGAGTAACA AGTATGATCC 300
 C  D  Y   N  R  D   G  D  S  Y   R  S  P   W  S  N   K  Y  D  P  81
TCCCCTGGAA GACGGTGCCA TGCCATCTGC TCGCCTGCGC AAGCTGGAGG TGGAAGCCAA 360
 P  L  E   D  G  A   M  P  S  A   R  L  R   K  L  E   V  E  A  N  101
CAATGCCTTT GACCAGTATA GAGACTTGTA TTTCGAAGGT GGAGTCTCCT CTGTCTACCT 420
 N  A  F   D  Q  Y   R  D  L  Y   F  E  G   G  V  S   S  V  Y  L  121
CTGGGATCTG GATCATGGTT TTGCCGGTGT GATCCTCATT AAGAAAGCTG GAGATGGATC 480
 W  D  L   D  H  G   F  A  G  V   I  L  I   K  K  A   G  D  G  S  141
AAAGAAGATT AAGGGATGCT GGGATTCCAT CCACGTGGTG GAGGTTCAGG AGAAATCCAG 540
 K  K  I   K  G  C   W  D  S  I   H  V  V   E  V  Q   E  K  S  S  161
CGGCCGTACT GCTCATTACA AGCTGACCTC CACAGTGATG CTGTGGCTGC AGACTAATAA 600
 G  R  T   A  H  Y   K  L  T  S   T  V  M   L  W  L   Q  T  N  K  181
AACTGGTTCT GGTACCATGA ACCTTGGCGG GAGCCTCACC AGACAGATGG AGAAAGATGA 660
 T  G  S   G  T  M   N  L  G  G   S  L  T   R  Q  M   E  K  D  E  201
GACTGTGAGC GACTCTTCTC CGCACATAGC CAATATTGGA CGCCTTGGTAG AGGACATGGA 720
 T  V  S   D  S  S   P  H  I  A   N  I  G   R  L  V   E  D  M  E  221
AAACAAAATC AGAAGTACAC TGAATGAGAT TTATTTTGGA AAAACAAAGG ACATCGTGAA 780
 N  K  I   R  S  T   L  N  E  I   Y  F  G   K  T  K   D  I  V  N  241
TGGGCTGAGG TCTGTGCAGA CTTTTGCAGA CAAATCAAAA CAAGAAGCTC TTAAAAATGA 840
 G  L  R   S  V  Q   T  F  A  D   K  S  K   Q  E  A   L  K  N  D  261
CCTGGTGGAG GCTTTGAAGA GAAAGCAGCA AAGTTAAAGT ACTCTTCATG CTAACAAGAC 900
 L  V  E   A  L  K   R  K  Q  Q   S  *  272
ATGCCATGCA CTCGTTAGAT TCCTTTCTTA GAAAACTCAT CCCCCTGACC TTTTCTGTTA 960
CGTCACGTCA CAATTTGCAT CCAATACTAT TCATGCAACG CCATCTTCTC CCCATGAATA 1020
AAGCTGTACA AACTTTAAAA AAAAAAAA 1048
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B

C

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GGAGAGCCAC AGACGCCGGG ACAGGGACCG TCGCCGCCGC CGCCACCATG AGCGATCAGC 60
                                                      M  S  D  Q  4
AGCTGGACTG CGCCTTGGAC CTGATGAGGC GCCTGCCTCC ACAGCAGATT GAGAAGAACC 120
 Q  L  D  C  A  L  D   L  M  R   R  L  P  P   Q  Q  I   E  K  N  24
TCAGCGATCT GATCGACCTG GTCCCCAGTC TGTGTGAAGA TCTCCTGTCA TCTGTTGACC 180
 L  S  D  L   I  D  L   V  P  S   L  C  E  D   L  L  S   S  V  D  44
AGCCCCTGAA AATTGCCAGA GACAAGGTGG TGGGCAAGGA TTACCTTTTG TGTGACTACA 240
 Q  P  L  K   I  A  R   D  K  V   V  G  K  D   Y  L  L   C  D  Y  64
ACAGACACGG GGACTCCTAT AGGTCACCGT GGAGTAACAA GTATGACCCT CCTTTGGAAG 300
 N  R  D  G   D  S  Y   R  S  P   W  S  N  K   Y  D  P   P  L  E  84
ATGGGGCCAT GCCATCTGCT CGGCTCAGAA GCTGGAGGT AGAGGCCAAC AATGCCTTCG 360
 D  G  A  M   P  S  A   R  L  R   K  L  E  V   E  A  N   N  A  F  104
ACCAATACCG AGACCTGTAT TTTGAAGGTG GGGTCTCATC AGTCTACCTC TGGGATCTTG 420
 D  Q  Y  R   D  L  Y   F  E  G   G  V  S  S   V  Y  L   W  D  L  124
ATCATGGCTT TGCTGGAGTG ATCCTCATAA AGAAAGCTGG AGATGGATCC AAAAAGATCA 480
 D  H  G  F   A  G  V   I  L  I   K  K  A  G   D  G  S   K  K  I  144
AAGGCTGCTG GGATTCCATC CACGTGGTGG AAGTGCAGGA GAAGTCCAGC GGCCGTACTG 540
 K  G  C  W   D  S  I   H  V  V   E  V  Q  E   K  S  S   G  R  T  164
CCCATTACAA GTTGACCTCC ACGGTGATGC TATGGCTGCA AACCAACAAA TCCGGCTCGG 600
 A  H  Y  K   L  T  S   T  V  M   L  W  L  Q   T  N  K   S  G  S  184
GCACCATGAA CCTGGGAGGC AGCCTAACCA GACAGATGGA GAAAGACGAA ACTGTGAGTG 660
 G  T  M  N   L  G  G   S  L  T   R  Q  M  E   K  D  E   T  V  S  204
ACTGTTCCCC ACACATAGCC AACATCGGGC GCCTGGTGGA GGACATGGAA AACAAAATCC 720
 D  C  S  P   H  I  A   N  I  G   R  L  V  E   D  M  E   N  K  I  224
GAAGCACGCT GAATGAGATC TACTTTGGAA AAACAAAGGA CATCGTCAAC GGGCTGAGAT 780
 R  S  T  L   N  E  I   Y  F  G   K  T  K  D   I  V  N   G  L  R  244
CTCTTGATGC TATCCCCGAC AACCACAAGT TTAAGCAGTT GCAGAGGGAA CTTTCTCAAG 840
 S  L  D  A   I  P  D   N  H  K   F  K  Q  L   Q  R  E   L  S  Q  264
TGCTGACCCA GCGCCAGGTC TACATCCAGC CTGATAATTG AGCCGACCCA GGTCTGTGCA 900
 V  L  T  Q   R  Q  V   Y  I  Q   P  D  N  *   277       S  V  Q
GACGTTTGCA GACAAATCAA AGCAAGAAGC GCTTAAGAAC GACCTGGTGG AGGCCTTGAA 960
 T  F  A  D   K  S  K   Q  E  A   L  K  N  D   L  V  E   A  L  K
GAGAAAGCAG CAGTGTTGAA GACCTCTGCT TCACGCTAAC CGGACACGCC ATGCACTCGT 1020
 R  K  Q  Q  *  272
TAGGTTCCTT TCTTTAGAAA ACTCGTTTTC TGCTCCTTTT CCCTCTTCCT TTCCCGCCCT 1080
GACAGGTCAC ATAACAGTTT GCATCGACCA CGCAGCGCCA TCTCTCCCCC AAAATAAAGT 1140
CCGATAACCA CCCTCCTCTG GCTCCAAGGC CTGCTTCCCA CGACGTTTCC ATAGAGACCG 1200
TGTGGTTTTG TTCGCCTGTC CCCCTTCCTT CCCTTGCCCA TTTATAGGCA CAGATACACT 1260
GTCTGACACC GCTCCCTCCA TCTTTTTGTT ACATTGGTGT AAAAAATGTA AAACAAAAAA 1320
TTTTATGAAC TAACTGGTGT GTGAGAGAAG AAACTGGAGA TCTGATCCGT GTGTGTGTGG 1380
GAGTTGCTTG GAGTCAGGGC TGGGGACAGG GGACAGCAGT GGAGGAAGGA GTGATGTCCT 1440
CAGCACTGTC CTGCACAGGT GGACCCTTGT CCCAAGGAGA CCATGCTGGG GTGGGGTGGG 1500
GCTGGCGACC GGCTGCTCTT CAGGCCAGGT GCTTTTCTGT CAATTTTTAT GGAATGCAAA 1560
AGGAGTTTTT TGTTTTATTT TGGTTTTTTT TTGTAAAGCT TAAACAGACA AAAAAATCTA 1620
CATCTTCTAC TTGAGCCTCC ATACTTAAAA AAAAAAAAAA A 1661
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Figure 3. (A) Consensus nucleotide sequence of the chicken β2 cDNA and the amino acid sequence of the β2 isoform. A consensus polyadenylation signal is at nucleotides 1017-1022; the stop codon is indicated by an asterisk. These sequences are in GenBank (accession number U07826). (B) Diagram showing a comparison of the β1 and β2 cDNAs. The positions of the start codon (ATG) and the stop codon (stop) are indicated. Nucleotides 758-870 of the β1 cDNA, which are missing from the β2 cDNA, are indicated by the dashed lines. (C) Consensus nucleotide sequence and predicted protein sequence of mouse β-subunit cDNAs. The nucleotide sequence of a β1 cDNA (GenBank accession number U10406), is shown with the predicted amino acid sequence underneath. A β2

Figure 4. Western blots of two-dimensional gels of chicken tissue extracts probed with a mixture of mAb 1B11, which detects primarily the A2 subunit, and affinity-purified β2-subunit specific antibodies. Panel *a* shows a drawing of the proteins that react with this mixture of antibodies. Tissue extract samples are: *b*, pectoral muscle; *c*, Brain; *d*, Skeletal Muscle; *e*, Liver; *f*, Gizzard. These blots were reacted with a mixture of mAb 1B11 and the β2-specific antibodies in order to observe the A2 α subunit as point of reference for the mobilities of the β-subunit isoforms. Only the portion of each blot containing capping protein is shown; the pH range is as indicated in Fig. 1 *a*.

nus predicted for the β2 protein no longer contains the epitope for mAb 1E5, which was mapped within 25 amino acids of the COOH terminus of the β1 protein (28); thus, the lack of reactivity of mAb 1E5 with the B2 protein on Western blots of 2D gels is consistent with the absence of the epitope. Second, the isoelectric point predicted for the protein encoded by the β2 cDNA is 5.8, which is close to the observed isoelectric point of the B2 protein on 2D gels, and different from the isoelectric point of 5.3 predicted from the sequence of the β1 protein and observed for the B1 protein on 2D gels. Third, the molecular mass predicted for the β2 protein is slightly smaller than that predicted for the β1 protein (30,613 kilodaltons for β2 vs 31,367 daltons for β1); this is consistent with the slightly faster mobility of the B2 protein in SDS gels.

cDNA (GenBank accession number U10407) differs from the sequence shown in that nucleotides 801-913, underlined here, are deleted. This deletion gives the alternative predicted protein sequence for the COOH terminus of the β2 isoform as indicated under the nucleotides 913-997. Stop codons are indicated with asterisks. In addition, the β2 cDNA has a slightly shorter 5' untranslated sequence, starting at nucleotide 52, and uses an alternative polyadenylation site after nucleotide 1177. A similar alternative polyadenylation site was also observed for some β1 cDNAs. A polyadenylation signal at 1155-1160 is underlined.

Verification That the β2 cDNA Encodes the B2 Protein

To verify that the β2 cDNA encodes the B2 protein, we prepared affinity-purified antibodies specific for the 27 amino acids predicted from the β2 cDNA to be at the COOH terminus of the β2 protein. These antibodies recognized only the B2 protein on Western blots of 2D gels of brain, liver, cardiac muscle, and gizzard tissue extracts (Fig. 4, c, d, e, and f). On the basis of its reaction with the β2-specific antibodies, we designate the B2 protein observed on the Western blots as the β2 isoform. The β2-specific antibody did not react with the B1 or B1' proteins; thus, no β subunit was detected in extracts of skeletal muscle using the β2-specific antibody (Fig. 4 b). In brain (Fig. 4 c), a minor species having a slightly more acidic isoelectric point than the B2 protein also was detected by the β2-specific antibody.

The minor forms of the β subunits, designated here as the B1' and B2' proteins, react with mAb 1E5 and the β2-specific antibodies, respectively. Most likely these subsets of β subunits (B1/B1' and B2/B2') are related by posttranslational modifications that result in forms of each protein that differ slightly in isoelectric point. Different levels of phosphorylation could explain the different isoelectric points, however, no phosphate (<0.1 mol/mol) was detected in purified capping protein of skeletal muscle, which contains the B1' protein (9).

Expression of the β2 mRNA Correlates with the Expression of the β2 Protein

To correlate the expression of the β2 mRNA and the β2 protein, we analyzed the expression of the β1 and β2 mRNAs in a variety of chicken tissues using RNAse protection analysis (Fig. 5). The RNA probes used in these experiments were designed to detect β-subunit mRNAs that either contained or lacked the 113 nucleotides that distinguish the β1 mRNA from the β2 mRNA (Fig. 5 C). Hybridization of probe 419 (Fig. 5 A) to β1 mRNA yields two fragments of 240 nt and 174 nt, and hybridization to β2 mRNA yields one fragment of 410 nt. Likewise, hybridization of probe 533 (Fig. 5 B) to β1 mRNA yields one fragment of 533 nt, and hybridization to β2 mRNA yields two fragments of 244 nt and 174
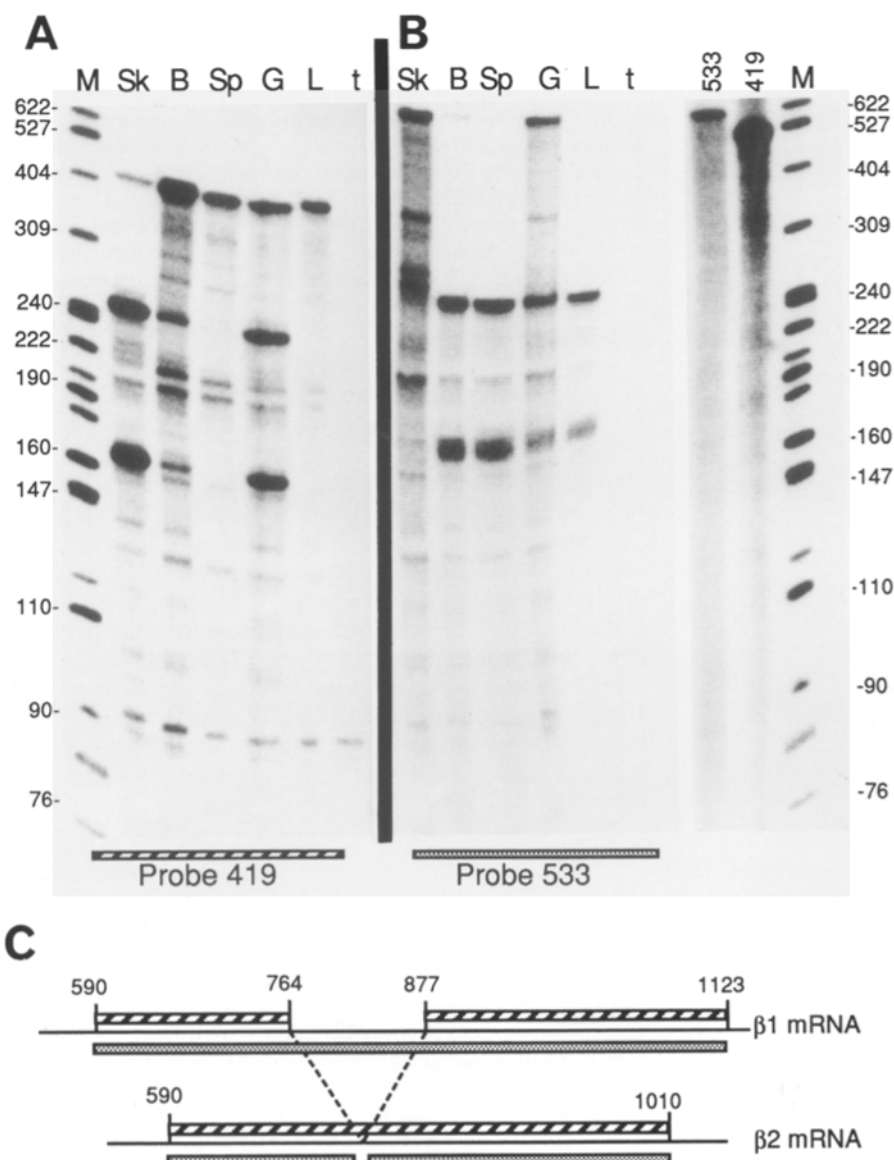
Figure 5. RNAse protection assay for identification of the β1 and β2 mRNAs in chicken tissues. Samples of poly(A⁺) RNA used in the assays are: *Sk*, Skeletal Muscle; *B*, brain; *Sp*, spleen; *G*, gizzard; *L*, liver; *t*, yeast tRNA. *A* shows samples hybridized with probe 419; *B* shows samples hybridized with probe 533. Lanes labeled 533 and 419 contain samples of probe 533 and probe 419, respectively. ³²P-labeled DNA size markers obtained from HpaII-digested pBR322 are in lanes marked *M* and their sizes in nucleotides are indicated on the side of each panel. (*C*) Diagram of the β1 and β2 mRNAs and their expected patterns of hybridization with probe 533 and probe 419. Probe 533 (*stippled bars*) hybridizes completely with β1 mRNA and protects ~533 nt; probe 533 partially hybridizes with β2 mRNA and two products of 174 nt and 244 nt are protected. Probe 419 (*hatched bars*) hybyridizes completely with β2 mRNA and protects a fragment of ~419 nt; probe 419 hybridizes with β1 mRNA such that fragments of 240 nt and 174 nt are protected.

nt. In striated muscles, such as pectoral muscle and cardiac muscle (not shown), an mRNA corresponding to the β1 mRNA was the major form of β-subunit mRNA expressed; a small amount of an mRNA corresponding to the β2 mRNA also was detected in these tissues. In gizzard, the ratio of β1 and β2 mRNAs was approximately 2:1. In liver, intestine (not shown), and spleen, only the β2 mRNA was detected; in brain, the β2 mRNA was the predominant transcript, but a small amount of β1 mRNA also was detected. In addition, several cDNAs previously isolated by us from chicken skeletal muscle (10), were sequenced in the relevant region to determine which β-subunit isoform each encoded; 9 of 10 were β1 cDNAs and one was a β2 cDNA based on the absence of the 113 nucleotides found exclusively in the β1 cDNA. Thus, the β2 mRN is expressed in non-muscle tissues and in varying amounts in muscle tissues, which primarily express the β1 mRNA.

Mouse and Human Capping Protein β Subunits Are Nearly Identical to the β-Subunit Isoforms of Chicken Capping Protein

We isolated several complete cDNAs for mouse capping protein β subunit from a newborn mouse skeletal muscle library by screening with a chicken β2 cDNA. Sequencing of these cDNAs indicated that two different types of β-subunit cDNAs were isolated: one was similar to the chicken β1 cDNA and the other was similar to the chicken β2 cDNA. The proposed splicing sites are the same in mouse and chicken. The sequences of the mouse β1 cDNA, the predicted protein sequence of the mouse β1 isoform, and that predicted for the COOH terminus of the mouse β2 isoform are shown in Fig. 3 C. In addition, two partial cDNA sequences for a putative capping protein β2 subunit of human liver and testis were identified in the Genbank data base by searching for proteins homologous to the β1 protein using BLASTX (2). Additional cDNAs for this human β subunit were isolated from a HepG2 cDNA library.

The COOH-termini predicted for the chicken β2 isoform, the mouse β2 isoform and the human β2 isoform are 96% identical; those for the human and mouse β2 isoform are 100% identical (Fig. 6 A). Likewise the COOH-termini predicted for the β1 isoforms of chicken and mouse are 88% identical (Fig. 6 B). In contrast, the COOH-termini of the chicken β1 and β2 isoforms are only 22% identical. This finding is particularly remarkable because the COOH-termini of capping protein β subunits also are not conserved in comparisons to lower species. In this region of the protein, vertebrates, Dictyostelium, budding yeast, and nematode share only 17–36% identity. However, outside this region, within the NH₂-terminal ∼245 of ∼275 amino acids, the β subunits are 47–90% identical among the different species.

Capping Protein Isoforms in the Dynactin Complex

Dynactin complex is composed of a number of polypeptides, including the dynactin polypeptides p160/p150 (24, 48), the actin-related protein, Arp1 (formerly named actin-RPV), (31, 42), and capping protein (46). Western blots of 2D gels demonstrate that capping protein of chick brain dynactin complex contains the β2 isoform (Fig. 7). mAb 1E5 did not react with any proteins of dynactin complex (data not shown).

A

Human	...LRSVQ-TFADKSKQEALKNDLVEALKRKQ-----QC*
Mouse β2	...LRSVQ-TFADKSKQEALKNDLVEALKRKQ-----QC*
Chick β2	...LRSVQ-TFADKSKQEALKNDLVEALKRKQ-----QS*
Chick β1	...LRSID-AIPDNQKYKQLQRELSQVLTQRQIYIQPDN*
Mouse β1	...LRSLD-AIPDNHKFKQLQRELSQVLTQRQVYIQPDN*
Nematode	...LRSTE-KQSELEKQDEIVRELNNAMANR------GN*
Dictyostelium	...LRNAT-GNSELEKRKNLSNQIGSAIGNR--------G*
Yeast	...TKNAAIASSAEEANKDAQAEVIRGL---------QSL*

B

Human	...LRSVQ-TFADKSKQEAIKNDIVEAIKRKQ-----QC*
Mouse β2	...LRSVQ-TFADKSKQEAIKNDIVEAIKRKQ-----QC*
Chick β2	...LRSVQ-TFADKSKQEAIKNDIVEAIKRKQ-----QS*
Chick β1	...LRSID-AIPDNQKYKQLQRELSQVLTQRQIYIQPDN*
Mouse β1	...LRSLD-AIPDNHKFKQLQRELSQVLTQRQVYIQPDN*
Nematode	...LRSTE-KQSELEKQDEIVRELNNAMANR------GN*
Dictyostelium	...LRNAT-GNSELEKRKNLSNQIGSAIGNR--------G*
Yeast	...TKNAAIASSAEEANKDAQAEVIRGL---------QSL*

Figure 6. Alignment of amino acid sequences of the COOH-terminal region of capping protein β subunits of different species. (A) Amino acid residues identical to the chicken β2 isoform are shown in inverted style (*white letter on a black background*). (B) Amino acid residues identical to the chicken β1 isoform are shown in inverted style. The arrow indicates the position where the sequence of the chicken β1 and β2 isoforms diverge due to alternative splicing; their NH₂-terminal sequences are identical preceding this position. The sequence alignments were produced by the CLUSTAL V method (26) using MegAlign (DNAstar). The stippled box above each set indicates a region that is strongly predicted to form an α helix in all the sequences as determined by the profile network prediction program v. 11.93 (43). The subset of the prediction with most accuracy (>82%) is reported. Note that the COOH-termini of the chicken, human, and mouse β2 isoforms are nearly identical (96% identity) as are the COOH-termini of the chicken and mouse β1 isoforms (88% identity). In contrast, this COOH-terminal region is only 17–36% identical in comparisons of the β1 and β2 isoforms and the β subunits of D. discoideum, S. cerevisiae, and C. elegans. In contrast, the NH₂-terminal regions of these β subunits are 47–90% identical (3, 25, 52).
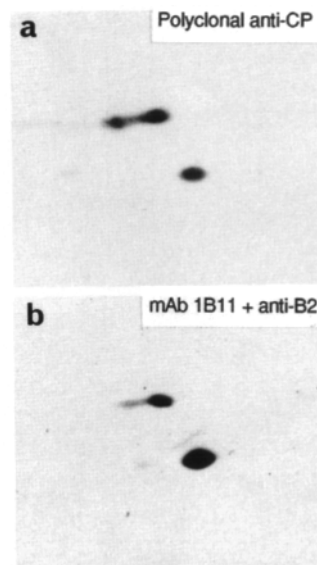
Figure 7. Western blots of two-dimensional gel of purified dynactin complex probed with (a) polyclonal anti-capping protein and (b) a mixture of mAb 1B11 and rabbit β2 subunit-specific antibodies. The capping protein of the dynactin complex is composed of the A1 and A2 subunits and the β2 subunit. Only the portion of the blot containing capping protein is shown; the pH range is as indicated in Fig. 1 a.

The β1 and β2 Isoforms Are Differentially Localized in Cardiac Myocytes

Cardiac muscle contains both capping protein β-subunit isoforms (Figs. 1, 2, and 4). We used mAb 1E5, which is specific for the β1 isoform, and affinity-purified rabbit β2-specific antibodies to determine the locations of the β-subunit isoforms in cryosections of adult chicken cardiac ventricle and in cultured cardiac myocytes. The specificity of these antibodies for proteins in whole cell extracts of chicken tissues and of cultured cardiac myocytes is shown in Western blots in Fig. 8. Only a single band corresponding to a capping protein β subunit was detected by these antibodies in all extracts tested. The β1 protein was enriched in skeletal and cardiac muscles, whereas, the β2 protein was enriched in brain and liver (Fig. 8 A). Both β-subunit isoforms were detected in extracts of cardiac myocytes grown in culture (Fig. 8 B).

In cryosections of adult chicken cardiac muscle, capping protein containing the β1 isoform was located at Z-discs (Fig. 9 a) and the β2 isoform was enriched at intercalated discs (arrowheads in Fig. 9 b). The localization of the β2 isoform at intercalated discs, which are the sites where the terminal sarcomere of each myofibril associates with the sarcolemma at the fascia adherens junction (21, 53), was confirmed by double-labeling with anti-vinculin (40) (arrowheads in Fig. 9, b and c). The β2 isoform also was detected in a punctate pattern throughout the cytoplasm, some of which was observed as bright, linearly arrayed foci (adjacent to bracket in Fig. 9 b). This punctate staining suggested that some β2 isoform was associated with the myofibril bundles. Some of the cytoplasmic staining also may correspond to capping protein of dynactin complex. Costameres, which also contain vinculin (41), were not intensely labeled with the β2-specific antibodies, however, we cannot rule out the possibility that some β2 isoform may be a minor component of costameres and that we do not detect it by immunolabeling. The pattern of immunolabeling obtained using the β2-specific antibodies was similar with preparations of affinity-purified antibody from two different rabbits; immunoglobulins from preimmune sera of these rabbits did not stain the cardiac tissue.

Many features of the subcellular distributions of the β-subunit isoforms in cardiac myocytes grown in culture were similar to their distributions in cardiac tissue. The β1 isoform was assembled at Z-discs of myofibrils (Fig. 10, a, c, e, and g); some β1 isoform was diffusely distributed throughout the cytoplasm. The β2 isoform was not at mature Z-discs, but was located at cell–cell junctions and at plaque-like structures that were typically located at the ends of the
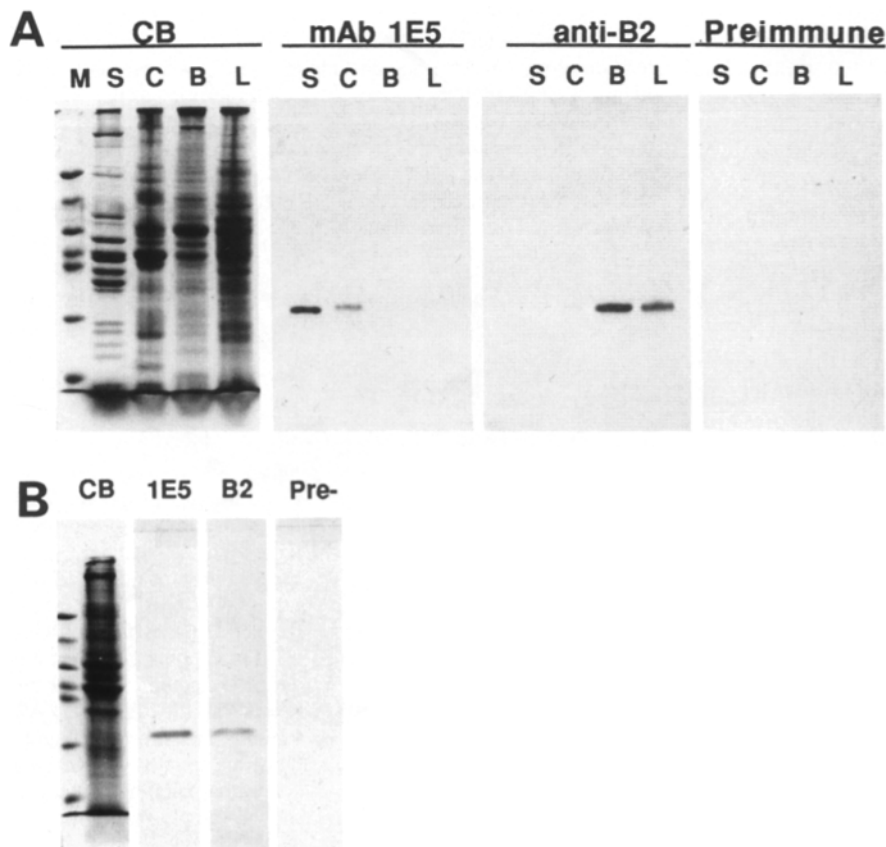
Figure 8. Characterization of the β1- and β2-specific antibodies on Western blots of one-dimensional SDS gels containing whole cell extracts of chicken pectoral muscle, cardiac muscle, brain, liver, and of extracts of cultured chick cardiac myocytes. (*A*) Blots of 10% SDS gels loaded with samples of chicken tissue extracts were probed with mAb 1E5, which identifies the β1 protein, affinity-purified β2-specific antibodies and immunoglobulins isolated from the preimmune serum from the rabbit that provided the anti-β2. Lanes are loaded as: pectoral muscle (lane *S*), cardiac muscle (lane *C*), brain (lane *B*), and liver (lane *L*); *M* indicates the molecular mass markers which include proteins of 97, 66, 55, 52, 40, 31, and 21 kD. The small amount of the β2 protein present in cardiac muscle is not detectable with the amount of total protein loaded in this lane of the one-dimensional gel. Note, however, that the two-dimension gels of Fig. 1 *d* and Fig. 4 *d* show the β2 isoform in cardiac muscle; we consistently observe a difference in the sensitivity of detection of β subunits on 1D and 2D gels. A Coomassie blue–stained gel of the proteins in the tissue extracts is labeled CB. (*B*) Samples of a whole cell extract of cultured cardiac myocytes was loaded on a 10% SDS gel and Western blots were probed with mAb 1E5 (*1E5*), β2-specific antibodies (*B2*), and preimmune immunoglobulin (Pre-). Molecular mass markers are the same as described in *A*.

anti-β1　　　anti-β2　　　anti-vinculin
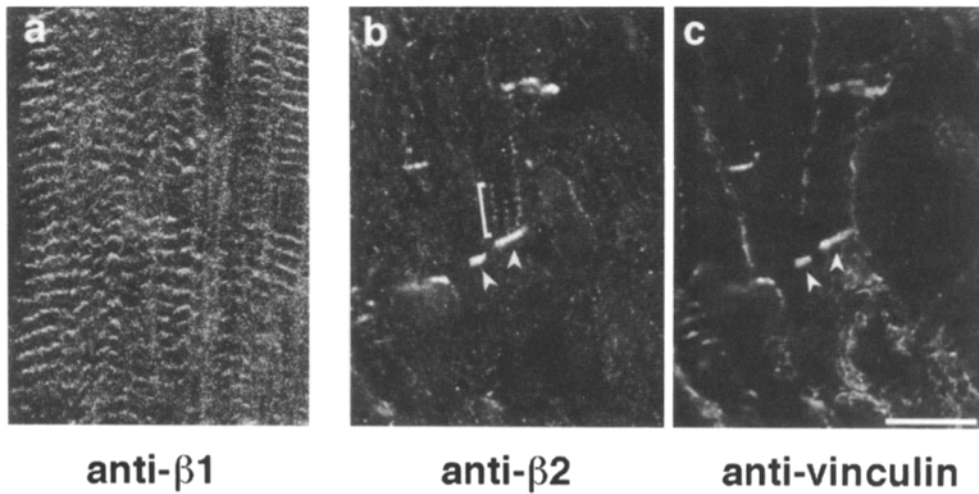
Figure 9. Immunofluorescence localization of the capping protein β1 and β2 isoforms in cryosections of adult chicken cardiac muscle. Sections were labeled with mAb 1E5, to detect the β1 protein (*a*) or β2-specific antibodies (*b*); the section shown in *b* was also labeled with anti-vinculin (*c*). The β1 protein is localized at Z-discs (*a*). The β2 protein is enriched at the intercalated discs (*arrowheads* in *b*) and is distributed in a punctate pattern throughout the cytoplasm; some punctate staining was in linearly arrayed foci (*bracket* in *b*). Double immunofluorescence labeling for vinculin confirms the identification of the intercalated disc (*arrowheads* in *b* and *c*). Bar, 10 μm.

myofibrils (Fig. 10, *b*, *d*, *f*, *h*, and *j*). In the most mature myocytes with many Z-discs, the β2 isoform was predominantly at cell–cell junctions (*arrows* in Fig. 10, *a*, *b*, *c* and *d*). In myocytes with fewer Z-discs, the β2 isoform was detected at plaque-like structures that are likely to be myofibril–membrane attachment sites. By observing different focal planes, it appeared that the plaque-like structures were located near the sarcolemma, most often at the bottom surface of the myocytes. The association of the β2 isoform at these sites varied depending on the maturity of the myofibrillar structures. In some myocytes, the β2 isoform was associated with irregularly spaced, plaque-like structures along myofibrils (*arrows* in Fig. 10, *g* and *h*) or along non-striated fibrillar structures that were continuous with striated myofibrils (*arrow* in Fig. 10, *e* and *f*). In myocytes that were just beginning to form Z-discs, the β2 isoform was continuously distributed along fibrils but was absent from regions where β1-containing Z-discs were forming (*arrows* in Fig. 10, *i* and *j*).

The localization of the β2 isoform at cell–cell junctions was confirmed by double labeling with anti-A-CAM (Fig. 11, *a* and *b*). In the myocyte shown in Fig. 11 *a*, the β2 isoform also was detected along the cell perimeter that was not in contact with other cells. The β2 isoform also was detected at periodically arrayed structures that are likely sites of contact between nascent myofibrils and the membrane (*small arrows* in Fig. 11 *a*). In myocytes with few myofibrils, the β2 isoform was observed at structures that resembled subsarcolemmal adhesion plaques (SAPs) which also contain vinculin (32) (*arrows* in Fig. 11, *c* and *d*). Interestingly, the β2 isoform was not a component of the vinculin-containing attachment plaques of cells in the myocyte cultures that lacked myofibrils (Fig. 11, *e* and *f*). In these cells, which are presumably fibroblasts or undifferentiated myocytes, the β2 isoform was distributed in a punctate pattern throughout the cytoplasm. A similar punctate distribution was observed for the β2 isoform in chick embryo fibroblasts isolated from skin; neither focal contacts nor stress fibers were labeled by anti-β2 in fibroblasts (not shown).

Discussion

Our analysis of chicken capping protein indicates that two β-subunit isoforms, named β1 and β2, are expressed and arise from alternatively spliced β-subunit mRNAs. The β1 isoform is the predominant, but not exclusive, isoform of muscle tissues and the β2 isoform is the predominant, but not exclusive, isoform of non-muscle tissues. In cardiac myocytes, which express both β-subunit isoforms, the isoforms are localized to different structures. The differential localization of the capping protein β-subunit isoforms in cardiac myocytes suggests that each isoform performs specific functions to organize the actin filaments of myocytes.

The two capping protein β-subunit isoforms differ only in amino acids at their COOH-termini, thus, any differences in the functions of these isoforms, including the differential sorting to structures in cardiac myocytes, may be attributed to this region of the molecule. From an evolutionary perspective, it is remarkable that the COOH terminus of the chicken β2 isoform is nearly identical to the COOH-termini of the homologous capping protein β subunit of humans and mice. Likewise, the COOH-termini of the chicken and mouse β1 isoforms are nearly identical. This region of the β subunit is of particular interest because the COOH terminus of the chicken β1 isoform is implicated in binding actin (28). In contrast, no significant similarity is found in comparisons of the COOH-termini of the β1 and β2 proteins to each other or to the known invertebrate β subunits, even though the NH₂-termini of the β subunits, which comprise the bulk of the molecule, are similar among all species. The very high similarities among the COOH-termini of the chicken, mouse and human β2 isoforms and between the chicken and mouse β1 isoforms, suggest that the β-subunit isoforms participate in cellular functions that are important and specific for vertebrates.

Double immunolabeling using isoform-specific antibodies showed that the β-subunit isoforms were distributed to distinct structures in cardiac muscle and myocytes. One possible trivial cause for the differential localizations of the two
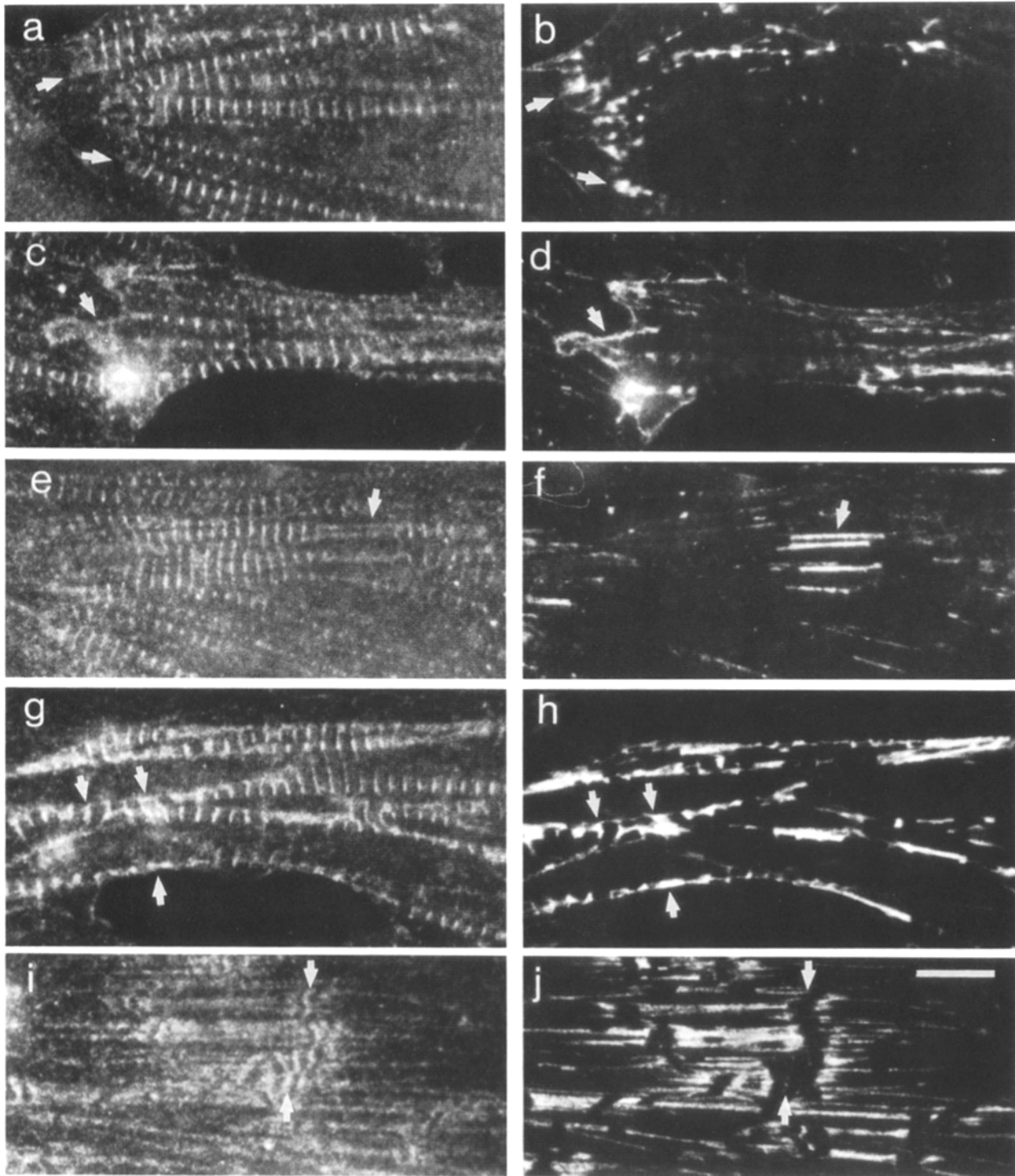
Figure 10. Double immunofluorescence localization of the β1 and β2 isoforms in cultured cardiac myocytes using mAb 1E5 to identify the β1 isoform (*a, c, e, g,* and *i*) and β2-specific antibodies to identify the β2 isoform (*b, d, f,* and *j*). The β1 isoform of capping protein was localized at Z-discs and was diffusely distributed in the cytoplasm. The β2 isoform was not a component of mature Z-discs but was enriched at cell–cell junctions (*arrows* in *b* and *d*) and at sites where myofibrils contacted the sarcolemma (*arrows* in *b* and *h*). The β2 isoform also was detected along non-striated fibrils that are contiguous with striated myofibrils (*arrow* in *e* and *f*) and at plaque-like structures along nascent myofibrils (*arrows* in *h*)). In some myocytes with few mature Z-discs, the β2 protein was distributed continuously along fibrillar structures (*i* and *j*); however, as Z-discs formed along these fibrils (*arrows* in *i*), the β2 protein appeared to be cleared from those regions with nascent Z-discs (*arrows* in *j*). Bar, 10 μm.

anti-β2   **anti-A-CAM**

anti-β2   **anti-vinculin**
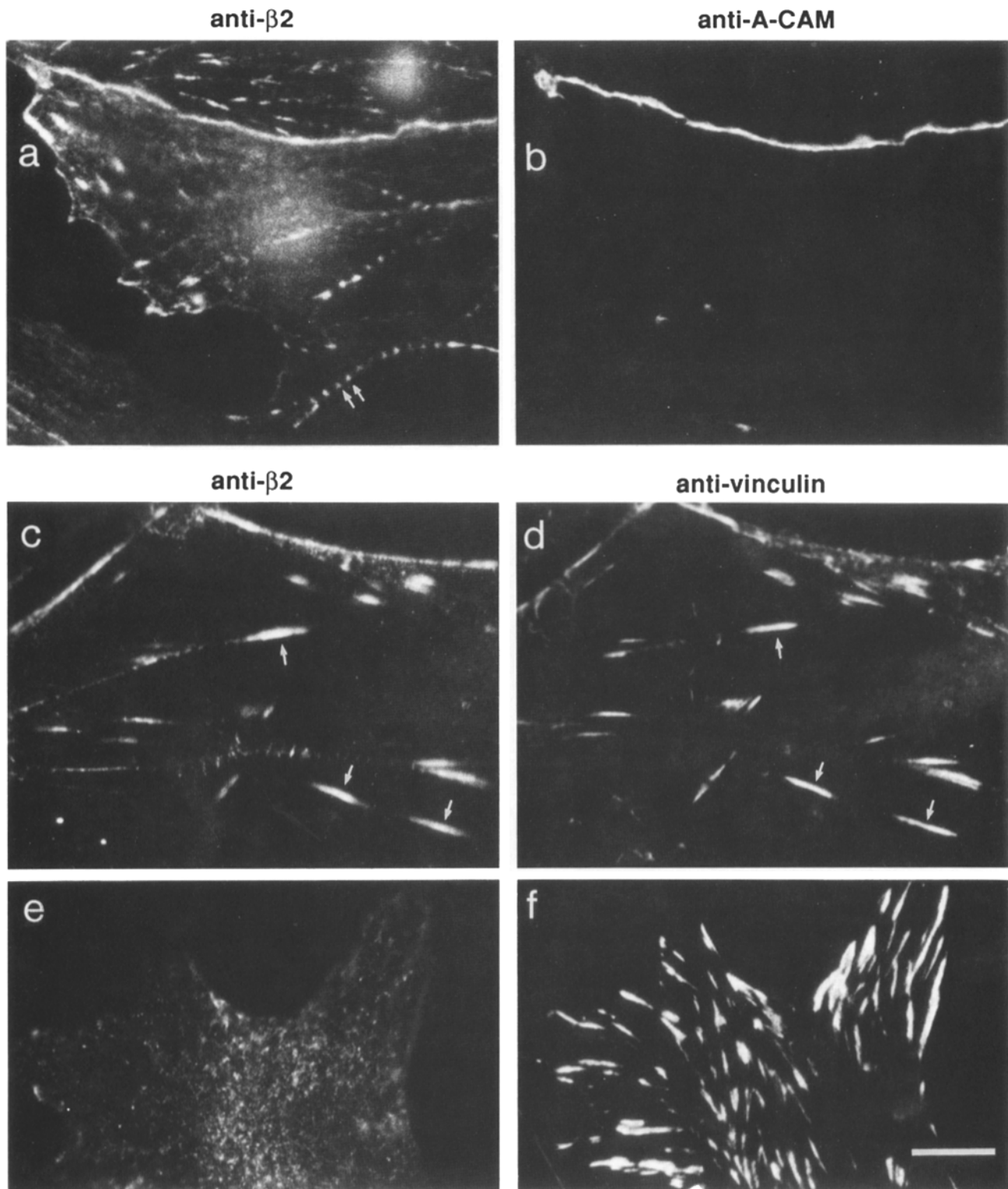
Figure 11. Double immunofluorescence localization of the β2 isoform (*a, c,* and *e*) and either A-CAM (*b*) or vinculin (*d* and *f*). The β2 isoform was distributed along cell–cell junctions labeled by anti-A-CAM (*a* and *b*). The β2 isoform also was found at regions along the cell perimeter that were not in contact with other cells; a nascent myofibril appeared to be forming along one region of the cell edge, where the β2 isoform was arrayed in a periodic pattern (*small arrows* in *a*). The β2 isoform was localized in myocytes with vinculin at sub-sarcolemmal adhesion plaques (SAPs) (*arrows* in *c* and *d*), which are thought to be sites where nascent myofibrils attach to the plasma membrane (32). The vinculin-containing focal contacts of cells in the myocyte cultures that are presumably fibroblasts do not contain the β2 isoform (*e* and *f*). In these cells, the β2 isoform was in a diffuse, punctate pattern. Bar, 10 μm.

β-subunit isoforms is that the isoform-specific antibodies may have varying accessibilities to their respective antigens at different cellular locations. To minimize this possibility, we tested several protocols for fixation and cell permeabilization before the double-labeling experiments. Under no condition was the β2 isoform detected at mature Z-discs in cardiac myocytes or the β1 isoform enriched at intercalated discs or cell–cell junctions. Furthermore, since the isoform-specific antibodies each bind epitopes in the unique, COOH terminus of their respective protein antigen, we expect that this region of each protein should have equal access to its specific antibody. However, we cannot exclude the possibility that a molecule may interact specifically with the COOH terminus of only one of the isoforms at a specific location, thereby blocking the binding of antibodies to that isoform at the location and preventing detection of the isoform by immunolabeling.

In cardiac myocytes, the β1 isoform is assembled at Z-discs, whereas the β2 isoform is located at cell–cell junctions, at sites of myofibril-sarcolemma attachment, particularly intercalated discs, and throughout the cytoplasm. One can speculate about possible reasons for the striking differential distribution of these isoforms. Actin filaments of sarcomeres and those of cell junctions may require capping protein isoforms with different functional properties. For example, actin filaments of cell junctions may be more dynamic than actin filaments of the sarcomere, and the capping protein isoform at cell junctions may bind actin less tightly or have a unique regulatory function not required of sarcomeric capping protein. The two α-subunit isoforms of capping protein prepared as α1β1 and α2β1 heterodimers by in vitro translation bind actin filaments with different affinities (11). Whether the α-subunit isoforms preferentially associate with specific β-subunit isoforms in vivo and whether the α1β2 and α2β2 heterodimers also show differential actin-binding properties are not known.

Since the COOH terminus of the β1 isoform was implicated as part of the actin-binding site for capping protein of skeletal muscle (28), differential binding of the β1 and β2 proteins to different actin isoforms is another possible reason for the differential localization of the capping protein isoforms. A common feature of the distribution of the β2 isoform is its localization at sites where actin filaments contact the plasma membrane. The β2 isoform may interact preferentially with the barbed end of filaments composed of non-muscle actin isoforms, which have been found to be enriched at the plasma membrane of a variety of cells. β-Actin is enriched in regions of the membranes of erythrocytes, endothelial cells, vascular pericytes and 3T3 fibroblasts (27), and in skeletal muscle, the cortical actin filaments are enriched in non-muscle γ-actin as compared with the myofibrils (16, 39). Preliminary actin polymerization assays indicate that capping protein composed of the β2 isoform interacts with conventional muscle α-actin (Schafer, D. A., and J. A. Cooper, unpublished) and human capping protein prepared as α2β2 by in vitro translation binds actin (Barron-Casella, E. A., M. A. Torres, S. W. Scherer, H. H. Q. Heng, L.-C. Tsui, and J. F. Casella, manuscript submitted for publication). Analysis of this question, including tests using different actin isoforms, will require additional work.

An alternative possible reason for the differential localization of the capping protein isoforms is that each isoform may specifically interact with other proteins that exist exclusively at one location or another. For example, the β2 isoform may interact with a component of cell junctions, such as cadherin (23, 51) or a catenin, and either nucleate the formation of actin filaments or stabilize the barbed end of the actin filaments at that location. Likewise, the β1 protein may bind to a component of the Z-disc, such as titin or nebulin, and, thereby, organize the actin filaments of the sarcomere.

Some of the cytoplasmic β2 isoform may be associated with dynactin complex, which also was distributed in a punctate pattern in chick embryo fibroblasts (24). The identification of the β2 isoform of capping protein in dynactin complex, and its location at one end of the short Arp1-containing filament that comprises part of dynactin complex (46), raises the possibility that capping protein also interacts with actin-related proteins. However, dynactin complex also contains 1 mol/mol conventional actin (46) and the capping protein may bind this molecule, which then interacts with Arp1 to form the short filament.

The association of the β2 isoform with nascent myofibrillar structures in cardiac myocytes in culture suggests a role for this capping protein isoform in the early stages of myofibrillogenesis. In myocytes in culture that contained abundant, mature myofibrils, the β2 isoform was concentrated at cell–cell junctions where myofibrils associated with the plasma membrane. However, in myocytes with fewer mature myofibrils, the β2 isoform also was associated with periodically arrayed structures that resembled nascent Z-bodies and with plaque-like structures that appeared to be sites of contact between the myofibrils and the sarcolemma. The detection of the β2 isoform at these vinculin-containing myofibril-substrate adhesion plaques, which have been called SAPs (32), and its absence from focal contacts of non-myogenic cells suggests that the β2 isoform functions to stabilize the barbed end of membrane-associated actin filaments at the ends of nascent myofibrils. Electron microscopic examination of similar myofibril-membrane attachment sites in rat cardiac myocytes shows that the filaments comprising these sites are not striated and contain thick actin cables that associate laterally with the membrane at electron-dense plaques (7). Capping protein may nucleate actin filament formation at these sites or may stabilize the barbed end of actin filaments at these sites.

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