Interspecies comparison of calmodulin binding proteins throughout the gastrointestinal tract: comparison with human colon adenomas and adenocarcinomas

T J McGarrity, L P Peiffer, M L Billingsley

Abstract
Calmodulin is an ubiquitous cytoplasmic protein which mediates many of the actions of calcium on intestinal tissue including regulation of growth and differentiation of normal and neoplastic cells. Using a biotinylated calmodulin overlay system, we compared the pattern of calmodulin binding proteins throughout the gastrointestinal tract of mice, rats, rabbits, and humans, and in human colonic adenomas and adenocarcinomas. A common calmodulin binding protein of 67 kDa was found in membrane and cytosolic fractions of oesophagus, stomach, proximal and distal small intestine, and colon from all four species. In human tissue this 67 kDa protein was present in greatest concentration in stomach tissue. Furthermore, a 67 kDa binding protein was the major calmodulin binding protein from human stomach and ileum as determined by ion exchange and calmodulin affinity chromatography. A similar pattern of binding proteins was noted between rabbit and human cytosolic fractions; proteins of 60/67 kDa and 105 kDa were present in stomach tissue. A 94 kDa protein was present in samples of rabbit and human ileum but not of mouse or rat. A similar pattern of calmodulin binding proteins was seen in normal and neoplastic large bowel tissue, apart from one of nine adenocarcinomas, where a distinct 54 kDa band was noted in both cytosolic and membrane fractions. The results of this study show interspecies and organ differences between calmodulin binding proteins, but suggest that a 67 kDa protein is the major binding protein present throughout normal gastrointestinal tract and neoplastic human tissue.

The divalent ion calcium has diverse functions in the gastrointestinal tract, including effects on motility, hormone release, and the regulation of cell growth and differentiation.1,2 Several calcium activated enzyme systems could be responsible for the actions of calcium on growth regulation in gastrointestinal tissue. Both protein kinase C and calmodulin dependent proteins have been implicated in the regulation of cellular growth and proliferation.3,4 Calmodulin is an ubiquitous multivalent calcium binding protein which mediates the action of calcium via calcium-calmodulin dependent proteins.5,6 Examples of calmodulin dependent enzymes include calmodulin dependent adenylate cyclase, cyclic nucleotide phosphodiesterase, and multifunctional protein kinase.

It has been suggested that dietary calcium may provide protection against the development of colorectal carcinoma.7-9 Enhanced cellular proliferation as measured by increased [3H]-thymidine incorporation into colonic crypt cells has been used as a biological marker in patients at increased risk for developing colorectal cancer.7-9 Supplemental dietary calcium induced a quiescent state of proliferation in normal appearing colonic crypt cells of patients with familial colon cancer.10 This effect of calcium on proliferation was not seen in adenomatous and carcinomatous tissue, suggesting that colorectal tumour growth is independent of calcium mediated growth regulation.9 Calmodulin antagonists have been shown to exert an antitumour effect on solid sarcoma cells11 and breast tumour cells12 in culture. Calmidazolium, a calmodulin antagonist, enhanced differentiation of the human colon cancer cell line HT-29.12 These studies, however, did not carefully document which calmodulin dependent systems were present or inhibited in cancerous cells. One hypothesis which might explain the effects of calcium on growth and differentiation of colonic cells is that increased extracellular calcium leads to increased transport of calcium across the cell membrane. Once calcium is internalised, calmodulin dependent systems may be activated.

The use of high affinity avidin-biotin interactions to detect biotinylated calmodulin and calmodulin binding proteins has proved to be a useful tool for screening multiple samples for the detection of calmodulin binding proteins after sodium dodecylsulphate-polyacrylamide gel electrophoresis and blotting of proteins.13 The purpose of this report was twofold. Firstly, we determined the normal pattern of calmodulin binding proteins in the gastrointestinal tract of several species commonly used as models for carcinogenesis studies. In this report, biotinylated calmodulin gel overlays and calmodulin affinity chromatography were used to identify major calmodulin binding proteins in normal oesophagus, stomach, duodenum, ileum, and colon of mouse, rat, rabbit, and human. Secondly, the pattern of the binding proteins was determined and compared between normal epithelium, premalignant adenomatous polyps, and adenocarcinomas of the large intestine.
ANIMAL TISSUE

Gastrointestinal tissue was obtained from non-fasting adult female CF-1 mice, male Sprague-Dawley rats, and an adult male New Zealand rabbit. Mice and rats were killed by cervical dislocation after ether anaesthesia and the rabbit was killed by an overdose of phenobarbitone (75 mg/kg, intraperitoneally). Whole tissue segments of mouse oesophagus, stomach, duodenum, ileum, and proximal and distal colon were excised and rinsed in cold saline. For the rat and rabbit tissue, mucosal samples were stripped from the adjacent muscle layer by blunt dissection. Tissues were rapidly frozen in liquid N₂ and stored at −70°C.

HUMAN TISSUE

Random biopsy samples of normal oesophagus to distal colon were obtained from patients undergoing elective endoscopy. Samples of adenomas were obtained from endoscopic polypectomy specimens and samples of adenocarcinomas were obtained from operative specimens. Samples of adjacent normal tissue more than 5 cm from the macroscopically neoplastic tissue were also obtained. Histology of all neoplasms was confirmed on routine haematoxylin and eosin stained sections. In patients undergoing endoscopy informed written consent was obtained, and this project had prior approval of the Clinical Investigation Committee of this hospital. Tissues were rapidly frozen in liquid N₂ and stored at −70°C.

SODIUM DODECYLSULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND PROTEIN BLOTTING PROCEDURES

Gastrointestinal tissue samples were maintained at 4°C and homogenised with a Polytro (4°C) in 10 mmol/l HEPES, pH 7-4, 0·1 mmol/l NaCl, 1 mmol/l ethylenediamine tetra-acetic acid, 1 mmol/l ethyleneglycol-bis-β-aminoethyl ether N₂,N₁-tetra-acetic acid, and 100 mmol/l leupeptin (homogenisation buffer). Homogenates were centrifuged at 1000 rpm×10 minutes and the pellet was discarded. The resulting supernant was centrifuged at 37000×g for 30 minutes and separated into crude cytosol (supernatant) and membrane (pellet) fractions. Protein content was determined using the Biorad protein assay (BioRad, Rockville Center, NY).

One dimensional SDS-PAGE was carried out as previously described, using pyronin-Y as a tracking dye. For the comparison study of gastrointestinal tissue across species, 100 µg of protein per sample was loaded onto each lane. In the study of normal, adenomatous, and carcinomatous colonic tissue 200 µg of protein sample was loaded. Gels were electroblotted onto nitrocellulose (Schleicher and Schuell, Keene, NH) for three hours at 50 volts. Efficiency of transfer was confirmed by staining the gel after blotting. The nitrocellulose blot was incubated in a blocking solution (5% non-fat dry milk, 50 mmol/l Tris-HCl, pH 7-4, 150 mmol/l NaCl, 1 mmol/l CaCl₂; 0·02% antifoam A). Calmodulin binding proteins were visualised using biotinylated calmodulin as previously described. Briefly, after blocking blots were incubated with biotinylated calmodulin (25 µg/5 ml of 50 mmol/l Tris-HCl, pH 7-4, 150 mmol/l NaCl, 1 mmol/l CaCl₂; buffer A) for 30 minutes followed by three 10 minute washes in buffer A. Biotinylated calmodulin was detected using preformed avidin-alkaline phosphatase complexes (Vector Labs), dissolved in buffer A; non-fat dry milk was excluded from the avidin detection systems because some milk preparations contain free biotin which interferes with detection of biotinylated calmodulin. After 30 minutes and three additional washes complexes were visualised using the nitrobluetetrazolium/5-bromo-4-chloro-3-indolylphosphate chromogen system.

The membrane sample was suspended in 1 ml buffer consisting of 10 mmol/l HEPES, pH 7-4, 150 mmol/l NaCl, 0·1% Triton X 100, 0·1% NP-40; samples were subjected to SDS-PAGE and

Methods

Distribution of calmodulin binding proteins in human, rat, rabbit, and murine tissues

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Figure 1: Calmodulin binding proteins from mouse (M), rat (R), rabbit (RB), and human (H) stomach. A 67 kDa calmodulin binding protein was detected in all samples. Several higher molecular weight proteins were noted in rabbit and human cytosol.
biotinylated calmodulin overlay analysis as described above. Molecular weights were determined by using known biotinylated molecular weight standards (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase) in the corresponding blots. Data from all blots were submitted to the reviewers. Several blots were scanned using a laser scanning densitometer (Molecular Dynamics) and relative absorbance quantitated using digital software from Protein Databases Inc.

CALMODULIN AFFINITY COLUMNS

In one experiment calmodulin binding proteins were isolated from human cytosolic tissue samples using ion exchange chromatography on DEAE-Trisacryl (0-25 mol/l NaCl fraction) and subsequent calmodulin-Sepharose affinity chromatography. Briefly, the sample was added to a DEAE-Trisacryl ion exchange column (20 ml bed vol) in homogenisation buffer. The column was washed with 5 bed volumes of homogenisation buffer, and calmodulin binding proteins were eluted from the DEAE column by using 2 bed volumes of 50 mmol/l Tris-Cl, pH 7-4, 0-25 mol/l NaCl, and 1-0 mmol/l CaCl2. The 0-25 mol/l NaCl fraction was adjusted to 3 mmol/l CaCl2 and loaded onto a calmodulin affinity column (20 ml bed vol, 2 mg calmodulin/ml of gel). The calmodulin-Sepharose was washed with 5 bed volumes of buffer A, and binding proteins eluted in buffer A lacking CaCl2 and containing 5 mmol/l ethyleneglycol-bis-β-aminoethylether N,N'-tetra-acetic acid (EGTA). Eluted fractions were concentrated by lyophilisation, dialysed, and subjected to SDS-PAGE as outlined above.

Results

DETECTION OF CALMODULIN BINDING PROTEIN USING BIOTINYLATED CALMODULIN IN NORMAL GASTROINTESTINAL TISSUE

The Table summarises the distribution of the binding proteins in the gastrointestinal tract using the biotinylated calmodulin overlay system. A common protein of 67 kDa was seen in all four species in both cytosol and membrane fractions. A 94 kDa protein was found in cytosol and membrane fractions of stomach and colon tissue from all species.

As Figure 1 shows the 67 kDa protein was present in both cytosolic and membrane fractions from stomach of all four species. Since equal amounts of protein were electrophoresed, the cytosolic samples from rat, rabbit, and human stomach seemed to have a greater amount of this 67 kDa binding protein relative to the corresponding samples from the oesophagus. Data on the relative amount of the 67 kDa protein from various tissues were determined by densitometric analysis (Fig 2). Highest levels were found in stomach and colonic tissues.

A second binding protein of approximately 70 kDa was seen in cytosol and membrane fraction from rabbit and human stomach. In addition, the cytosolic sample from human and rabbit stomach showed a higher molecular weight protein of approximately 105 kDa. This protein was not seen in other species.

The duodenal and ileal tissue samples showed the 67 kDa binding protein. A 94 kDa protein was noted in both ileal fractions of rabbit and the human cytosol. Rabbit ileum and stomach had a higher molecular weight binding protein of approximately 105 kDa.

The colon samples from all species showed the presence of the 67 kDa binding protein (Fig 3). The mouse cytosol and membrane, reminiscent of the pattern in mouse oesophagus and stomach, contained a protein of 94 kDa. A protein of approximately 105–108 kDa was seen in human colon cytosol. The pattern of calmodulin binding proteins was similar in human stomach and colon samples. Across species, patterns of binding protein in the human and rabbit samples were most similar throughout the gastrointestinal tract.

All of the proteins bound biotinylated calmodulin in a calcium dependent manner. Incubations in the presence of EGTA showed no detectable signal after biotinylated calmodulin overlays (data not shown).

DETECTION OF CALMODULIN BINDING PROTEIN IN NORMAL EPITHELIUM, ADENOMAS, AND ADENOCARCINOMAS OF THE LARGE INTESTINE USING BIOTINYLATED CALMODULIN

Samples of large bowel adenocarcinomas (n=9), adenomas (n=2), and adjacent normal epithe-
CaM affinity chromatography

Figure 3: Calmodulin binding protein in colon samples. A 67 kDa protein was prominent in all samples. This protein band appears as a doublet in rat, rabbit, and human cytosol fractions, and a 94 kDa protein was seen in cytosol of all species.

Figure 4: Calmodulin binding proteins of normal tissue (N) and adenocarcinomas (A) of human large intestine. 200 μg of protein was added per lane. A 67 kDa protein was seen in cytosol and membrane fraction; a 43 and 35 kDa band was present in cytosol.

Figure 5: Calmodulin (CaM) affinity chromatography of human ileum and stomach cytosol. Samples were chromatographed as described in Methods, and calmodulin binding proteins eluted with 5 mmol/l ethylene glycol bis-β-aminoethyl ether N,N′-tetra-acetic acid subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated with biotinylated calmodulin. Both stomach (lane 1) and ileum (lane 2) contained a 67 kDa binding protein, confirming results of biotinylated calmodulin overlay analysis.

Discussion
The key result of the present study is that there is...
a 67 kDa calmodulin binding protein throughout the gastrointestinal tract of the four species tested and it is present in normal and neoplastic large bowel. This 67 kDa protein was detected in cytosolic and membrane fractions and was present in the greatest amounts in human and rabbit stomach samples. The results of calmodulin affinity chromatography further confirmed that the 67 kDa was a prominent calmodulin binding protein in human colon, ileum, and stomach. Although the identity of the 67 kDa protein is not known, possibilities include sub-units of calmodulin kinase II, calcineurin, or a novel binding protein found primarily in gastrointestinal tissue.

Our study also showed a similar pattern of binding proteins in cytosolic and membrane fractions of normal epithelium, adenomas, and adenocarcinomas of the human large bowel. A distinct 54 kDa protein was noted in only one of nine adenocarcinomas sampled and partitioned in both cytosolic and membrane fractions. Thus the modulating effects of calcium on growth of normal but not adenomatous or carcinomatous large bowel tissue seems to be unrelated to any gross change or expression of novel calmodulin binding proteins during malignant transformation. It is likely that the regulatory effects of calcium on growth in normal and transformed colon tissue are related to other calcium dependent enzymes such as protein kinase C and oncomodulin.

A protein of similar molecular weight was also seen in the cytosol of the human stomach. A 94 kDa protein was detected in the cytosol fraction from stomach and colon of all four species, and mouse oesophagus. A higher molecular weight protein of approximately 105 kDa was present only in human cytosol from stomach and colon. This protein band was not seen in human duodenal and ileal samples. The human small intestine tissue showed the faint presence of the 67 kDa protein and the 94 kDa protein in ileum. Future studies will focus on antibody based identification of these unknown human calmodulin binding proteins.

Several studies have investigated the presence of calmodulin binding proteins in the small and large intestine. Rochette-Egly and colleagues have studied developmental patterns of calmodulin binding proteins in rat1 and human2 small intestine using an 125I-calmodulin overlay technique. In the rat, at fetal day 13, two calmodulin binding proteins with molecular weights of 145 and 135 kDa were present. By day 19, additional bands at 240 and 110 kDa were seen, but, at birth and in the adult small intestine the 135 kDa protein is the major binding protein of the intestinal brush border microfilaments.3 The 100 kDa protein–calmodulin complex has been studied in chicken intestines by Mooscker and Coleman.4 This complex forms an important part of the infrastructure of the brush border cytoskeleton. In the presence of free calcium, 110 kDa protein calmodulin complexes moved along actin cables, showing that the complex is a possible mechanoezyme. The 110 kDa protein has been termed ‘brush border myosin I’.5

By the eighth week of human fetal life only one 125I-calmodulin binding protein was detachable (145–135 kDa) in small intestine homogenates.6 At 12 weeks additional faint bands corresponding to 160 kDa were detected. As opposed to the adult rat small intestine, where a 110 and 90 kDa binding protein is present, in the human only the 90 kDa protein was present.7 This corresponds with our result which showed a protein of approximately 94 kDa in our human ileal samples. Although the identity of this protein is not known, this 94 kDa peptide may be an isoform of caldesmon or the brush border 110 kDa binding protein.

Rochette-Egly and Haffen have also studied the intracellular migration of calmodulin during development.8 During fetal development of the small intestine a diffuse cytoplasmic staining pattern of calmodulin was seen, whereas in the mature enterocyte intense calmodulin staining was localised to the apical membrane.9 The developmental pattern was also seen in the large intestine but was delayed in comparison with small bowel. At fetal week 14, a 110 kDa protein seen in proximal and distal small bowel specimens was undetectable in the proximal and distal colon.10 Immunoblotting experiments using affinity purified antibodies against the avian 110 kDa calmodulin binding protein showed staining of an 80–90 kDa protein in adult human jejunum and colon samples. Thus the 94 kDa peptide observed in this study may represent the 110 kDa brush border calmodulin binding protein, or alternatively, an isoform of caldesmon.

The present study shows the use of the biotinylated calmodulin overlay technique to detect and compare calmodulin binding proteins throughout the gastrointestinal tract and between species. Given the many functions of calmodulin and its binding proteins, the ability to analyse variation in these proteins provides a major tool for continued investigation in normal and diseased gastrointestinal tissue. In this study there were no major changes in the spectrum of binding proteins between normal and neoplastic human colon tissue.

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