Identification, Localization, and Primary Structure of CAP-23, a Particle-bound Cytosolic Protein of Early Development

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Abstract. We report the identification of CAP-23, a novel particle-bound cytosolic protein associated with developing cells in both mammalian and avian tissues. CAP-23 was a substrate for purified protein kinase C (PKC) in vitro, and the protein was phosphorylated in a PMA-sensitive manner in cultured cells, indicating that it is a PKC substrate in situ. cDNA coding for chick CAP-23 was isolated. The deduced sequence revealed an unusual amino acid composition that strikingly resembled that of rat GAP-43, a growth-associated neuron-specific PKC substrate. Further predicted features of CAP-23 included a PKC phosphorylation site at Ser-6, and the presence of basic NH_2- and COOH-terminal domains. CAP-23 was encoded by an mRNA of ~1.5 kb, whose distribution during chick development resembled that of the corresponding protein. Southern blot analysis revealed the presence of a single main hybridizing species in the chick genome.

The distribution of CAP-23 during development was analyzed with Western blots and by immunofluorescence on tissue sections. In cultured cells the protein appeared to be distributed in a regular spotted pattern below the entire cell surface. In early chick embryos (E2), CAP-23 was present in most if not all cells. The protein then became progressively restricted to only some developing tissues and to only certain cells in these tissues. In most tissues CAP-23 levels fell below detection limits between E15 and E19. Highest levels of the protein were found in the nervous system, where CAP-23 levels peaked around E18, and where elevated levels were still detectable at birth.

INTERACTIONS between cells during the development of multicellular organisms require molecular mechanisms to connect the extracellular environment to underlying intracellular sites. A prominent role in this process is played by the junction between cell membrane and cortical cytoskeleton. To date, only a few molecular components of such junctions have been described, and many of them, including vinculin, talin, and alpha-actinin are found mainly at focal adhesion points (Burridge et al., 1988). Less localized components of the cortical cytoskeleton include the network-forming spectrins and the potential membrane-anchoring proteins, ankyrins (Lazarides and Woods, 1989). A unique type of cortical cytoskeleton and cell membrane-associated protein, growth-associated protein of M_r 43,000 (GAP-43)[1], has been found in neurons (Benowitz and Routtenberg, 1987; Skene, 1989; Allsopp and Moss, 1989). In contrast to other known components of the cell membrane–cytoskeleton region, GAP-43 levels vary with a specific cellular function, i.e., axonal growth (Benowitz and Routtenberg, 1987; Skene, 1989). GAP-43 is restricted to neurons, where it is found predominantly in axonal growth cones and nerve terminals (Skene et al., 1986; Jacobson et al., 1986; Goslin et al., 1988). In the latter, it constitutes ~1% of total protein and is a main protein kinase (PKC) substrate (Meiri et al., 1986; Nelson et al., 1989). Molecular properties of GAP-43 are highly conserved among vertebrates and include a conserved NH_2-terminal domain of 50 amino acids, resistance to denaturation, presumed lack of tertiary structure, phosphorylation-sensitive and calcium-independent calmodulin binding, and activation of GTP-γ-S binding by G_o (Skene, 1989; Strittmatter et al., 1990). GAP-43 does not share significant sequence homologies with other known proteins (Basi et al., 1987; Skene, 1989). While a direct involvement of GAP-43 in the process of axonal growth is most likely, no conclusive data on GAP-43 function have been obtained so far. With the purpose of identifying novel accessory components of the junction between cell membrane and cytoskeleton, we have searched for proteins with properties similar to those of GAP-43. We assumed that such proteins might also be found in nonneuronal cells and that shared properties might include cellular locations (e.g., association with cortical cytoskeleton enriched fractions), and unusual physical properties, including high hydrophilicity and resistance to denaturation. We report here the identification of CAP-23 (cortical cytoskeleton–associated protein of approximate molecular mass 23 kD), a novel highly hydrophilic cytosolic protein with an unusual amino acid composition very similar to that of GAP-43.

1. *Abbreviations used in this paper*: CAP-23, cortical cytoskeleton-associated protein of approximate molecular mass 23 kD; GAP-43, growth-associated protein of M_r 43,000; PKC, protein kinase C.

Materials and Methods

A protein fraction enriched in GAP-43 and in CAP-23 was prepared as described (Zwiers et al., 1985). Briefly, E16 chick brains were homogenized with a glass-teflon homogenizer in 5 mM EDTA, 100 U/ml trasylol, and 0.01% β-mercaptoethanol. All centrifugations were for 20 min, at 48,000 *g*, and at 4°C. A first pellet was extracted at pH 11, and most solubized proteins were removed by subsequent precipitation steps at pH 5.5, after incubation at 70°C (pH 5.5), and upon precipitation in 57% ammonium sulfate. Proteins precipitating between 57 and 82% ammonium sulfate were collected (ASP fraction).

CAP-23 was purified from the ASP fraction by two subsequent SDS-PAGE separations at different acrylamide concentrations. The procedure took advantage of the abnormal behavior of CAP-23 on SDS-PAGE. Proteins were first run on a 7% gel. After staining of the proteins with Coomassie brilliant blue, a gel stripe with proteins of 70–74 kD was removed, equilibrated in 25% stacking gel buffer (Laemmli, 1970) with 0.1% SDS and traces of bromphenol blue, and placed on top of a 15% gel with a large 5% stacking gel. On the second gel, CAP-23 ran as a protein of ~50 kD, thereby migrating away from contaminating 70–74-kD proteins. Gel containing Coomasie-stained CAP-23 was collected and protein was electroeluted from the gel into dialysis membrane (Spectrapore, Spectrum Med. Ind. Inc., Los Angeles, CA) in 0.3% Tris-base, 1.44% glycine, and 0.05% SDS. Gel-purified chick GAP-43 was prepared from an E13 chick brain ASP fraction by an analogous procedure.

Biochemical Characterization of CAP-23

IEF/SDS-PAGE two-dimensional gels (2D-IEF; O'Farrell, 1975) were run with ampholyte (Sigma Chemical Co., St. Louis, MO) gradients of pH 2.5–5 in the first dimension. Two-dimensional SDS-PAGE based on the fractionation of proteins on gels of different acrylamide concentrations was performed essentially as described in the previous paragraph: samples were first run on 7% gels, vertical lane stripes were then layered horizontally on a second gel with large 5% stacking gel and 15% running gel, and proteins were electrophoresed out of the 7% gel and through the 15% gel(7/15 gels).

In vitro phosphorylation of ASP proteins (2 mg/ml) was performed in 2.5 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.25% NP-40 containing 10 μM γ-^{32}P-ATP upon addition of 8 μg/ml (final concentration) of E13 chick brain homogenate protein (to provide the kinase activities), or in the presence of 0.4 μg of protease-activated purified PKC (isoform mixture, kind gift of P. Parker, Ludwig Institute, London, U.K.) as described (Gonzatti-Haces and Traugh, 1986). After 15 min at 30°C, phosphorylation was interrupted by boiling in sample buffer. Proteins were then separated by either 2D-IEF/SDS-PAGE (phosphorylation with brain homogenate kinases) or on 7/15 gels (phosphorylation with isolated PKC), and analyzed for incorporated ^{32}P by autoradiography on x-ray film (Eastman Kodak Co., Rochester, NY).

For in situ phosphorylation experiments (Meiri et al., 1986), cells were preincubated in culture medium supplemented with 2 mCi/ml^{32}P-Na-phosphate for 1 h. Phosphorylation was allowed to proceed for 10 more min in the presence or in the absence of 10 nM PMA (Sigma Chemical Co.). Cells were then rapidly rinsed three times with ice-cold PBS, scraped from the culture dishes in PBS with 5 mM EDTA, and cellular proteins were analyzed for incorporated ^{32}P as described.

Antibodies

BALBc mice were immunized by injecting (i.p.) 20 μg of gel-purified chick CAP-23 in complete Freund adjuvant, followed by boosts after 3 and 5 wk (10 μg of antigen in incomplete Freund, i.p.). Mice with strong specific immune response were either used for hybridoma production following established procedures (Koehler and Milstein, 1975), or were used to obtain serum. In the latter case, mice were sensitized with 0.5 ml of incomplete Freund adjuvant, and production of ascitic fluid was induced by the injection of 3×10^6 myeloma cells (Lacy and Voss, 1986). Hybridomas secreting antibodies against CAP-23 were selected first by solid phase RIA with ASP proteins as antigen. Specificity was then verified on Western blots of 7/15 gels, with proteins from the ASP fraction and from chick brain homogenate as antigens. mAb 15C1 specifically bound to CAP-23. An antiserum against chick GAP-43 used in the initial part of this study was a kind gift of D. Bray and D. Moss (Medical Research Council, Kings College, London, U.K.). The specific mAb against chick GAP-43 (antibody 5F10) was produced in our laboratory by a procedure analogous to the one described for CAP-23.

Blotting of CAP-23 and of GAP-43 onto nitrocellulose was performed in the presence of 0.05% SDS, after prefixation and preincubation of the gels in the presence of 1% SDS as described (Jacobson et al., 1986). Bound antibody was detected either with ^{125}I-rabbit-anti-mouse IgG (Milan Analytica, La Roche, Switzerland) or by phosphatase-coupled antimouse antibody (Milan Analytica, La Roche, Switzerland).

For immunofluorescence, cultured cells were fixed with 4% paraformaldehyde in 0.1 M K-phosphate, pH 7.4 (37°C, 30 min), and then permeabilized for 1 h at RT with 0.5% NP-40 in 20 mM Tris, 160 mM NaCl, 5% BSA, pH 7.4. Alternatively, fixation was in ice-cold methanol for 5 min, and the subsequent permeabilization step in NP-40 was omitted. Permeabilized cells were then exposed for 4 h at RT to mAbs (1:100 dilutions of ascitic fluids in detergent-free buffer containing 1% BSA). Bound antibody was detected with FITC-labeled sheep-anti-mouse IgG (Serotec, Oxford, U.K.). Labeling of unpermeabilized living cells was performed for 45 min at 4°C in culture medium supplemented with 1:100 diluted 15C1 ascites. All of the following manipulations were performed in culture medium at 4°C. Cells were washed three times, incubated for 30 min in the presence of FITC-labeled antimouse antibody as described above, and washed free of unbound antibodies (three times). Finally, antibody-exposed cells were fixed in paraformaldehyde and examined under FITC illumination. To label unfixed and unpermeabilized tissue, E6 chick brain was homogenized mildly (potter) in PBS. Tissue debris were washed at 800 *g* for 5 min and incubated with antibodies as described above for living cultured cells. Washes were performed at low speed as described above, and labeled, unfixed tissue fragments were finally mounted between cover glasses for microscopy. In double labeling experiments, 1:500 dilutions of rabbit-anti-chick vinculin antiserum (kind gift of M. Burger, Friedrich Miescher Institute, Basel, Switzerland) were included in the first antibody incubation mixture, and bound antivinculin antibody was detected with RITC-labeled goat-antirabbit antiserum (Milan Analytica). Fluorescent signals were detected with a Zeiss Axiovert-10 microscope (Carl Zeiss, Oberkochen, FRG) equipped with appropriate filters. To detect CAP-23 and GAP-43 immunoreactivity on 20-μm cryostat sections of 4% paraformaldehyde-fixed chick tissues, sections were processed as described above for tissue culture cells.

cDNA Cloning and Analysis

Screening of an E10 chick embryo λgt11 library (Clonetech Laboratories Inc., Palo Alto, CA) with specific monoclonal (15C1) and polyclonal antibodies to CAP-23 was according to established procedures (Sambrook et al., 1989). Plaques positive for both 15C1 and anti-CAP-23 antiserum were purified, and corresponding inserts were analyzed by restriction enzyme digestion and found to yield partially overlapping patterns. An Eco RI/Taq I fragment from the largest clone (~ 0.9-kb insert) was labeled by the random priming method (Feinberg and Vogelstein, 1984) and used to confirm the presence of overlapping segments on the antibody positive clones (3 out of ~200,000 screened). The same fragment was also used to rescreen the λgt11 library. Clone 22i (insert length of 1,042 bp) was finally selected and sequenced by the chain termination method, using the sequenase II kit (United States Biochemical Corp., Cleveland, Ohio). For in vitro transcription and translation the insert was cloned into vector pGEM-3 (Promega Biotec, Madison, WI) and transcription was catalyzed by T7 polymerase. In vitro synthetized RNA was translated using a reticulocyte lysate kit (Amersham International, Amersham, U.K.) in the presence of ^{35}S-methionine (final concentration, 1 mCi/ml; Du Pont, Regensdorf, Switzerland), according to the manufacturer's recommendations. Synthetized protein was analyzed for abnormal migration behavior on SDS-PAGE as described above. The migration position of authentic CAP-23 was determined on corresponding 15C1 Western blots of chick brain homogenate fractions, and alignment was achieved with the help of molecular weight standards (Bio-Rad Laboratories, Richmond, CA).

For Northern analysis, total RNA from chick tissues was isolated by a rapid extraction method (Lizardi, 1983), and 10 μg of total RNA were applied per lane of 1% agarose-5% formaldehyde gels (Sambrook et al., 1989). Separated RNAs were blotted onto Genescreen (Du Pont) and hybridized to ^{32}P-labeled 22i insert (random priming method, ~10^9 cpm/μg of DNA; 10^6 cpm/ml of hybridization mixture) according to the recommendations of the manufacturers. To verify that comparable amounts of RNA had been applied to the blot lanes, ethidium bromide was included in the formaldehyde gel, and genescreen-bound RNA was examined under appropriate UV illumination (data not shown). After high stringency washing (68°C, 0.2× SSC, 0.5% SDS), blots were exposed to x-ray film (Eastman Kodak Co., Rochester, NY), and films were developed after 1 d of exposure.

Genomic DNA was isolated from P45 chick liver (Sambrook et al., 1989) and digested to completion in the presence of appropriate restriction enzymes. 5 μg of digested DNA were applied per lane of a 0.8% agarose gel, separated DNA was blotted onto Genescreen, and hybridization was

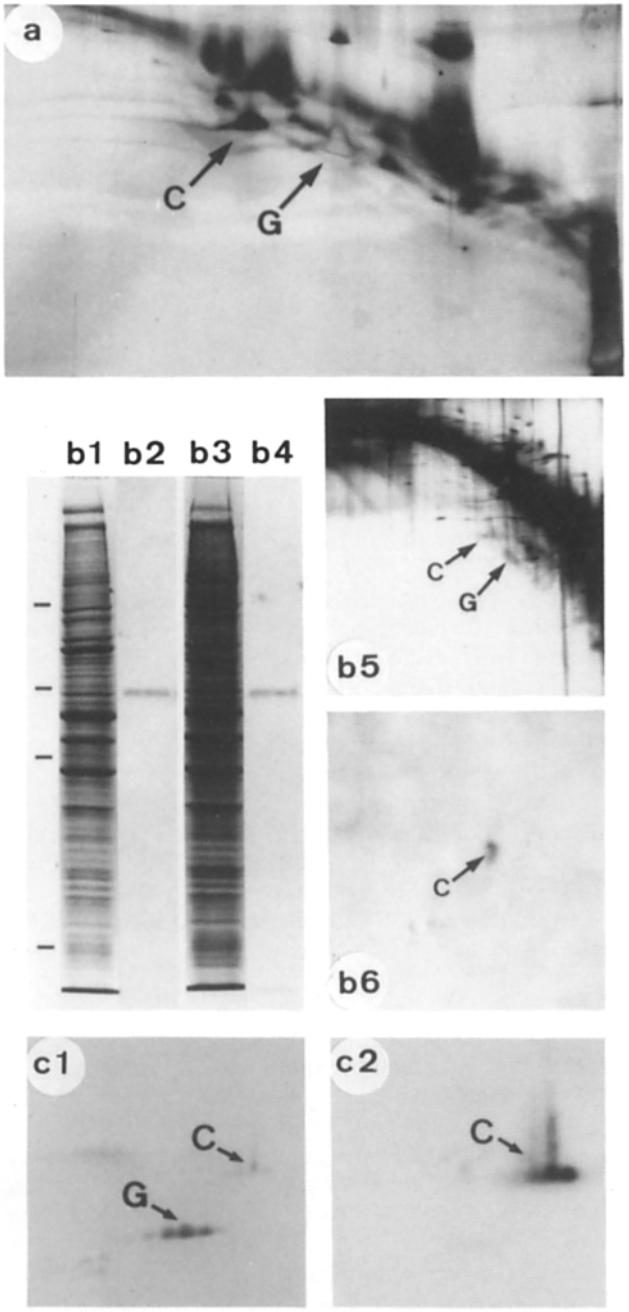


Figure 1. Identification of CAP-23: gel migration behavior and specific antibodies. (*a*) ASP proteins of abnormal migration behavior on SDS-PAGE. E13 chick brain ASP fraction proteins were separated by two-dimensional SDS-PAGE. Gels were 7% acrylamide in the first and 15% acrylamide in the second dimension. Proteins were stained with silver. CAP-23 (*C*) and GAP-43 (*G*) were prominent abnormally migrating proteins. (*b*) Specific detection of CAP-23 by mAb 15C1. (*b1–b4*) 10% SDS-PAGE of E7 chick brain homogenate (*b1* and *b2*) and hind limb homogenate (*b3* and *b4*) protein (50 μg/lane). Contiguous lanes were either stained with Coomassie blue (*b1* and *b3*) or probed for 15C1 binding antigen after transfer on nitrocellulose (*b2* and *b4*). The migration position of molecular mass standards is indicated on the margin of lane b1 (from top to bottom: 97, 66, 45, and 31 kD). (*b5* and *b6*) 7/15 gels of E13 chick brain homogenate protein (50 μg); gels were run in parallel and proteins were either stained with silver (*b5*) or blotted and probed with 15C1 (*b6*). The blot in b6 was stained with India Ink (Pelikan) for allignment of transferred proteins with silver stained proteins (*b5*). (*arrow*) Migration position of CAP-23. (*c*) Identification and behavior of CAP-23 on 2D-IEF gels. ASP fraction proteins (20 μg) from E13 chick brain were fractionated by equilibrium IEF (pH 2.5 to 5 gradients) in the first dimension, and on 10% SDS-PAGE in the second dimension. The gel was then stained with Coomassie blue (*c1*). After staining, proteins were destained and transferred to nitrocellulose for Western blotting. (*c2*) 15C1 blot of same gel as shown in *c1*. The fractionation range shown in the figure is ~ pH 3.4–5. *C*, CAP-23; *G*, GAP-43.

performed as described above. After high stringency washing, blots were exposed for 3 d to x-ray film.

Other Methods

Cells from chick embryo tissues were kept in enriched L15 (Mains and Patterson, 1973) with 5% rat serum. Dissociated tissues were incubated for 30 min at 37°C in L15 with 0.1% trypsin, washed in culture medium, and further dissociated with a siliconized Pasteur pipette. Washed cell suspensions were added to collagen-coated tissue culture dishes, with the exception of E6 DRG neurons, which were added to laminin-coated tissue culture dishes in the presence of 20 ng/ml of nerve growth factor. Protein was determined by the filter binding assay (Schaffner and Weissmann, 1973) with BSA (Sigma Chemical Co.) as standard.

Results

Identification of CAP-23 as an Intracellular, Surface-associated PKC Substrate

To search for proteins with physical properties and subcellular distribution similar to those of GAP-43, we took advantage of (*a*) the strongly selective properties of an isolation scheme devised to produce enriched cortical cytoskeleton–associated protein fractions, including GAP-43; and (*b*) the abnormal migration behavior of GAP-43 on SDS-PAGE. The enrichment protocol was derived from a cortical cytoskeleton preparation from rat liver (Hubbard and Ma, 1983), with the additional final selection for presumably small and highly soluble proteins requiring between 57 and 82% ammonium sulfate to be precipitated (the ASP fraction; Zwiers et al., 1985).

ASP fraction proteins were subsequently selected for GAP-43-like anomalous SDS-PAGE behavior, i.e., for slower relative migration in gels of lower acrylamide concentration. This unusual behavior probably reflects poor SDS binding, due to the extremely high hydrophilicity of the protein (Skene, 1989). We have devised a simple two-dimensional SDS-PAGE procedure (7/15 gel) to identify anomalously migrating proteins (see Materials and Methods), and the resulting fractionation pattern for E13 chick brain ASP fraction proteins is shown in Fig. 1 *a*. Upon 7/15 gel fractionation, most proteins migrated along a (logarithmic) line. GAP-43 (*G*) and a small number of additional proteins migrated distinctly below the line, indicating that these proteins displayed the anomalous gel behavior as described above. One fairly abundant protein (*C*), designated in the following as CAP-23, was enriched in the ASP fraction and migrated well below the 7/15 gel protein line. A protein with the same fractionation and migration properties could also be detected in E10 chick liver fractions (data not shown).

To determine whether CAP-23 corresponded to a distinct molecular species and to produce specific molecular re-

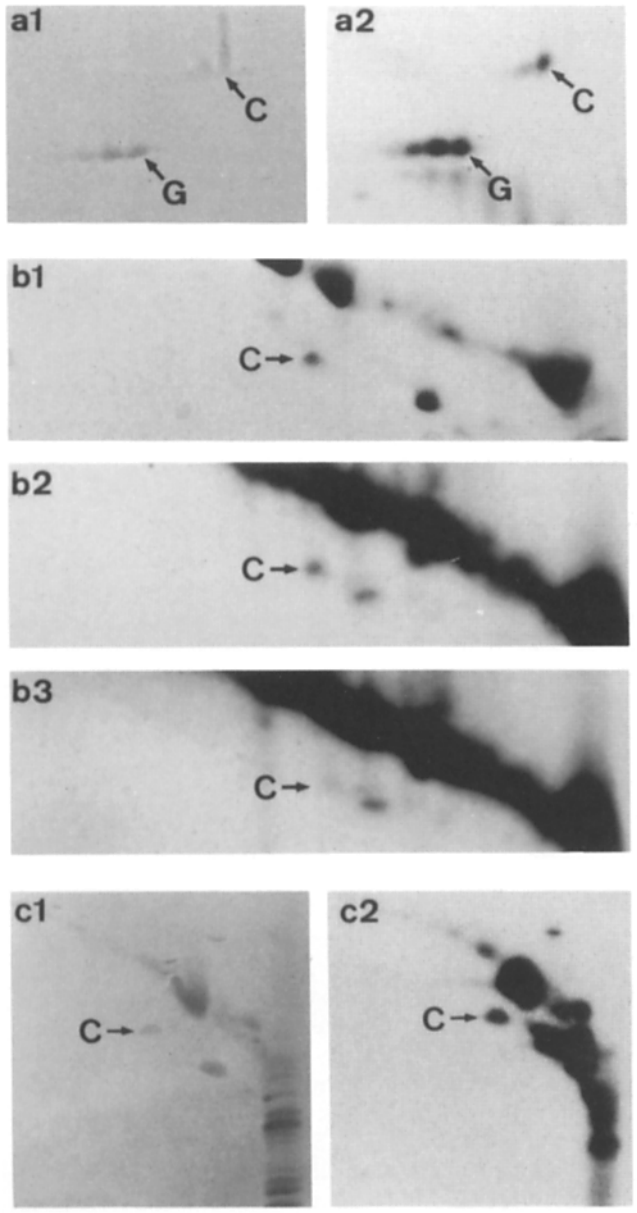


Figure 2. Phosphorylation of CAP-23, in situ and in vitro. (*a1* and *a2*) Phosphorylation of CAP-23 in vitro by brain homogenate kinases. Coomassie-stained gel (*a1*) and corresponding autoradiogram (*a2*) of ^{32}P-phosphorylated ASP proteins after separation by 2D-IEF/SDS-PAGE. (*b1–3*) PMA-sensitive phosphorylation of CAP-23 in situ. E6 spinal cord-derived cells (1d in vitro) were exposed to ^{32}P in the presence (*b2*) and in the absence (*b3*) of 10 nM PMA. Autoradiograms of the corresponding 7/15 gels (10 μg of cell derived homogenate protein) are shown. 10 μg of ASP protein were added to the samples before electrophoresis to directly determine the migration position of CAP-23. In control experiments, the migration position of ^{32}P-labeled proteins was not affected by the addition of ASP protein. (*b1*) Coomassie staining of gel shown in *b2*. (*c1* and *c2*) Phosphorylation of CAP-23 by purified PKC. 20 μg of E13 chick brain ASP protein were phosphorylated with trypsin-activated purified PKC, in the presence of γ-^{32}P-ATP, and separated on a 7/15 gel. The resulting Coomassie blue-stained gel (*c1*), and the corresponding autoradiogram (*c2*) are shown. In control experiments without added PKC, no ^{32}P-labeled ASP proteins could be detected (six times longer exposure of dried gel to x-ray film). *C*, CAP-23; *G*, GAP-43.

agents, antibodies were raised against 7/15 gel-purified CAP-23 from chick brain ASP fractions. As shown in Fig. 1 *b*, mAb 15C1 specifically bound to CAP-23 on Western blots of brain (E7 and E13) and hind limb (E7) homogenate proteins.

Like GAP-43, CAP-23 displayed microheterogeneity on 2D-IEF gels (Fig. 1 *c*). The second most basic spot (isoelectric point of ~4.6) was consistently found to be the major species and displayed a characteristic concentration-dependent smearing pattern in the second fractionation dimension. As such smearing, which is reminiscent of the behavior of beta-tubulin on similar gel systems, was not observed upon one-dimensional SDS-PAGE, it seems plausible that it might be due to concentration-dependent self-aggregation in the absence of SDS. Remarkably, a smearing pattern was never observed for the most basic spot of CAP-23.

We then determined whether CAP-23 was a substrate for protein kinases in situ and in vitro. As shown in Fig. 2 *a*, CAP-23 was phosphorylated by kinases present in chick brain homogenate. The most basic spot of CAP-23 did not contain incorporated ^{32}P, suggesting that it represented a dephosphorylated form of the protein. Basal phosphorylation of embryonic chick spinal cord cells in vitro did not lead to CAP-23 phosphorylation (Fig. 2 *b3*). The latter was, however, obvious upon addition of phorbol ester to the culture (Fig. 2 *b2*), suggesting that CAP-23 might be a substrate for PKC in situ. This interpretation was further substantiated by the demonstration of purified PKC-mediated phosphorylation of CAP-23 in vitro (Fig. 2 *c*). Therefore, like GAP-43, CAP-23 is an acidic PKC substrate.

We used antibody 15C1 to determine the location of the corresponding antigen in embryonic chick cells. As shown in Fig. 3 (*a* and *b*), the antibody failed to bind to the surface of intact living cells, indicating that the corresponding epitope was intracellular. Permeabilized cells displayed strong granular labeling. In control experiments, 1:100 dilutions of ascites containing mAb IN-1 against rat oligodendrocyte inhibitors of neurite growth (Caroni and Schwab, 1988) were used as first antibody. Under the conditions and sensitivity required for CAP-23 immunocytochemistry, we did not detect specific binding of IN-1 to antigens in our chick-derived cultures.

In double-labeling experiments, the pattern observed with 15C1 was similar to the one obtained with antibodies to the surface-associated intracellular protein vinculin (Fig. 3, *e* and *f*). Careful comparison of 15C1 with vinculin labeling patterns revealed that 15C1 immunoreactivity was distributed in a much finer granular pattern than its vinculin counterpart (Fig. 3, *e* and *f*), and that focal adhesion points were not emphasized by antibody 15C1. The fine granular pattern was consistently observed on 15C1 positive cells, and spot spacing tended to increase with cell spreading. The characteristic pattern was observed in formaldehyde-, as well as in methanol-fixed cells. A similar granular staining pattern was observed when indirect immunofluorescence was performed on homogenized chick tissues (Widmer, F., and P. Caroni, unpublished results), suggesting that the granular distribution of CAP-23 immunoreactive material was neither due to fixation, nor to solubilization in the presence of detergents.

In addition, 15C1 consistently bound to nuclear structures (see Fig. 3, *c* and *f*). As the bulk of 15C1 immunoreactive

material did not fractionate with nuclei (data not shown), it is not clear whether the observed nuclear staining pattern was due to actual localization of the antigen to nuclear structures. Fig. 3 (*g* and *h*) shows the localization of 15C1 immunoreactivity in E2 whole embryo and in E13 gut sections. 15C1 immunoreactivity appeared to be predominantly localized along cell surfaces. Therefore, indirect immunomicroscopy at the light microscope level yielded a labeling pattern consistent with an intracellular, particle-bound, and possibly subplasmalemmal localization of CAP-23.

Primary Structure of Chick CAP-23

To isolate cDNA clones coding for chick CAP-23 we screened a bacterial expression library with a specific mAb and with an antiserum against gel-purified chick CAP-23. Approximately 200,000 clones of an E10 chick embryo λgt11 cDNA library were screened and three plaques were identified that specifically bound both antibodies. The isolated clones displayed similar restriction enzyme digestion patterns and were found to overlap extensively. Additional hybridizing clones were selected from the same library, and the longest clone (22i) was used as a probe on Northern blots of embryonic chick tissues. The resulting distribution of hybridizing mRNA was similar to the observed distribution of CAP-23 protein, therefore, suggesting that clone 22i might code for CAP-23. Sequencing revealed an insert of 1,042 bp, including an open reading frame of 627 bp, flanked by a start codon satisfying Kozak's criteria for eukaryotic translation initiation sites, by a polyadenylation sequence, and by a polyA tail of 40 residues. Insert 22i was therefore cloned into vector pGEM3 for in vitro transcription and translation. As shown in Fig. 4, clone 22i coded for a protein whose migration behavior on SDS-PAGE was undistinguishable from that of CAP-23. Fig. 4 demonstrates that the in vitro expressed protein not only had an apparent molecular weight identical to that of CAP-23, but also exhibited an identical retardation behavior in gels of decreasing acrylamide concentration. We therefore concluded that clone 22i coded for chick CAP-23.

The nucleotide sequence of clone 22i and the deduced amino acid sequence are shown in Fig. 5. The nucleotide sequence was confirmed by sequencing of two additional independent, partially overlapping clones, which were found to yield identical sequences. In agreement with its putative intracellular location and with its solubility properties, CAP-23 lacked a signal sequence at its amino terminal and did not possess significant hydrophobic domains. As shown in Fig. 6 *a*, CAP-23 is an extremely hydrophilic protein with a high content of Lys and of Glu. This high hydrophilicity is consistent with the observed abnormal gel behavior of the protein, which is probably due to poor SDS binding (see also Skene, 1989).

Extensive comparison with sequences presently (April 1990) available in the EMBL and Genbank Databases revealed no significant homologies with proteins of known primary structure. The most homologous sequences found were the ones for middle (Myers et al., 1987) and for large (Lees et al., 1988) neurofilament protein (residues 480–725 of human NF-M; 45% similarity, 31% identity, 9 gaps), and for rat (Basi et al., 1987) and mouse (Cimler et al., 1987) GAP-43 (23% identity in 157 amino acid overlap with rat sequence). In both cases, sequence homologies were probably mainly due to shared unusual amino acid compositions, as similar homology and identity values were obtained when major frameshifts between compared proteins were introduced (see Fig. 6 *b*, and its legend). Fig. 6 *a* shows the very high degree of similarity between the amino acid compositions of CAP-23 and of GAP-43. Particularly striking common features include the extremely low content of hydrophobic residues, unusually high contents of Lys and of Glu, and concomitantly low proportions of Arg. In addition, CAP-23 has no Cys, while the only two Cys in GAP-43 are found at positions two and three, where they have been shown to be involved in membrane anchoring of GAP-43 (Zuber et al., 1989).

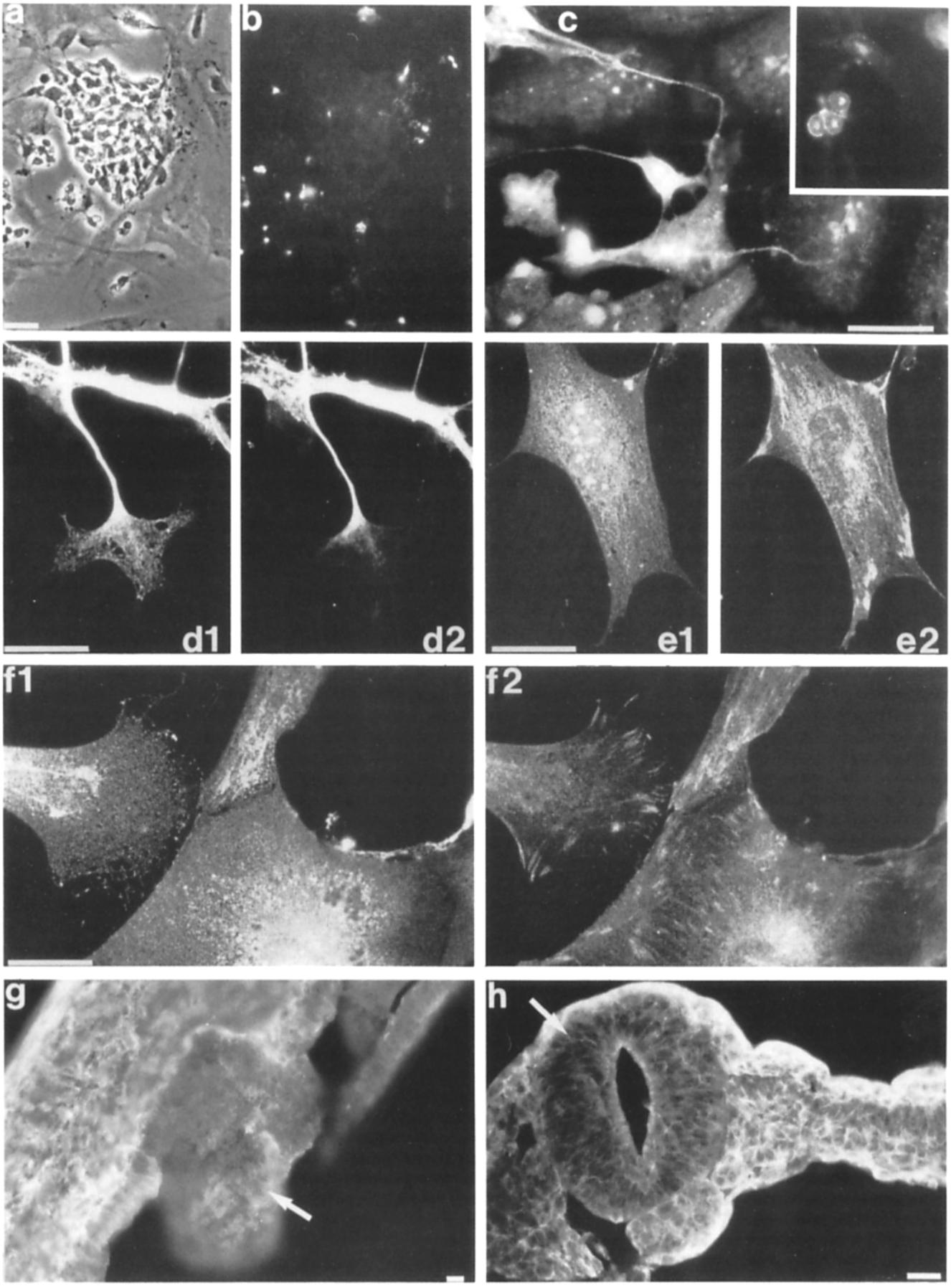
The predicted isoelectric point of CAP-23 (5.1) was slightly less acidic than the observed value for the protein on IEF gels (4.6–4.8). Examination of the sequence for potential PKC phosphorylation sites indicated that only Ser-6 fitted the consensus criteria (clusters of basic residues flanking a Ser or Thr; no acidic residues in the vicinity of the phosphorylation site; and basic amino acid at position +2; Woodgett et al., 1986). As, in addition, Ser-6 appeared to be a particularly favorable phosphorylation site, we consider it most likely that the observed PKC-mediated phosphorylation of CAP-23 in situ and in vitro (Fig. 2) involves Ser-6. An additional potential phosphorylation site was identified at Thr-31, as the sequence TEEEE might be a target for casein-II-type kinases (Edelman et al., 1987). Therefore, in agreement with its observed microheterogeneity on 2D-IEF gels, CAP-23 might be phosphorylated on at least two positions, including one very favorable PKC site.

Chou-Fasman plots of CAP-23 indicated the potential for extensive alpha-helical domains, the complete absence of beta sheets, a clustering of turns in the second half of the protein, and an almost complete lack of hydrophobic stretches. A comparison of the predicted structural features with the primary structure of the protein indicated that CAP-23 might be subdivided into four domains of markedly different composition. The NH_2-terminal part of the protein (first 25 residues) has a highly basic composition (pI = 10.73, net charge of +6), no Glu residues, and the only potential PKC phosphorylation site of CAP-23. The next 70 residues define an acidic, extremely hydrophilic domain with 30% Glu content, a pI of 4.16, and a net charge of −13. The stretch between residues 97 and 185 is Pro-rich, extremely hydrophilic, and of approximately balanced distribution between basic and acidic residues (pI of 9.04, net charge of +1). Finally, the last 25 residues of CAP-23 diverge from the extremely hydrophilic composition of the protein and constitute a basic (pI of 10.38, net charge of +1) COOH-terminal region.

We determined the behavior of CAP-23 hybridizing species on Southern blots of chick genomic DNA. As shown in Fig. 7, hybridization under high stringency conditions resulted in a set of major single bands for a number of restriction enzymes tested, thus, indicating that CAP-23 is in all likelihood encoded by one single gene in the chick genome. An additional second set of less intensely labeled single bands might indicate the presence of an additional, possibly related gene.

Distribution of CAP-23 Protein and mRNA during Chick Development: Progressive Restriction According to a Tissue-specific Schedule

Aspects of the distribution of CAP-23 during chick develop-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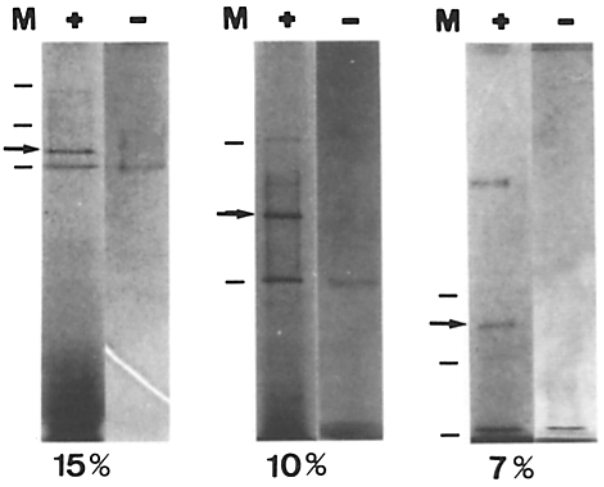

Figure 4. Migration behavior of in vitro translated 22i-clone product on gels of different acrylamide concentration. Autoradiograms of ^{35}S-labeled reticulocyte lysate translation reaction product with (+) or without (−) 22i-RNA are shown. 22i translation product migrated faster in gels of higher acrylamide concentration, as shown by its migration relative to both ^{35}S-labeled background bands, and molecular mass standards (from top to bottom: 97, 66, and 45 kD). The migration position of CAP-23 as determined on corresponding Western blots of chick brain homogenate protein are indicated by the arrows. Note that CAP-23 and 22i product comigrated in all gels, indicating that clone 22i contained the entire coding sequence of CAP-23. The additional 22i-specific ^{35}S-labeled band probably represented CAP-23 dimer.

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1                                                    GCCGCGGTAGCGGCGGCAGTGCTCCAGCAGCAGC
35  GCGAGGCACTCCGCGCCCGGCGAGCGAGCGGCAGCCGCAGCGCACAGCGGCTCCGGGCTGCGC
98  GGGGCGGGCGGCAGCGCGGCACAGGCACAGAGAGCGGCAGCGGCAGCACGCGGGAGCTCCAAC

161 ATG GGA GGC AAA CTG AGC AAG AAG AAG AAG GGG TAC AGC GTC AAT GAT
    met gly gly lys leu ser lys lys lys lys gly tyr ser val asn asp   16
    └D1
209 GAA AAA GCT AAA GAC AAA GAC AAG AAG GCT GAA GGA GCA GCA ACT GAG
    glu lys ala lys asp lys asp lys lys ala glu gly ala ala thr glu   32
                                       └D2
257 GAA GAG GAG ACT CCA AAG GAG GCT GAG GAT GCC CAG CAA ACC ACA GAG
    glu glu glu thr pro lys glu ala glu asp ala gln gln thr thr glu   48

305 ACC ACA GAA GTG AAG GAG AAC AAT AAA GAG GAG AAG GTT GAG AAG GAT
    thr thr glu val lys glu asn asn lys glu glu lys val glu lys asp   64

353 GCT CAG GTC TCT GCC AAT AAG ACA GAA GAA AAA GAA GGG GAG AAA GAG
    ala gln val ser ala asn lys thr glu glu lys glu gly glu lys glu   80

401 AAA ACA GTG ACC CAA GAA GAA GCC CAG AAA GCA GAA CCG GAG AAG TCA
    lys thr val thr gln glu glu ala gln lys ala glu pro glu lys ser   96

449 GAG GCT GTT GTC GAT GCA AAA GTA GAG CCA CAG AAG AAC AAT GAA CAG
    glu ala val val asp ala lys val glu pro gln lys asn asn glu gln  112
    └D3
497 GCA CCC AAG CAA GAG GAG CCA GCT GCA GCC TCT CGT CCT GCT GCC AGT
    ala pro lys gln glu glu pro ala ala ala ser arg pro ala ala ser  128

545 AGC GAA CGA CCC AAA ACT TCT GAG CCT AGC AGC GAT GCA AAA GCT TCC
    ser glu arg pro lys thr ser glu pro ser ser asp ala lys ala ser  144

593 CAG CCT TCA GAA GCC ACA GCT CCC AGC AAA GCA GAT GAC AAG AGC AAA
    gln pro ser glu ala thr ala pro ser lys ala asp asp lys ser lys  160

641 GAG GAA GGG GAA GCC AAA AAG ACT GAG GCT CCC GCA ACG CCT GCA GCC
    glu glu gly glu ala lys lys thr glu ala pro ala thr pro ala ala  176

689 AAG AAA CTA AAA GCG AAG TGG CCC CAG CTT CAG ACT CAA AAC CTA GCA
    lys lys leu lys ala lys trp pro gln leu gln thr gln asn leu ala  192
                                       └D4
737 GCA GCG AGG CTC ACC TTC TTC CAA GGA GAC CGT AGC AGC CAC AGC AGC
    ala ala arg leu thr phe phe gln gly asp arg ser ser his ser ser  208

785 ACC TAG TTCCACTGCCAAGGCCTCGGACCCGTCAGCCCCACCAGAGGAAGCGAAACCTTCT
    thr  *                                                       209

846 GAAGCCCCAGCGACTAATTCGGATACAACCATAGCAGTGCAAGATTAAATTGGACAGCCTGTT
909 GAAACAACCACTTAAAACAACCTGTGTCTTTTTTTTATTTTTTCAGCTATACTAACTCATTTC
972 AGATCTGAAATAACAAAAAGTATTGCCCAGA41
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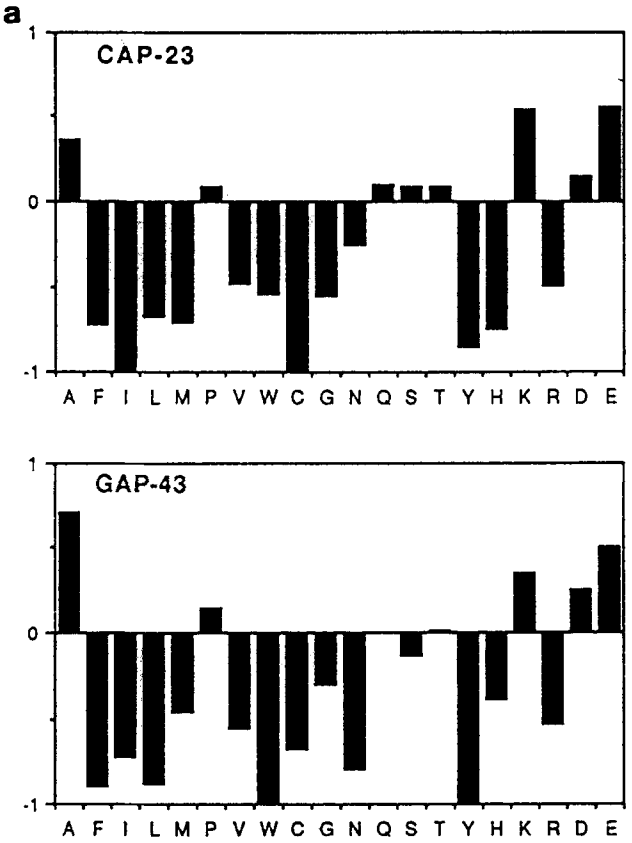
Figure 5. Nucleotide sequence and deduced amino acid sequence of CAP-23-coding clone 22i. The potential PKC phosphorylation site (Ser-6) and the polyadenylation sequence are highlighted. Also pointed out are the boundaries between the four stretches of markedly different aminoacid composition as discussed in the text (D1–4). These sequence data are available from EMBL/GenBank/DDBJ under accession number X54861.

ment are shown in Figs. 8 and 9. The Western blots of Fig. 8 *a* demonstrate that CAP-23 could already be detected at E3, and that overall contents of the protein in liver and in skeletal muscle declined to undetectable levels around E12 and E18, respectively. In the intestine, CAP-23 was still detectable at birth. Homogenate levels of the protein in the nervous system were comparable to the ones from other tissues until around E10, when they increased to reach maximal levels around E18. A slight decrease in total brain CAP-23 levels was observed after E19, but brain contents at birth was still distinctly above peak values in other tissues. Tissue homogenate data therefore suggested that CAP-23 might be a protein of developing tissues. The data also demonstrated that antibody 15C1 detected the same molecular species in the different developing chick tissues. High molecular mass crossreacting species were sometimes observed as two bands larger than 200 kD in nervous system samples containing high levels of CAP-23. As these crossreacting species were only observed when tissues were homogenized in hypotonic, low salt–containing media, they probably represent nondissociated CAP-23-containing complexes.

Fig. 8 *b* shows aspects of the distribution of CAP-23 mRNA during chick development. A main mRNA species of ~1.5 kb specifically hybridized to insert 22i. An additional less prominent species migrating slightly faster than

Figure 3. 15C1 immunoreactive material in cultured cells and in tissue sections from chick tissues. Cells shown in the figure were labeled after 1 d in vitro. No antibody binding could be detected when nonpermeabilized E3 whole embryo cells were exposed to 15C1, indicating that the corresponding epitope was intracellular. (*a*) Phase contrast; (*b*) corresponding 15C1 labeling. In permeabilized cells, the distribution of 15C1 immunoreactivity was granular and surface associated. (*c*) Surface-associated 15C1 labeling of E3-derived neurons. (*d–f*) Double labeling experiments with antibody 15C1 (*dl, el, fl*) and with antichick vinculin (*d2, e2, f2*). (*d*) Granular 15C1 labeling of E6 DRG neuronal growth cone, and corresponding virtual absence of labeling of this structure by antivinculin; (*e* and *f*): Distribution of 15C1-positive patches along the surface region of E3-derived cells; note that 15C1 did not colocalize with vinculin-positive focal adhesion points, and that 15C1-positive patches had a fine structure distinct from the one observed for vinculin-positive regions. (*g* and *h*) Localization of 15C1 immunoreactive material in the vicinity of the cell surface was also observed on sections of chick tissues; (*g*) E13 intestine; (*h*) E2 neural tube. (*g, arrow*) Epithelial cell layer; (*h*, arrow) neural tube. Bar, 20 μm.



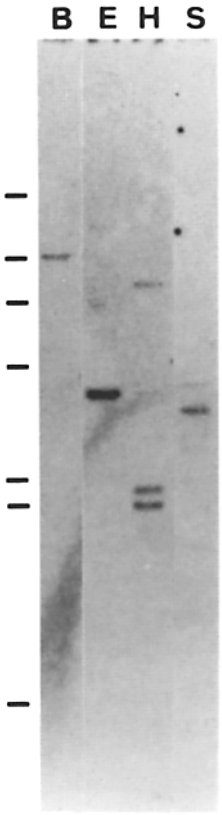


Figure 7. CAP-23 is probably encoded by one gene in the chick genome. Southern blot analysis at high stringency of chick genomic DNA digested with the following enzymes: *B*, Bgl II; *E*, Eco RI; *H*, Hind III; *S*, Sac I (clone 22i contained one Hind III and one Sac I cleavage site). In several cases single hybridizing species were detected, implying the presence of a single CAP-23-coding gene in the chick genome. A second, less intense band might indicate the presence of a related gene. Molecular size markers were 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb.

b

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  6 SKKKKGYSVNDEKAKDKD.KKAEGAATEEEETPKEAEDAQQTTETTEVKE
    .| . :::.: .: | || ||:::::|.|.|...|:.....:..|..|..:
 36 TKIQASFRGHITKKKLKDEKKGDAPAAEAEAKEKDDAPVADGVEKKEGDG
    .          .          .          .          .
 55 NNKEE.......KVEKDAQVSANKTEEKEGEKEKTVTQEEAQKAEPEKSE
    ....:       |.|.....:....|||.|| : ...:|.|..||.| |.
 86 SATTDAAPATSPKAEEPSKAGDAPSEEKKGEGDAAPSEEKAGSAETE.SA
    .          .          .          .          .
 98 AVVDAKVEPQNNEQAPKQEEPAAASRPAASSERPKTSEPSSD.RKASQP
    | .... .| ...|::| .||| .|. ||| .: : |..:..|  ||.||
135 AKATTDNSPSSKAEDGPAKEEPKQADVPAAVTDAAATTPAAEDAAKAAQP
    .          .          .          .          .
147 SEATAPSKADDKSKEEGEAKKTEAPATPAAKKLKAKWPQLQT 188 CAP
    ...||.|...:..||..:. |....| ... | .:. .| :.
185 PTETAESSQAEEEKEAVDEAKPKESARQDEGKEDPEADQEHA 226 GAP
```

Figure 6. Comparison of CAP-23 with the neuronal growth-associated protein GAP-43. (*a*) Similar unusual amino acid compositions of CAP-23 and GAP-43. Mole percentages N(a) for each amino acid were determined and values p(a) for average vertebrate proteins (von Heijne, 1986) were subtracted. Differences were normalized by forming the quotients (N(a)-p(a))/p(a). Negative values varied from zero (no difference) to −1 (amino acid not found in CAP-23, or in GAP-43); positive differences had values ranging from zero (no difference) to +1 (defined for N[a] = 25%; this value was larger than the largest occurring N[a]). Amino acids are designated by their one letter code. Note extremely high hydrophilicity of both proteins, shared elevated relative levels of Ala, Pro, Lys, Asp, and Glu, and unusual Lys/Arg ratios. (*b*) Lack of significant overall sequence homologies between chick CAP-23 and rat GAP-43. The highest scoring Bestfit alignment is shown (length, 192; gaps, 4; percent similarity, 41.2; percent identity, 28.0). The following Bestfits with frameshifted sequences yielded comparable score values (rGAP43 1–210 to cCAP50 4–208) length, 213; gaps, 4; similarity, 41.2; identity, 21.8; (rGAP43 47–210 to cCAP50 73–208) length, 164; gaps, 4; similarity, 44.1; identity, 27.9; (rGAP43 6–107 to cCAP50 95–194) length, 102; gaps, 1; similarity, 41.0; identity, 23.0. Therefore, sequence matches between chick CAP-23 and rat GAP-43 are probably due to the similar amino acid compositions of the two proteins, rather than to homologous sequence motifs.

28S ribosomal RNA also hybridized to insert 22i. The distribution of both hybridizing species was similar, suggesting that they might have originated from the same transcript. In agreement with the Western blot data, CAP-23 mRNA was present in E5 chick embryos, declined to undetectable levels according to a tissue-specific schedule, and was highest in the nervous system. The data therefore suggest that the developmental pattern observed for CAP-23 protein contents might be governed by the levels of CAP-23 mRNA.

Fig. 9 shows that 15C1 immunoreactivity could be detected in essentially all cells of E2 chick embryos. As development progressed, staining became restricted to only certain organs, and later to only certain cells in those organs. In agreement with the data of Fig. 8, CAP-23 levels were particularly high in the developing nervous system. A comparison with the distribution of GAP-43 indicated that CAP-23 might not be restricted to GAP-43-positive cells in the developing spinal cord, and in peripheral nerves. Fig. 9 also clearly demonstrates that the developmental profile of CAP-23 was different in various tissues. In addition, levels of CAP-23 were not related to cell proliferation. While it remains a challenge to identify aspects of the development of cells and/or tissues that might correlate with changes in the levels of CAP-23, it is tempting to speculate that this protein might play a specific role in the development of tissues.

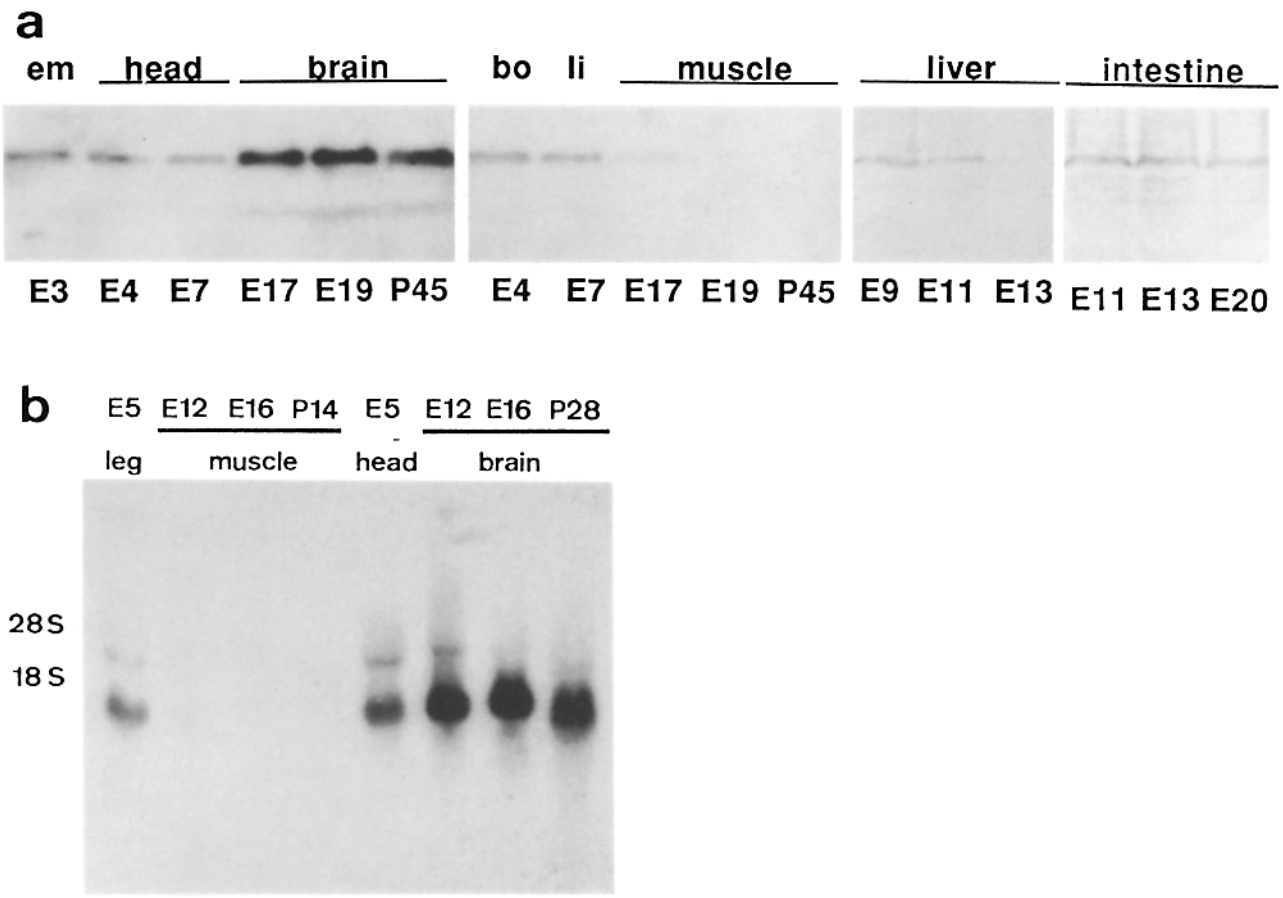


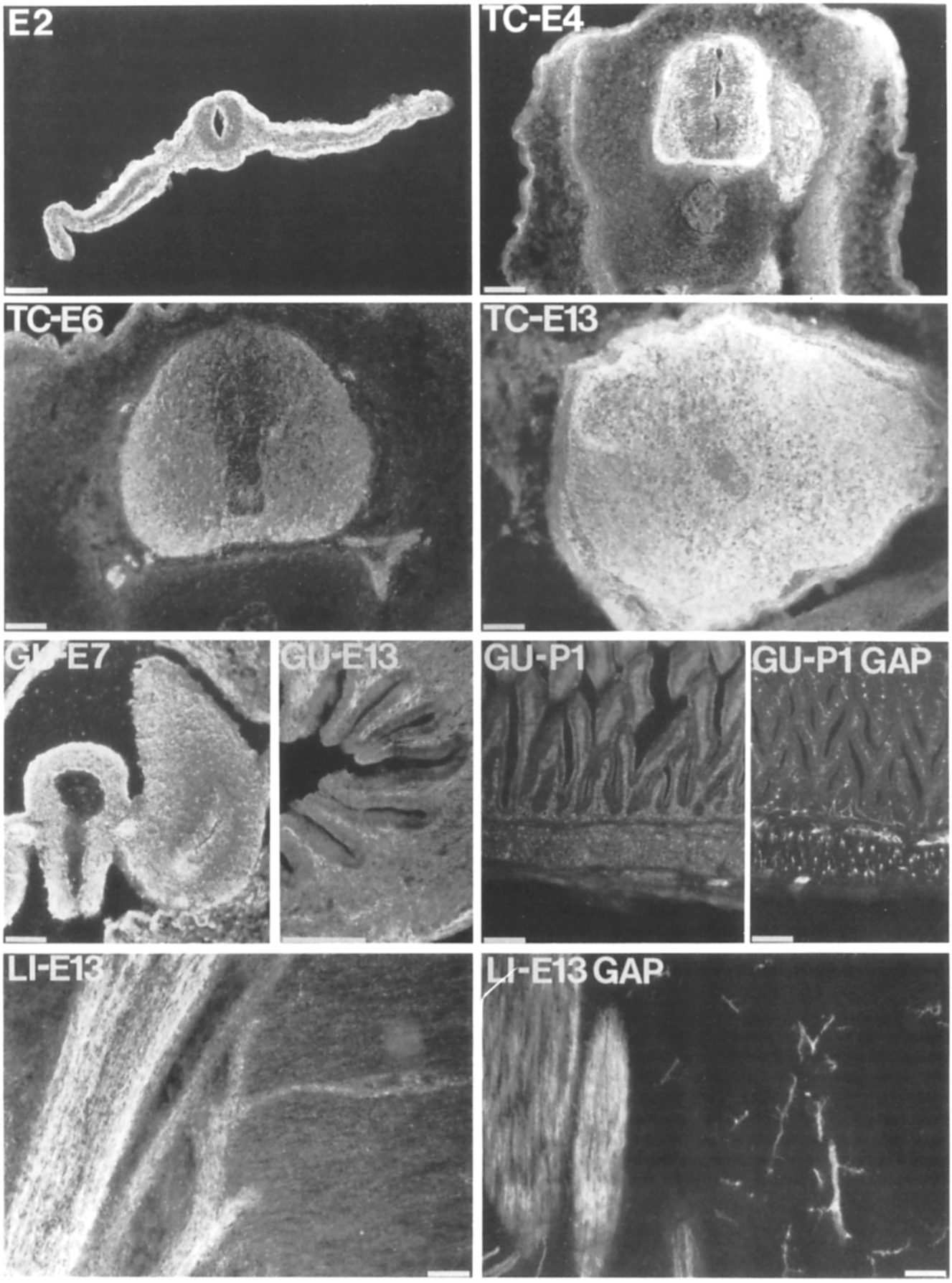
Figure 8. CAP-23 protein and mRNA distribution during chick development. (*a*) CAP-23 contents of chick tissue homogenates. Equal amounts of homogenate protein (100 μg) were separated by 10% SDS-PAGE and analyzed on 15C1 Western blots as shown in Fig. 1. CAP-23 was either detected with iodinated (brain and muscle), or with alkaline phosphatase-coupled (liver and gut) second antibody. When detected by the same method, CAP-23 contents of E7 head, and of E9 liver were approximately equal. (*b*) Distribution of CAP-23 mRNA in the chick. 10 μg of total RNA were applied to each gel lane. In control experiments, the same hybridizing RNA species were detected when polyA+-RNA (E13 brain) was blotted. The size of the main hybridizing species was estimated to be ~ 1.5 kb. An additional minor hybridizing species migrating ahead of 28S rRNA codistributed with the 1.5-kb species, indicating that it probably originated from the same transcript. Overall distribution of CAP-23 mRNA was very similar to the one observed for the corresponding protein.

Discussion

To identify subplasmalemmal proteins involved in the communication of cells with their environment during development, we have searched for proteins with physical properties and subcellular localizations similar to those of the neuron-specific GAP-43. Our search has led to the identification of CAP-23, a novel particle-bound cytosolic protein. In CAP-23-positive cells the protein was distributed homogeneously and in a granular pattern underneath the cell surface. During early chick development CAP-23 was found in most if not all cells. As development proceeded the protein was detected in an increasingly narrower range of tissues and of cells. CAP-23 was the most prominent protein that fulfilled our screening criteria. However, close examination of data similar to those shown in Fig. 1 *a* suggested that additional proteins with similar subcellular localization and physical properties might be identified by the same procedure.

Biochemical Properties of CAP-23

CAP-23 possessed several characteristic biochemical properties that facilitated its identification. The relative migration velocity of CAP-23 on SDS-PAGE varied with the concentration of acrylamide: the protein migrated faster in gels of higher acrylamide concentration. This behavior was similar to that of GAP-43, where it has been attributed to poor binding of SDS, probably due to an extremely high hydrophilicity of the protein (Skene, 1989). CAP-23 fractionated into at least four spots upon IEF (mean isoelectric point of ~4.6), suggesting that it might be a phosphoprotein. This was confirmed by phosphorylation experiments in situ and in vitro, which demonstrated PKC-mediated phosphorylation of CAP-23. The most basic CAP-23 species carried no incorporated ^{32}P-phosphate and probably represented a dephosphorylated form of the protein. The other more acidic CAP-23 species characteristically smeared in the second dimension of 2D-IEF gels. This behavior depended on the concentration of the protein and was not observed when low amounts of CAP-23 were loaded on the gel system. Therefore, phosphorylated forms of CAP-23 might have a tendency to aggregate. The characteristic smearing behavior of CAP-23 on 2D-IEF gels was also observed for rat CAP-23 (data


E2
TC-E4
TC-E6
TC-E13
GU-E7
GU-E13
GU-P1
GU-P1 GAP
LI-E13
LI-E13 GAP

not shown); this, together with the abnormal migration behavior of the protein on SDS-PAGE, constitutes a reliable identification criterion for this unusually hydrophilic phosphoprotein.

Primary Structure of Chick CAP-23

Determination of the primary structure of CAP-23 revealed that it was a novel, highly hydrophilic protein with no significant sequence homologies to presently known proteins. In agreement with biochemical data, CAP-23 possessed a favorable potential PKC phosphorylation site, at least one additional potential casein-II kinase site, and no significant hydrophobic domains. Chick CAP-23 was coded by an mRNA of ~1.5 kb whose distribution and abundance during development correlated well with the protein data obtained with specific antibodies, suggesting that the distribution of the protein might be regulated at the level of its mRNA. Finally, Southern blot analysis indicated that CAP-23 was probably coded by a single gene in the chick genome.

CAP-23 has a very unusual amino acid composition. Hydrophobic residues are either markedly underrepresented or absent, and charged residues are disproportionally often Lys and Glu. The latter feature is also found in the variable tail domain of neurofilament proteins (Myers et al., 1987), and in GAP-43 (Skene, 1989). Glu and Lys are the most extended and most flexible among the basic and acidic residues. Conceivably, this might facilitate intra- and intermolecular ionic interactions. Such interactions are thought to contribute to the formation of oligomers and to interactions with other axonal proteins in neurofilaments (Julien et al., 1987). CAP-23 has a Lys-rich NH_2-terminal domain of 25 residues, followed by an extremely Glu-rich stretch of ~70 residues. These two regions, and in particular two sequences of four Lys (residues 7–10) and of four Glu (residues 32–35) might conceivably interact with each other, either within the same protein chain, or in the formation of (antiparallel) oligomers. PKC-mediated phosphorylation at Ser-6 might interfere with these interactions by introducing two net negative charges at the center of the basic NH_2-terminal. That phosphorylation might influence the intermolecular interactions of CAP-23 is suggested by the behavior of CAP-23 on IEF gels: characteristic concentration-dependent smearing of CAP-23 in the second gel dimension was not observed for the most basic protein spot, which probably represents a dephosphorylated form of the protein. It is therefore conceivable, that PKC-directed phosphorylation of CAP-23 might affect binding of CAP-23 to either itself or to a hypothetical ligand.

Both ends of CAP-23 are predominantly basic, with amino acid compositions diverging from those of the central 160 residues of the protein. High density of charged residues and extremely low contents of hydrophobic residues might give the protein an asymmetrical, rod-like three-dimensional structure flanked by two slightly less hydrophilic and markedly basic ends of ~25 residues each. The ends might conceivably function as binding sites for homo- or heterophilic interactions. However, although it might be anticipated that such interactions could contribute to the localization of the protein in the cell and possibly to its association with ligand(s), such considerations are presently only speculations.

CAP-23 was isolated as a protein resembling GAP-43 in physical properties and in its associations with subcellular fractions. In immunofluorescence experiments on cultured cells, CAP-23 and GAP-43 displayed very similar characteristic granular labeling patterns of neuronal growth cones and of neurites. The distribution of CAP-23 immunoreactivity in nonneuronal cells, where GAP-43 was absent, was granular, suggesting that one property shared by these two proteins might be their peculiar localization. Close comparison of the amino acid sequences of CAP-23 and of GAP-43 failed to reveal homologies that could not be rationalized by the very similar amino acid compositions of the two proteins (Fig. 6). Therefore, it is possible that common molecular features of the two proteins might be limited to their similar physical properties and amino acid composition. Experiments with chimeric proteins and a precise description of CAP-23 distributions, both at the ultrastructural level and in the developing nervous system, will help to determine what functional properties, if any, might be shared by the two proteins.

Subcellular Localization of CAP-23

The majority of E13 chick brain homogenate CAP-23 could be recovered in a soluble fraction enriched in cortical cytoskeleton–associated proteins (data not shown). Labeling of cultured cells with a specific antibody demonstrated that CAP-23 was an intracellular, presumably cytosolic protein. An association of CAP-23 with particulate, possibly surface-associated structures was suggested by the characteristic distribution of CAP-23 immunoreactivity in fixed and permeabilized cultured cells. In addition, cell surface association of CAP-23 immunoreactivity was also suggested by labeling experiments on tissue sections. We therefore speculate that CAP-23 might bind to cortical cytoskeleton, and, directly or indirectly, to the surface membrane of cells.

CAP-23 appeared to be a ubiquitous protein of early chick embryos, and the protein could be detected in a number of developing chick tissues. ASP fractions from embryonic chick liver and skeletal muscle, like their brain counterpart, were enriched in CAP-23, indicating that this protein associated with similar subcellular components in different tis-

Figure 9. Distribution of CAP-23 immunoreactivity in the developing chick. The sections and photographs shown in the figure were all processed in the same way, allowing direct comparison of fluorescent signals. Most if not all cells of E2 embryos (cross section) contained high levels of CAP-23 immunoreactivity. Thoracal-cervical sections (*TC*) at E4, E6, and E13 revealed strong CAP-23 immunoreactivity in the spinal cord and in nerves, while most surrounding regions already displayed low labeling levels at E4. In the intestine (*GU*), CAP-23 was apparently present in all cells at E7, whereas it was mainly found in the central region of villi at E13. When the distributions of CAP-23 and of GAP-43 (a protein exclusively found in neurons) were compared, similar overall patterns were observed in P1 intestine and in E13 hind limb (*LI*). CAP-23, however, was consistently detected in a broader region of cells in the vicinity of GAP-43 positive axons. In addition, CAP-23 immunoreactivity could still be detected on muscle cells of E13 hind limb. *GAP*, sections labeled with mAb against GAP-43. Bars, 100 μm.

sues. Also, CAP-23-positive cells in culture invariantly displayed the same characteristic labeling pattern. Finally, a protein with similar biochemical properties and tissue distribution during development was detected in the rat. Rat CAP-23 further resembled chick CAP-23 in that antibody 15C1 detected an antigen of similar migration behavior, and of similar distribution in cultured cells from both species (Widmer, F., and P. Caroni, manuscript in preparation). Therefore, CAP-23 appears to be a widespread protein of embryonic chick and rat tissues. Considering the evolutionary distance between these species, it seems likely that CAP-23 might be found in most, if not all vertebrate embryos.

Distribution of CAP-23 during Chick Development

The distribution of CAP-23 varied strongly during chick development. Overall, a progressive reduction of CAP-23-containing tissues and cells was observed. While most, and possibly all cells in E2 embryos contained high levels of the protein, major regional differences were already detectable at E4. Levels of the protein stayed high during the initial formation of organs like liver, heart, intestine, and limbs, but extensive regions of the embryo already contained very low levels of the protein at E4 (Fig. 9). Disappearance of CAP-23 immunoreactivity was according to a tissue- and cell-specific schedule, and significant levels of the protein could still be detected at birth in the intestine and in the nervous system. The latter appeared to be the predominant source of CAP-23 after E3. Preliminary observations in the developing hind limb, liver, and intestine indicated that the protein became restricted to a progressively narrower range of cells of these developing organs, and then became undetectable at a time when major tissue growth was still proceeding. Therefore, although a precise study of the developmental events associated with major changes in CAP-23 levels still has to be performed, it is already clear that CAP-23 is a protein of early embryos and of developing tissues, and that the presence of CAP-23 does not correlate with tissue growth. Future experiments will be aimed at determining whether CAP-23 levels might correlate with the differentiation of cells. Proteins expressed exclusively during development often are replaced by adult forms with only slightly modified properties. We could, however, not detect proteins with properties similar to those of CAP-23 on silver-stained 2D-IEF gels of adult liver and skeletal muscle chick proteins (Widmer, F., and P. Caroni, unpublished observations). Therefore, it seems unlikely that CAP-23 might be the juvenile form of a protein present at all developmental stages. On the other hand, an exclusive association of CAP-23 with development, and possibly with aspects of the differentiation of cells would suggest that this unique and widespread subplasmalemmal protein might play a specific role in development. A role of CAP-23 in specialized cellular functions also appears to be likely if one considers its striking similarities with a unique protein exclusively associated with growing axons and with developing nerve terminals, i.e., GAP-43 (Benowitz and Routtenberg, 1987; Skene, 1989).

Conclusions

We have identified a unique and widespread protein associated with aspects of development. To our knowledge, CAP-23 is a novel protein. This was also supported by sequence data obtained with a CAP-23 encoding cDNA. CAP-23 might, however, be identical to a protein enriched in rat brain-derived cell membrane fractions (Steisslinger et al., 1987), which strikingly resembled CAP-23 on 2D-IEF gels. While this protein was not further characterized, a second cell membrane–associated protein identified in the same study was shown to be identical to GAP-43 (Steisslinger et al., 1987).

Our finding that a unique and widespread particle-bound cytosolic protein associated with development resembles the neuronal GAP-43 in its subcellular associations and physical properties suggests that these proteins might have related cellular functions. Determining the function of CAP-23 might provide valuable information on the roles played by the subplasmalemmal region of cells in developmental processes, possibly including morphogenesis and intercellular communication.

We are grateful to D. Bray and D. J. Moss (antichick GAP-43 antiserum; Medical Research Council, Kings College, London, U.K.), to P. Parker (purified PKC; Ludwig Institute, London, U.K.), and to J. C. Perriard and R. Chiquet-Ehrismann (expression library; Swiss Federal Institute of Technology, Zurich, Switzerland, and Friedrich Miescher Institute, Basel, Switzerland) for gifts of reagents; and to Drs. R. Chiquet-Ehrismann, I. Levitan, and A. Matus for critically reading the manuscript.

Received for publication 6 June 1990 and in revised form 7 September 1990.

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