β -Centractin: Characterization and Distribution of a New Member of the Centractin Family of Actin-related Proteins

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An examination of human-expressed sequence tags indicated the existence of an isoform of centractin, an actin-related protein localized to microtubule-associated structures. Using one of these tags, we isolated and determined the nucleotide sequence of a full-length cDNA clone. The protein encoded represents the first example of multiple isoforms of an actin-related protein in a single organism. Northern analysis using centractin-specific probes revealed three species of mRNA in HeLa cells that could encode centractin isoforms. One mRNA encodes the previously-identified centractin (now referred to as α -centractin). The full-length cDNA clone isolated using the expressed sequence tag encodes a new member of the centractin family, β -centractin. A probe specific for α -centractin hybridized to the third species of mRNA observed (referred to as γ -centractin). Comparisons of Northern blots of human tissues indicated that α -centractin and β -centractin mRNAs are equally distributed in all populations of mRNA examined, whereas the expression of γ -centractin appears to be tissue specific. The amino acid sequence of β -centractin, deduced from the cDNA, indicates a 91% identity with α -centractin, increasing to 96% similarity when conservative amino acid changes are taken into account. As antibodies previously raised against α -centractin reacted only poorly with β -centractin, new antibodies were produced and combined with two-dimensional gel electrophoresis to discriminate the two isoforms. Using this system, the subcellular distribution of the α - and β -isoforms were determined. Both isoforms were found predominantly in the cytosolic fraction as a part of a previously identified 20S complex (referred to as the dynactin complex) with no evidence for a free pool of either isoform. The isoforms were found in a constant ratio of $\sim 15:1$ ($\alpha:\beta$) in the dynactin complex.

INTRODUCTION

Actin is represented in vertebrates by as many as six isoforms (Firtel, 1981). Although the functional differences between muscle and nonmuscle forms have been

well characterized, the need for multiple cytoplasmic isoforms in a single cell is only beginning to be understood (Herman, 1993). Centractin, also referred to as Arp1 (Schafer *et al.*, 1994) or Actin-RPV (Lees-Miller *et al.*, 1992), is a recently-identified and -characterized actin-related protein (Clark and Meyer, 1992, 1993; Paschal *et al.*, 1993). At the amino acid level centractin is

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53% identical to mammalian actin, and the level of similarity is >70% when conservative amino acid substitutions are included. Conserved residues between these proteins appear to be largely limited to those regions forming the internal scaffold elements of the actin molecule, including the nucleotide and cation-binding cleft (Kabsch *et al.*, 1990). Conversely, those residues connecting these elements are less well conserved, suggesting centractin and actin may interact with a different subset of proteins. Centractin itself is highly conserved because the amino acid sequences of canine and human centractin are identical (Clark and Meyer, 1992; Lees-Miller *et al.*, 1992).

Centractin has been localized to at least three subcellular compartments. Both immunocytochemistry and cell fractionation have demonstrated its presence in centrosomes, whereas immunofluorescence has indicated its presence on vesicular structures in the cytoplasm of canine fibroblasts (Clark and Meyer, 1992, 1993). A substantial fraction of centractin has been detected as part of the dynactin complex in cytosolic fractions of bovine (Paschal *et al.*, 1993) and chick embryonic brain (Lees-Miller *et al.*, 1992). The evidence to date has not clarified whether centractin is present as part of the dynactin complex in all of these locations, although immunocytochemistry, using antibodies raised against a component of the dynactin complex, p150^{Glued}, shows an overlapping pattern of vesicular and centrosomal staining (Paschal *et al.*, 1993).

We previously suggested that centractin, like actin, may exist as multiple isoforms (Clark and Meyer, 1992, 1993). In the original description of centractin, we presented amino acid-sequence comparisons between full-length canine centractin and ones deduced from two partial cDNA clones that were isolated as human-expressed sequence tags (ESTs) and present in the GenBank database (Adams *et al.*, 1992). Although the C-terminal EST was indistinguishable from our canine centractin sequence, there were significant differences between the N-terminal EST and centractin. Because it was clear that canine and human centractins were identical, we concluded that the N-terminal EST represents a novel isoform. Using the N-terminal EST and our original centractin clone, we have identified two novel centractin-related mRNAs. The complete cDNA sequence of a bona fide isoform of centractin, which we propose to call β -centractin, has been determined. We believe this to be the first report of actin-related protein isoforms in a single organism. Antisera raised against this new isoform have allowed us to determine the subcellular distribution of centractin isoforms and their relationship to one another.

MATERIALS AND METHODS

Probes

All DNA fragments used as probes (except β -actin) were first agarose gel purified; then the fragment was cut out and run in a Low-melting

point-agarose gel from which it was isolated using Gelase (Epicenter Technologies, Madison, WI). For the HeLa Northern blot, a γ -centractin lane, cDNA clone R14C1 (canine α -centractin) (Clark and Meyer, 1992) was liberated from the vector with *EcoRV* and *BamHI* yielding a 935-base pairs (bp) fragment. For the HeLa northern blot, β -centractin lane, and for cloning human β -centractin, EST1218 (also known as HHCPJ76, ATCC# 7896) (human β -centractin) was released from the vector by digestion at *EcoRI* yielding a 1-kilobase (kb) fragment. For the human multiple-tissue Northern, cDNA clone PC3 (human β -centractin) was liberated from the vector with *EcoRV* yielding a 1022-bp fragment, and cDNA clone FC25 (human α -centractin) was liberated from the vector with *EcoRV* and *BamHI* yielding a 940-bp fragment. For cloning of human α - and γ -centractin, clone R14C1 (canine α -centractin) was liberated from the vector with *EcoRV* and *BamHI* yielding a 935-bp fragment. The β -actin fragment was provided with the multiple-tissue Northern (Clontech, Palo Alto, CA). All probes were labeled by random priming (United States Biochemical, Cleveland, PA) using [α^{32} P]dCTP (New England Nuclear, Boston, MA) and purified away from unincorporated nucleotide by spin chromatography through G-50 (Pharmacia, Piscataway, NJ) according to Sambrook *et al.* (1989).

Northern Blot Analysis

The human-multiple tissue poly(A)⁺ Northern was obtained from Clontech, and hybridization and stripping were conducted according to the manufacturer. This single blot was stripped and reprobed sequentially with β -centractin, α -centractin, and β -actin probes. Final washing was in 0.2× SSC at 65°C. Poly(A)⁺ RNA for the HeLa Northern was obtained using FastTrack 3.0 (Invitrogen, San Diego, CA). Five micrograms of RNA were added to three volumes of 59% formamide/1.2× 3-(*N*-morpholino) propane sulfonic acid (MOPS)/18% formaldehyde, heated 5 min at 68°C, then separated on 15% formaldehyde/agarose gels in 1× MOPS. Blotting was conducted by capillary transfer to ZetaProbeGT membranes (BioRad, Richmond, CA). Gel preparation, transfer, blot fixation, hybridization, and stripping on were carried out according to the manufacturer. Blots were washed twice for 5 min at 65°C in 0.2× SSC/5% sodium dodecyl sulfate (SDS) then twice for 30 min at 65°C in 0.05× SSC/1% SDS (human β -centractin on HeLa mRNA) or 0.2× SSC/1% SDS (canine α -centractin on HeLa mRNA). All blots were exposed to X-OMAT film (Kodak, Rochester, NY) with a single intensifying screen at −80°C.

Library Screening for Human α/γ - and β -Centractin cDNA

A human testicular oligo-dT primed λ gt11 cDNA library (Clontech HL1010b) was probed with both EST1218 (β -centractin) and canine α -centractin. Large scale preps of phage isolates were by the plate lysis method (Sambrook *et al.*, 1989). Purified phage DNA was digested with *EcoRI* and liberated fragments were subcloned into pBluescriptKS- (Stratagene, La Jolla, CA). All clones were classified by end sequencing and restriction digestion analysis.

DNA Sequencing

DNA sequence was determined for human α -centractin, human β -centractin, and EST1218 on both strands by double-stranded sequencing (United States Biochemical) of exonuclease III-generated nested deletions (Sambrook *et al.*, 1989).

Subcellular Fractionation of Canine Testis

Canine testes were minced, pressed through an embryonic tissue press, and homogenized as a 25% homogenate in STKM (0.25 M sucrose, 50 mM Tris (hydroxymethyl) aminomethane [Tris]-HCl pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) with 10 strokes of a loose-fitting glass homogenizer. The homogenate was fractionated by centrifugation for 10 min at 700

$\times$ g (Sorvall SS-34, Newton, CT, 2500 rpm, 4°C) to yield the nuclear pellet, for 20 min at 10 000 $\times$ g (SS-34, 9150 rpm) to yield the 10 K pellet (mitochondrial), and for 1 h at 100 000 $\times$ g (Beckman Ti-60, Fullerton, CA, 38 000 rpm) to yield the 100 K pellet (microsomal) and the 100 K supernatant (cytosol). To reduce the possibility of contamination with cytosol, pellets were washed once with a volume of STKM equal to that of the initial supernatant and finally suspended in the same volume of STKM. Sucrose density gradient centrifugation of the cytosol was performed as previously described (Paschal *et al.*, 1991). Protein quantitation was based on A_{280} measurements. Centriosomes were prepared by the method of Bornens *et al.* (1987) from HeLa cells as described previously (Clark and Meyer, 1992).

Immunochemical Analyses

Subconfluent normal canine fibroblasts (TM 03978, National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Camden, NJ) and HeLa cells were metabolically labeled by growth for 1 h in Dulbecco's minimal essential medium (minus methionine) followed by 4 h in the same medium plus 50 μ Ci/ml 35 S-Translabel (ICN, Irvine, CA). After chasing for 30 min with cold media with methionine, cells were washed twice with phosphate-buffered saline then harvested from the plate by addition of IP buffer (50 mM Tris-HCl pH 8.0/150 mM NaCl/1% NP-40, 2.5 ml/160 cm² plate). Lysates were given 10 strokes in a tight-fitting glass homogenizer, and insoluble material was removed by centrifugation at 5000 $\times$ g for 30 min. Immunoprecipitation was carried out in IP buffer. Samples were incubated with primary antibody for either 4 h (canine fibroblast) or overnight (HeLa cells) at 4°C. For monoclonal antibodies, 5 μ g of rabbit anti-mouse IgG (Jackson Laboratories, West Grove, PA) was added after 1 h. Immune complexes were collected by addition of Protein A Sepharose (Pharmacia) for an additional hour then washed three times in IP buffer. Immune complexes were released by incubation of washed beads directly in the gel-loading buffer. For immunoblotting unlabeled canine testes fractions or purified HeLa centrosomes were fractionated as indicated above, transferred to nitrocellulose, and probed with either the general centractin antibody or the α -specific monoclonal antibody. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibody and visualized using a chemiluminescent system (Renaissance, New England Nuclear).

Two-Dimensional (2-D) Electrophoresis

2-D gels (O'Farrell, 1975) were prepared as previously described (Clark and Meyer, 1992), except 21-cm first dimension tubes were used with an ampholyte composition of 0.5% Ampholyte pH 3-10/0.5% Ampholyte pH 4-6/1.4% Ampholyte pH 5-7 (BioRad). The 20S cytosolic fraction was trichloroacetic acid (TCA)-precipitated together with 50 μ g of bovine serum albumin as carrier. 2-D gels were either transferred to nitrocellulose (Towbin *et al.*, 1979), silver stained (Ansorge, 1985), or prepared for fluorography using ENTENSIFY (New England Nuclear). Radioactivity was quantified by computer-assisted Geiger densitometer using AMBIS Quant-Probe Software (San Diego, CA) and adjusted for the additional methionine residue in the β -isoform.

Construction, Expression, and Purification of Glutathione-S-Transferase (GST)- α -Centractin and GST- β -Centractin Fusion Proteins

α - and β -centractin cDNAs were cloned into the *Nco* I/*Hind*III or *Nco* I/*Xho* I sites, respectively, of the GST fusion protein vector, pGEX-KG (Guan and Dixon, 1991). Induction of fusion protein and purification on glutathione-agarose (Sigma, St. Louis, MO) was according to the method of Frangioni and Neel (1993).

Transcription Constructs and In Vitro Transcription/Translation

For in vitro transcription of α - and β -centractin, constructs were prepared in the vector, pGEM-4Z (Promega, Madison, WI), into which

canine 3'-untranslated region (3'UTR)-deleted α -centractin (retaining $\sim$ 180 bp of 3'UTR) and the human cDNA encoding full-length β -centractin were inserted at *Eco*RI/*Bam*HI and *Eco*RI, respectively. Transcription was performed with SP6 RNA polymerase (Promega) according to the manufacturer. RNA was digested with RNase-free DNaseI (Boehringer Mannheim Biochemicals, Indianapolis, IN) and quantified on nondenaturing agarose gels with ethidium bromide. Translation was conducted in rabbit reticulocyte lysate (Promega) according to the manufacturer.

Affinity-purified Antibody Production

For antibody production, six histidine residues were added to the C-terminus of human β -centractin by polymerase chain reaction. β -centractin(His)₆ was cloned into the inducible expression vector pET8c (Studier and Moffatt, 1986) at *Nco* I/*Bam*HI. Expression and purification by NTA-Ni²⁺ chromatography was as described for canine α -centractin (Clark and Meyer, 1992). Antiserum was produced in rabbits by CoCalico (Reamstown, PA). An IgG fraction from the rabbit serum was obtained by affinity chromatography on Affinica Protein A agarose (Schleicher & Schuell, Keene, NH). The IgG fraction was then affinity purified on GST- β -centractin coupled to affigel-10 as previously described for canine α -centractin (Clark and Meyer, 1992). Isoform specificity was tested by immunoblotting serial dilutions of known quantities of recombinant α - and β -centractin as GST-fusion proteins. This antibody, W15 (referred to as the general centractin antibody in the text), was found to be equally reactive with both the α - and β -isoform.

Rabbit anti-p150^{Glued} antibody (UP236) was raised against the amino terminal half of rat p150^{Glued} expressed in *Escherichia coli* as a fusion protein using the vector pET15b (Novagen, Madison, WI). The antiserum was affinity-purified using the purified antigen. Characterization of the antibody is described in Melloni, Tokito, and Holzbaur (unpublished data).

The anti-ATP/ADP carrier antibody has been described previously (Buchanan *et al.*, 1976).

RESULTS

Multiple Centractin-related Transcripts Are Expressed in HeLa Cells

A comparison of two human-expressed sequence tags (Adams *et al.*, 1992) from the GenBank database with the published amino acid sequence of centractin suggested that these ESTs were not derived from the same gene (Clark and Meyer, 1992). Accordingly, we obtained the human EST1218 that did not match centractin (referred to as β) and together with our original canine clone (referred to as α) probed Northern blots of HeLa cell poly(A)⁺ RNA. As can be seen in Figure 1, at high stringency the α probe labeled two species of mRNA, one at 3.0 kb (α) and another at 1.3 kb (γ). In contrast, the β probe labeled a single species at 2.0 kb. It is also clear that the α probe did not cross-hybridize to the β -specific mRNA nor were either the α or γ species detected with the β probe. Northern analysis at lower stringency corroborated the existence of some cross-hybridization.

γ -Centractin mRNA May Be a Truncated α -Centractin mRNA

A partial cDNA representing a human α -centractin homologue was previously identified by Lees-Miller *et al.*

(1992). For precise comparison of centractin isoforms on human RNA Northern blots, we isolated a full-length cDNA representing human α -centractin. Using our original canine α -centractin probe (Clark and Meyer, 1992), we isolated 12 clones from a human testicular, oligo-dT-primed cDNA library. Sequencing indicated several differences in the 3'UTR between our human clone and that of Lees-Miller *et al.* (1992). Of greater interest was the sequence of one polyadenylated cDNA, corresponding to the length of the γ -centractin message. Aside from a shorter 3'UTR, this clone had a sequence identical to that of the α -isoform. However, an alternative polyadenylation signal (Wickens, 1990), which could specify this shorter mRNA, was not found in this clone nor in the full-length α -centractin cDNA. Thus, it is not clear at this time whether the shorter clone is an artifact of library construction or a cDNA clone corresponding to the γ -centractin isoform.

β -Centractin Is a Novel, Closely Related Centractin Isoform

Using the β -centractin EST as a probe, a human testis cDNA library was screened. β -specific clones were identified and sequenced. Two silent nucleotide changes were found between EST1218 and the testicular cDNA clones. Shown in Figure 2 is the deduced protein sequence of β -centractin, indicated as changes relative to the α -isoform. This comparison reveals that the two isoforms are of equal length and share an amino acid identity of 90.4%, with a similarity of 95.7%. The regions of highest similarity between α -centractin and actin are also completely conserved in the β -centractin molecule. Moreover, the few differences between the α - and β -isoforms are not limited to a particular region of the primary or tertiary sequence, based on the crystal structure of actin (Kabsch *et al.*, 1990); rather similarities are distributed over the length of the centractin molecule. Finding multiple centractin isoforms suggests the possibility that different forms have different subcellular localizations and potentially different functions. Alter-

Centractin Isoforms

Figure 1. Centractin-related poly(A)⁺ mRNAs in HeLa cells. Using a canine α -centractin probe and EST1218 as a human β -centractin probe, HeLa poly(A)⁺ mRNA contains three centractin messages. The β -centractin probe hybridizes to a single RNA species, whereas the α -centractin probe hybridizes to the previously characterized α -isoform and a smaller message, just large enough to encode a centractin. Higher stringency washes could not remove the γ -message without an equivalent decrease in α -centractin.

COMPARISON OF CENTRACTIN ISOFORMS

α -	MESYDVIANQFWIDNGSGVTKAGFAGDQIPKYCFPNYVGRPKHVRVMAG	50
β -	-----I-----M-----	
α -	ALEGDFIGPKAEHRGLLSIRYPMEHGIVKDWNDMERIWQVYVSKDQLQ	100
β -	-----L-----T-----V-R-----	
α -	TFSEEHVLLTEAPINPRKNRERAAEVFFETFNVPALFISMQAVLSLYAT	150
β -	-----S-----K-----	
α -	GRITGVVLDSDGVTHAVPIYEGFAMPHSIMRIDAGFVSRFLRLYLRLK	200
β -	-----V-----Y-----L-----	
α -	EGYDFHSSSEFEIVKAIKERACYLSINPKDEITLETEKAQVYLPDGSSTIE	250
β -	-----V-----T-A-----V-RT-----A-----V-----T-----ID-----	
α -	IGPSRFRAPELLFRPDIGEESGEGIEHVLVFAIQKSDMLRRTLFSSNIVL	300
β -	V-A-----Q-----V-D-----L-----VA-----H-----A-----	
α -	SGGSTLFKGFGRLLSEVKKLAPKDVKIRISAPQERLYSTWIGGSILASL	350
β -	-----I-----K-----	
α -	DTFKMWSKKEYEEDGARSIRKTF	376
β -	-----S-A-----	

Figure 2. Deduced protein sequences of human α - and β -centractin. The complete human α -centractin protein sequence is shown with changes found in the β -isoform indicated below the α -centractin sequence. These sequences are available from EMBL under accession numbers X82206 for human α -centractin, X82207 for human β -centractin, and X82208 for EST1218.

natively, like α - and β -tubulin, they may be assembled together into a single structure.

α - and β -Centractin mRNAs Have Similar Steady-State Levels in Human Tissues

As a first step toward gaining an understanding of why multiple centractin isoforms exist, a multiple-tissue Northern analysis was carried out on brain, heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Figure 3). Each lane in Figure 3 was loaded with an equal quantity of RNA. As each tissue expresses a unique profile of mRNAs, we chose to monitor the abundance of β -actin mRNA as a reference point for comparison of centractin mRNA levels. As with any message, this choice has limitations. In particular, β -actin mRNA is down regulated in tissues expressing muscle-specific isoforms (Buckingham, 1985). Our probe does not recognize the α -actin isoform of cardiac muscle but does recognize the smaller mRNA of an actin isoform specific to skeletal muscle. Thus, in these muscle tissues, the centractin mRNAs can be compared only on the basis of the total mRNA population. With that caveat in mind, there are still notable features of centractin expression that can be gleaned from the Northern data. Using α - and β -specific cDNA probes, several sig-

Figure 3. Centractin mRNA levels in multiple human tissues. The same Northern was repeatedly stripped and reprobed with a human β -centractin, α -centractin, and finally with a β -actin probe to normalize for loading differences. The α - and β -isoforms are equally expressed with the exception of a lower β mRNA level in lung. γ -centractin, however, has a notable tissue-specific expression (compare α/γ in heart and brain).

nificant features of centractin expression were revealed. First, there does not appear to be any tissue specificity in the expression of α - and β -centractin. α - and β -centractin are expressed at roughly equal ratios in all of the aforementioned tissues, except lung, where relatively lower quantities of β -mRNA were observed when normalized to β -actin expression. Second, both the α - and β -centractin mRNAs are notably more abundant in brain and pancreas than in lung or, particularly, liver when corrected for β -actin mRNA levels. In contrast to α - and β -centractin, the smaller α -related mRNA (γ) does display a tissue-specific expression pattern; the highest γ to α ratio is seen in muscle, the lowest in brain. In Northern blots of HeLa cell mRNA, the α - to γ -mRNA ratio is maintained at all stringencies. This suggests that in contrast to β -centractin, α -, and γ -centractin could arise from a single gene by alternative splicing or alter-

native polyadenylation. On the other hand, γ -centractin may be a separate gene, having a very similar nucleotide sequence to α -centractin.

α - and β -Centractin Can Be Resolved Using 2-D Gel Electrophoresis

Antibodies that were previously raised against recombinant α -centractin (Clark and Meyer, 1992) were both incapable of efficiently detecting recombinant β -centractin on immunoblots as well as immunoprecipitating in vitro-translated β -centractin. However, antibodies raised against recombinant β -centractin(His)₆ were immunoreactive against β -centractin and remained equally immunoreactive with α -centractin after affinity purification on immobilized GST- β -centractin fusion protein. The similar molecular weights of α - and β -centractin, 42.6 and 42.3 kDa, respectively, precluded resolution of the isoforms by one-dimensional (1-D) electrophoresis. To conduct studies on the expression of the isoforms, we took advantage of the 0.25 U difference in isoelectric point predicted by the amino acid sequences of the two isoforms and the finding that an existing centractin monoclonal antibody (13D2E5) (Hortsch *et al.*, 1986; Clark and Meyer, 1992) reacts with the α - but not the β -isoform. Using the same 2-D system that we used in the analysis of centractin in centrosomes (Clark and Meyer, 1992), we found that in vitro-translated centractin isoforms could be resolved (Figure 4). In addition to the spots on the 2-D gels that clearly migrate as predicted, minor spots can also be seen, particularly in the bottom panel. Because the lysate was programed with synthetic mRNAs transcribed from cDNA, they cannot represent other isoforms. However, they may represent transient or minor posttranslational modifications of centractins. Because we found no evidence for such species in vivo (see below), we have not pursued the nature of these additional spots. Our standard procedure for detection of α - and β -centractin in subcellular fractions consisted of resolving the polypeptides by 2-D gel electrophoresis then immunoblotting, first with an affinity-purified anti- β -centractin antibody recognizing both isoforms equally well (general centractin antibody) and then, after stripping the blot, with the α -specific monoclonal antibody (13D2E5). Radiolabeled in vitro-translated centractins, when added to fractions containing HeLa cell and canine testicular centractins, were found to comigrate, as determined by immunoblotting. This finding suggests, first, that centractins are not subject to posttranslational modifications which alter protein charge and second, that canine β -centractin is as conserved as the human and canine α -isoforms.

β -Centractin Is a Stoichiometric Component of the Dynactin Complex

Previous examination of centractins in the dynactin complex by sequencing of four tryptic peptides indicated

Figure 4. α - and β -centractin can be resolved by 2-D gel electrophoresis. Synthetic α - and β -centractin RNAs were separately transcribed and translated in vitro then analyzed either separately or together on 2-D gels. Top, α -centractin; middle, β -centractin; bottom, α - and β -centractin. The isoforms can be resolved by this method and were found to comigrate with endogenous centractins detected in cellular fractions by immunoblotting.

the presence of only the α -isoform (Paschal *et al.*, 1993). To directly examine the centractin composition of this macromolecular complex, we isolated cytosol from canine testes, a tissue known to be rich source of microtubule-associated proteins (Collins and Vallee, 1989), and further fractionated the cytosol on sucrose gradients. We then examined the gradient by immunoblotting with anti-p150^{Glued} and anti-p50 antibodies (Paschal *et al.*, 1993) to identify those fractions containing the dynactin complex. To identify centractins,

we utilized the general centractin antibody. As previously shown (Lees-Miller *et al.*, 1992; Paschal *et al.*, 1993), centractin cosedimented with the dynactin complex at 20S. Although a slight shift of centractin toward higher sedimentation could be observed, it is most notable that no centractin (including the β -isoform) was found in a free, cytosolic pool at the top of the gradient (Figure 5A and see DISCUSSION). To determine conclusively if the β -isoform is also present in a 20S complex, we examined the 20S fraction by 2-D electrophoresis and immunoblotting. Based on both its immunoreactivity with the general centractin antibody, lack of reactivity with the α -specific monoclonal antibody, and isoelectric point, we conclude that β -centractin is found associated with a 20S cytosolic complex (Figure 5B).

To determine whether the β , like the α -isoform, is an integral component of the dynactin complex, we im-

Figure 5. Centractins cosediment with p50 and p150^{Glued} at 20S in sucrose-gradient fractionated canine testicular cytosol. A 100 000 $\times$ g supernatant from a canine testes homogenate was further fractionated on linear 5–20% sucrose gradients. (A) Samples were collected from the bottom and analyzed on 1-D SDS-polyacrylamide gel electrophoresis (PAGE) gels by immunoblotting with antibodies against the 50 and 150 kDa components of the dynactin complex in addition to the general centractin antibody. (B) Fraction 5 was TCA-precipitated and analyzed by 2-D gel electrophoresis and either silver-stained (top) or immunoblotted (bottom) with the general centractin antibody. Note that centractins peak in fraction 5, whereas p50 and p150^{Glued} peak in fraction 6.

munoprecipitated the complex with a monoclonal antibody against the 50-kDa component (Paschal *et al.*, 1993). Metabolically labeled canine fibroblast extracts were used as a source of radiolabeled proteins. Immunoprecipitates, subjected to 1- (Figure 6A) and 2-D (Figure 6B) electrophoretic analysis, indicated that both α - and β -centractin were immunoprecipitated with the anti-p50 antibody. Thus, whether immunoprecipitated from cytosol or derived from its fractionation on sucrose gradients, β -centractin was consistently found to have at least a 10- to 20-fold lower abundance than α -centractin (see Figures 5B and 6B). Because Northern analysis indicated nearly equal cellular mRNA levels of α - and β -centractin (Figure 3), we addressed the possibility that the bulk of β -centractin could exist somewhere in the cell other than the cytosol.

Centractins Are Predominantly in Cytosolic Fractions

To biochemically establish the subcellular distribution of centractins, canine testes were homogenized and subjected to differential centrifugation yielding material sedimenting at 700, 10 000, and 100 000 $\times g$, as well as a 100 000 $\times g$ supernatant. Fractions were analyzed on 1-D gels loaded by normalizing either for volumes adjusted to that of the subcellular compartment or for equal protein (Figure 7). The fractionation was assessed by immunoblotting with markers of mitochondria (ATP/ADP-carrier) and rough endoplasmic reticulum (ribophorin I). Using the general centractin antibody, this simple fractionation and detection scheme indicated that centractins are exclusively cytosolic. When examined on a per milligram protein basis, however, some centractin was found associated with the mitochondrial and microsomal fractions, even after washing in buffer. This distribution is in contrast with the largely vesicular localization of centractin and other components of the dynactin complex determined by immunofluorescence (Gill *et al.*, 1991; Clark and Meyer, 1992, 1993; Paschal *et al.*, 1993). It is particularly interesting to note that another component of the dynactin complex, p150^{Glued}, was distributed identically to centractin in these studies.

We originally localized centractin to centrosomes (Clark and Meyer, 1992). It was therefore of great interest to determine if both isoforms are present in this cellular structure, and if so, at what relative proportion. Accordingly, we examined the ratio of α - and β -centractin in HeLa centrosomes canine testis cytosol on 2-D gels (Figure 8). The ratio of α -: β -centractin found in the dynactin complex (see Figures 5 and 6) is maintained in the total cytosolic pool of centractins as well as in isolated centrosomes. This is consistent with our finding that all cytosolic centractin cosediments with the dynactin complex (Figure 5).

Figure 6. α - and β -centractin are associated with the dynactin complex. Metabolically labeled canine fibroblasts were immunoprecipitated with the anti-p50 monoclonal antibody. (A) Immunoprecipitated proteins were resolved by 1-D SDS-PAGE and developed by fluorography. (B) Immunoprecipitated centractin isoforms as in A were resolved on 2-D gels and prepared for fluorography. Also resolved by this analysis are two putative isoforms of p50 and a polypeptide comigrating with cytoplasmic actin isoforms (pI 5.2). In a similar experiment using metabolically-labeled HeLa cells, the radioactivity in the centractin spots was determined using a computer-assisted Geiger densitometer. The ratio between α - and β -isoform radioactivity was determined after adjustment for the additional methionine in the β -isoform. The ratio between the α - and the β -spot was determined to be $\sim 15:1$ ($\alpha:\beta$).

In HeLa cells we have estimated the quantity of α -centractin to be $\sim 0.006\%$ of the total protein. As the ratio between components of the dynactin complex and centractins are known (Gill *et al.*, 1991; Paschal *et al.*, 1993; Schafer *et al.*, 1994), we chose to obtain an exact ratio of the centractin isoforms. We determined this ratio

in the complex by its immunoprecipitation from metabolically labeled HeLa cell cytosol using anti-p50 antibody. The derived ratio, taking into account the additional methionine residue in β -centractin, was 1:15 (β : α). The result was consistent with estimates obtained from silver-stained and immunoblotted fractions (Figure 8).

DISCUSSION

The focus of this study is to understand the relationship between the β - and α -centractin isoforms in terms of overall expression and spatial distribution. In the course of investigating the first centractin isoform, now designated α -centractin, we uncovered two additional members of the centractin-related family. The identification of these additional centractin-related mRNAs raised several questions regarding the nature of centractin isoforms. The smaller α -related message, tentatively designated γ -centractin, is of particular interest as it is expressed in a tissue-specific manner. Our search for a full-length human α -centractin cDNA revealed a polyadenylated cDNA whose size roughly corresponds to that of γ -centractin mRNA visualized by Northern blotting. It is not clear at present whether this clone is derived from the γ -mRNA. However, the possibility of a tissue-specific α -centractin-related transcript is intriguing. Differences in translational efficiency of full-length α - and β -centractin isoforms examined *in vitro* were not observed and thus did not provide an expla-

nation for differences in their endogenous protein levels; however, it may point to a role for the 3'UTR in control of translation, because removal of the 3'UTR increased translation of both isoforms *in vitro*. If the γ -isoform in fact represents α -centractin without most of its 3'UTR, expression of the γ -mRNA might be a means of increasing cellular levels of the α -centractin protein. In this regard it is interesting to note that the α -centractin 3'UTR is highly conserved (83.5%) between canine and human homologues. This similarity is not simply due to the small number of possible bases at a given position, as 10 randomizations using the BESTFIT program (Genetics Computer Group, 1991) reduced the quality ratio from 8.7×10^2 to 8.8×10^{-3} . On the other hand it is also possible that β -centractin is less stable within the cell than the α -isoform.

In marked contrast, a cDNA representing β -centractin clearly represents a new isoform. Unlike the γ -centractin message, the β -centractin mRNA is expressed at levels comparable to that of the α -centractin mRNA, with neither exhibiting a particular tissue-specific expression. A previous study had indicated the cytosolic α -centractin isoform was exclusively found in a macromolecular complex with two other major constituents of 50 and 150 kDa (Paschal *et al.*, 1993). However, this study did not examine subcellular fractions other than cytosol and microtubule-associated proteins. Two critical findings in the current study resulted from our investigation of isoform distribution elsewhere in the cell. First, despite similar mRNA levels, the β -isoform is consistently ex-

Figure 7. Subcellular distribution of centractins. Whole canine testicular homogenate and subcellular fractions obtained by differential centrifugation were loaded on the basis of equal volume (left) or equal protein quantity (100 mU of A_{280}) (right). After separation by 1-D SDS-PAGE and transfer to nitrocellulose, centractins were visualized with the general centractin antibody. Anti-ATP/ADP carrier polyclonal antibody was used as a marker for mitochondria (Buchanan *et al.*, 1976) and anti-ribophorin I monoclonal (18A4) (Hortsch *et al.*, 1986) was used as a marker for rough endoplasmic reticulum.

Figure 8. Resolution of centractin isoforms in subcellular fractions. Preparations of canine testis cytosol and HeLa centrosomes were analyzed on 2-D gels. Centractin isoforms were detected with the general centractin antibody, and the position of α -centractin was confirmed with the α -specific monoclonal antibody.

pressed at a ~ 15 -fold lower level than α -centractin, whether examined in the dynactin complex purified on sucrose gradients or in isolated centrosomes (see also Figure 4) (Clark and Meyer, 1992). The dynactin complex has been shown to be composed of three major proteins (p150, p50, and centractin) and several minor components (Gill *et al.*, 1991; Paschal *et al.*, 1993; Schafer *et al.*, 1994). The stoichiometric ratios of these major components in the complex are $\sim 2:5:13$ for p150, p50, and centractin, respectively (Gill *et al.*, 1991; Paschal *et al.*, 1993; Schafer *et al.*, 1994). The calculated mass of the complex is 1.1×10^6 Da. This corresponds well to the $1.11 \pm 0.12 \times 10^6$ Da that has been determined by scanning electron microscopy (Schafer *et al.*, 1994) for the dynactin complex isolated from chicken embryonic brain. Because we have found β - and α -centractin in a ratio of $\sim 1:15$, this suggests the presence of one β -centractin molecule per complex. This conclusion is supported by our finding that the ratio is maintained when the complex is immunoprecipitated with antibody raised against the 50-kDa component. In this context it is interesting to note that in the dynactin complex isolated from chicken, a protein with comparable size and isoelectric point to β -centractin is found at a similarly low level of expression (Schafer *et al.*, 1994) (Figure 1).

It remains formally possible that the dynactin complex is heterogeneous with respect to centractin isoforms, with some free of β -centractin or some having additional copies of the β -isoform. Most likely, such complexes would still be immunoprecipitated with anti-p50 antibody, and the similar weight of centractin isoforms is not likely to grossly affect sedimentation characteristics unless isoform substitution results in a change in complex morphology. In this context it is worth noting that centractins have a small, but reproducible, difference in sedimentation characteristics compared to p50 and p150 (see also Paschal *et al.*, 1993) (Figure 5). Because both

isoforms of centractin can be immunoprecipitated with the anti-p50 antibody, it is not unreasonable to assume that there are at least two different centractin-containing complexes in the cytosol, one of which contains a relatively larger number of centractin molecules than the dynactin complex sedimenting at 20S. It would be most informative to immunoprecipitate centractin-containing complexes of both sedimentation characteristics to examine their composition. Unfortunately, our antisera, which quantitatively immunoprecipitate *in vitro*-translated centractins, fail to immunoprecipitate centractin in the context of the dynactin complex, potentially because of hidden epitopes.

The finding of an abundant cytosolic pool of centractin is in seeming conflict with immunofluorescence data. Immunofluorescence has indicated that centractin (Clark and Meyer, 1992, 1993) and p150^{Glued} (Gill *et al.*, 1991; Paschal *et al.*, 1993) are localized to numerous cytoplasmic vesicles and the centrosome. Our fractionation studies further support the centrosomal association; however, only a small quantity of centractins were found associated with membrane fractions, most being in the $100,000 \times g$ supernatant. The low level of membrane-associated centractin may be a consequence of our fractionation procedure in which the inherent dilution of homogenization leads to the dissociation of the dynactin complex from the membrane.

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