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ARTICLES

Identification of Calreticulin as a Nuclear Matrix Protein Associated With Human Colon Cancer

Gisela Brünagel,[2] Uzma Shah,[1] Robert E. Schoen,[2] and Robert H. Getzenberg[1]*

[1]Department of Urology, University of Pittsburgh School of Medicine and University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania
[2] Division of Gastroenterology, Department of Medicine, University of Pittsburgh School of Medicine and University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

Abstract Colon cancer is one of the most common malignancies among populations in the United States and Western Europe, and one of the leading causes of worldwide morbidity and mortality due to cancer. The early detection of colon cancer is central to the effective treatment of this disease and early detection markers are needed. We have demonstrated that high-resolution two-dimensional gel analysis of nuclear matrix proteins (NMPs) demonstrated a specific oncological fingerprint of colon cancer. Utilizing this approach, four proteins specific for colon cancer were identified. Additionally, one protein was expressed much more strongly in colon cancer compared to adjacent and normal donor tissue. The amino acid composition of this protein revealed sequence similarity with calreticulin. The multi-functional protein, calreticulin, is normally found in the lumen of the endoplasmic reticulum although some reports have described a nuclear localization of the protein. The aim of this study was to confirm the identity of the protein as calreticulin as well as to evaluate the localization of calreticulin in the nuclear matrix of colon cancer tissue. J. Cell. Biochem. 89: 238–243, 2003.

Key words: nuclear structure; neoplasia; tumor marker

Colon cancer is one of the most common malignancies among populations in the United States and Western Europe, and one of the leading causes of worldwide morbidity and mortality due to cancer. Although this disease is curable when diagnosed at an early stage, frequently the tumor is metastatic by the time a patient presents to the physician. The identification of an early tumor marker that would allow reliable early cancer detection could lead to a diagnostic assay that would greatly aid in the management of this disease.

In 1974, Berezney and Coffey first described the nuclear matrix as the structural framework scaffolding of the nucleus consisting of the peripheral lamins, protein complexes, an internal ribonucleic protein network, and residual nucleoli [Berezney and Coffey, 1974]. The nuclear framework consists of approximately 10% of the nuclear proteins, and is virtually devoid of lipids, DNA, and histones [Fey et al., 1991]. Most of the nuclear matrix proteins (NMPs) identified to date are common to all cell types, but several identified NMPs are tissue and cell line specific [Getzenberg, 1994]. NMPs have been demonstrated to participate in many vital cellular functions, such as steroid hormone binding, gene transcription, and translation [Getzenberg et al., 1990; Brancolini and Schneider, 1991; Ruh et al., 1996; Martelli et al., 1997]. Given that the nuclear matrix plays an important role in these vital cellular functions, changes in nuclear matrix structures could result in altered DNA topology and alterations in the interaction of various genes with the matrix, which could then participate in a cascade of events.

Using two-dimensional gel electrophoresis, we have analyzed NMP composition of ten colon cancers, adjacent normal tissues, normal donor tissues, and two colon cancer cell lines. Four proteins specific for colon cancer were identified

*Correspondence to: Robert H. Getzenberg, PhD, Department of Urology Research Laboratories, University of Pittsburgh, Suite G40, Shadyside Medical Center, 5200 Centre Avenue, Pittsburgh, PA 15232.
E-mail: getzenbergrh@msx.upmc.edu

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(CC2, CC3, CC4, CC5). In addition to the four proteins only present in the colon cancer samples, one protein (CC1) was expressed much more strongly in the colon cancers in comparison to the adjacent normal and donor tissue. As previously described, the amino acid composition of this NMP in colon cancer tissue revealed sequence similarity with calreticulin [Brunagel et al., 2002]. Calreticulin, is a highly conserved, ubiquitously expressed intracellular protein, which was first identified as a calcium binding protein of the endoplasmic reticulum in skeletal muscle [Ostwald and MacLennan, 1974]. Calreticulin is a multi-functional protein, which is normally found within the lumen of the endoplasmic reticulum [Krause and Michalak, 1997]. Despite the fact that calreticulin is typically associated with the endoplasmic reticulum a number of studies have demonstrated the nuclear localization of this protein. Yoon et al. [2000] reported calreticulin in the nuclear matrix of hepatocellular carcinomas. In addition it has been shown to be a nuclear export factor [Holaska et al., 2001]. The aim of this current study is to determine if in fact, the nuclear matrix protein that we associated with colon cancer, CC1, is calreticulin. Furthermore we determined if calreticulin is indeed differentially expressed in colon cancer and therefore may be a marker for the disease.

MATERIALS AND METHODS

Tissue Processing

Colon adenocarcinoma samples and matched adjacent normal tissues were collected through the Early Detection Research Network (EDRN) of the University of Pittsburgh Medical Center. Normal colon tissue was obtained from trauma victims. Diagnosis was obtained from pathology reports, which accompanied each specimen and was confirmed histologically. The tissues were stored at -80°C prior to processing. All tissue samples were collected and utilized under an IRB approved protocol.

Nuclear Matrix Preparation

NMPs were extracted from colon cancer tissue, adjacent tissue from these individuals, and donor patients according to the method of Getzenberg et al. [1991]. In summary, these tissue samples were finely minced and homogenized with a Teflon pestle on ice with 0.5% Triton X-100 in a solution containing 2 mM vanadyl ribonucleoside (RNAse inhibitor) to release the lipids and soluble proteins. The homogenized tissue was then filtered through a 350 μm nylon mesh. DNAse and RNAse treatments were used to remove the soluble chromatin. The remaining fraction contained intermediate filaments and NMPs. This fraction was then disassembled with 8 M urea and the insoluble components consisting of carbohydrates and extracellular matrix were pelleted. After dialyzing the urea out, the intermediate filaments were allowed to reassemble and were subsequently removed by centrifugation. The NMPs were then precipitated in ethanol. The protein concentration was determined by resuspending the pellet in 2D sample buffer consisting 9 M urea, 65 nM 3-((3-cholamidopropyl)-dimethyl-ammonio)-1propane-sulfonate, 2.2% amhpolytes, and 140 mM DTT and in PBS (phosphatase buffered salt solution), and quantitated by Coomassie Plus protein assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard. The final pellet containing these proteins represent $<1\%$ of the total cellular proteins.

Nuclear and Cytoplasmic Extraction

NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Co.) were used for the preparation of nuclear and cytoplasmic extracts. The protein concentration was quantitated by Coomassie Plus protein assay (Pierce Chemical Co.) with bovine serum albumin as a standard.

High Resolution, Two-Dimensional Electrophoresis

High resolution, two-dimensional electrophoresis was performed using the Investigator 2-D gel system (Genomic Solutions, Ann Arbor, MI) as described previously [Patton et al., 1990; Getzenberg et al., 1991]. One hundred μg of protein was loaded per gel onto a capillary size IEF column. One dimensional isoelectric focusing was carried out for 18,000 volt-h using 1 mm $\times$ 18 inch tube gels after 1.5 h of prefocusing. The tube gels were extruded and placed on top of 1 mm SDS Duracryl (Genomic Solutions) high tensile strength PAGE slab gels. The gels were electrophoresed at 12°C constant temperature for 4.5–5 h. Gels were fixed with 50% methanol and 10% acetid acid. After thorough rinsing and rehydration, gels were treated with

5% glutaraldehyde and 5 mM DTT after buffering with 50 mM phosphate (pH 7.2). The gels were stained with silver stain using the method of Wray et al. [1981] (Accurate Chemical Co., Westbury, NY). Molecular weights of the colon NMPs were identified using standards provided by Genomic Solutions. Isoelectric points (PI's) were determined using carbamylated standards (BDH-distributed by Gallard-Schlessinger, Carle Place, NY and Sigma Chemical Co., St. Louis, MO). Multiple gels were run for each sample and multiple samples were run at different times. Only protein spots clearly and reproducibly identical in all the gels of a sample type were taken into account as those representing the described NMP's. The gels were analyzed using the BioImage 2D Electrophoresis Analysis System (BioImage, Ann Arbor, MI), which matches protein spots between gels and sorts the gels and protein spots into a database.

One-Dimesional Immunoblot

One-dimesional immunoblot analysis was performed according to standard established protocols. Ten μg of each sample of extracted NMPs was suspend in PBS (phosphatase buffered salt solution) and nuclear and cytoplasmic extracts suspend in nuclear extraction reagent (NER) or in cytoplasmic extraction reagent (CERII) were separated by 12% SDS–PAGE. Ten μl of Rainbow markers (Amersham Life Sciences, Arlington Heights, IL) were also loaded. Proteins were then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and the membrane was blocked overnight in 4% nonfat dry milk in PBS with 0.2% Tween20 at 4°C. The membrane was then washed with PBS and 0.2% Tween20, followed by a 1-h incubation with a 1:2000 dilution of anti-calreticulin IgG (Research Diagnostics, Inc., Flanders, NJ) and 2% nonfat dry milk with 0.2% Tween20 in PBS. The membrane was further washed with PBS and 0.2% Tween20 and incubated for 1 h in a 1:5000 dilution of goat anti-rabbit IgG (Amersham Life Sciences) secondary antibody conjugated with horseradish peroxidase (Amersham Life Sciences). For determination of the relative purity of each protein extraction, the membranes were probed with a monoclonal α-tubulin mouse antibody (1:500) (specific for cytoplasmic tubulin) and 2% nonfat dry milk with 0.2% Tween20 in PBS. These membranes were further washed with PBS and 0.2% Tween20 and incubated for 1 h in a 1:5000 dilution of goat anti-mouse IgG (Amersham Life Sciences) conjugated with horseradish peroxidase. The membranes were washed again with PBS and 0.2% Tween20 and the proteins detected by chemiluminescence reaction using the ECL immunoblot kit (Amersham Life Sciences).

Two-Dimesional Immunoblot

After performing 2-D electrophoresis described above, the area of the gel where the spot was located and which the peptide sequence resulted in identification as calreticulin was removed and was transferred to a polyvinylidene difluoride membrane (Millipore). This area of the gel was utilized instead of the entire gel, because the large gel format makes blotting and processing difficult. Thereafter, the same procedure described above for the one-dimensional immunoblot was followed by a 1-h incubation with a 1:2000 dilution of anti-calreticulin IgG (Research Diagnostics, Inc.) with 2% nonfat dry milk with 0.2% Tween20 in PBS. The membrane was further washed with PBS and 0.2% Tween20 and incubated for 1 h in a 1:5000 dilution of goat anti-rabbit IgG (Amersham Life Sciences) secondary antibody conjugated with horseradish peroxidase (Amersham Life Sciences). The membrane was washed again with PBS and 0.2% Tween20 and proteins were detected by a chemiluminescence reaction using the ECL immunoblot kit (Amersham Life Sciences).

Protein Sequencing

We have been successful in developing a technique for sequencing of proteins isolated from spots in two-dimensional gels as previously described [Brunagel et al., 2002].

RESULTS

Using protein spots obtained from the two-dimensional gels, we identified four peptide sequences in CC1 all of which corresponding to calreticulin (Table I; Fig. 1). Since all of these sequences corresponded to calreticulin we utilized a commercial anti-calreticulin antibody (Research Diagnostics, Inc.) as a probe for these studies.

Our first step was to determine if the CC1 spot that we sequenced was recognized by an anti-calreticulin antibody, utilizing a two-dimensional immunoblot. The results verified that

TABLE I. Using Proteins Spots (CC1) From Two-Dimensional Gels From Human Colon Cancer Tissues, Four Peptides Sequences of the Protein all of Which Corresponding to Human Calreticulin (AAB51176: Accession No. National Center for Biotechnology Information, NCBI)

SpotCC1	AA amount	AA sequence	Protein homologies
Peptide 1	15	pavyfkeqfldgdgw	Calreticulin, *Homo sapiens* 15/15
Peptide 2	13	tlivrpdntyevk	Calreticulin, *Homo sapiens* 13/13
Peptide 3	14	qidnpdykgtxihpe	Calreticulin, *Homo sapiens* 14/14
Peptide 4	20	(y)tifdnflitndeayaeefg	Calreticulin, *Homo sapiens* 19/20

the spot removed for sequencing and indicated as CC1 in our studies is indeed identified by the anti-calreticulin antibody (Fig. 2A,B).

To rule out the possibility of contamination of the NMP extraction with cytoplamic proteins we performed one-dimensional immunoblot analysis of colon cancer, normal adjacent and normal donor tissues in the cytoplamic, nuclear, and NMP fractions (Fig. 3). A monoclonal α-tubulin mouse antibody specific for cytoplasmic α-tubulin (approximately 51 kD) was used as a control and identified the protein exclusively in the cytoplamic protein fraction of all the samples. The nuclear and the NMP fractions did not contain a band recognized by the α-tubulin antibody. These results suggest that the contamination of these fractions with cytoplasmic proteins is unlikely.

Using the anti-calreticulin antibody a band of approximately 60 kD was identified in human colon cancer tissue, adjacent normal tissue and normal donor tissue in all three different protein fractions. These results support the existence of calreticulin in the nuclear protein fraction and more specifically in the NMP fraction.

The abundance of calreticulin in the NMP fraction in the different samples reveals that the normal adjacent and normal donor colon tissue expressed a much lower abundance of calreticulin in comparison to the colon cancers. This observation confirmed the results we describe previously, that calreticulin is expressed in the nuclear matrix at a stronger level in colon cancer in comparison with normal adjacent and normal donor colon samples [Brunagel et al., 2002].

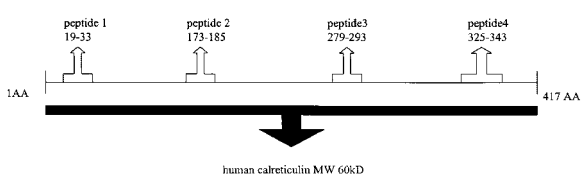

Fig. 1. Schematic diagram showing sequence homology between identified peptide sequence from CC1 and human calreticulin (AAB51176: Accession No. National Center for Biotechnology Information, NCBI).

DISCUSSION

The results outlined contain unexpected data indicating the presence of calreticulin in the nuclear matrix of human colon cancer at significantly higher levels than in normal adjacent and normal donor colon tissues. Calreticulin is principally considered localized in the endoplasmic reticulum where it serves as a calcium binding protein and a chaperone. Additionally, it has important regulatory functions such as modulation of steroid hormone receptors and retinoic acid receptors. It is also involved in the regulation of cell adhesion via interaction with the α subunits of integrins. These functions necessitate its presence at cellular locations other than the endoplasmic reticulum [Burns et al., 1994; Dedhar et al., 1994; Ito et al., 2001]. In 1991 the presence of calreticulin in the nucleus was demonstrated by both immunocytochemistry and sub-cellular fractionation [Opas et al., 1991; Dedhar et al., 1994]. Despite those findings, staining and fixation artifacts and the possibility of contamination in subcellular fractionation raised doubt about the presence of calreticulin in the nucleus. Experiments with the GFP (green fluorescent protein)-fusion protein also demonstrated the presence of calreticulin in the nucleus [Roderick et al., 1997]. Also the group of Paschal et al. have shown, that calreticulin is present in the nucleus and is involved in the nuclear export of nuclear hormone receptors, including steroid hormone, non-steroid hormone, and orphan receptors [Black et al., 2001; Holaska et al., 2001]. It has been reported that the transcriptional activation by glucocorticoid, androgen, retinoic acid, and vitamin D3 receptors is modulated in vivo in cells over expressing calreticulin

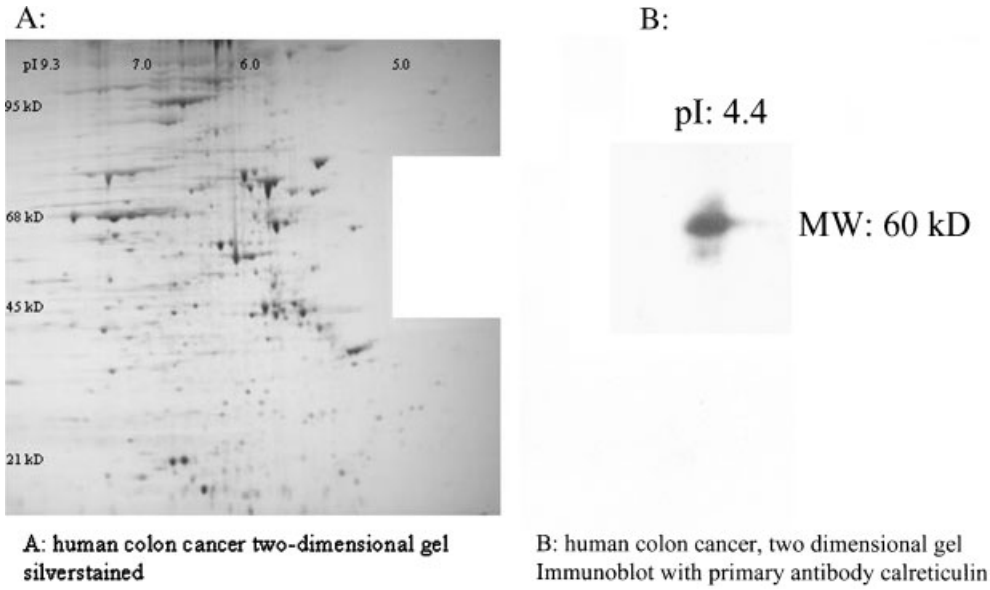

Fig. 2. Two-dimensional silver stained gel from NMPs from human colon cancer tissue (**A**); two-dimensional immunoblot analysis for human calreticulin from the nuclear matrix extracts of human colon cancer tissues (**B**).

[Burns et al., 1994, 1997; Dedhar et al., 1994; Michalak et al., 1996].

Other groups have reported an increase of calreticulin in the NMP of hepatocellular carcinoma [Yoon et al., 2000]. Additionally a proteomic analysis of colonic crypts from normal, multiple intestinal neoplasia (MIN), and p53-null mice demonstrated a significant increase of calreticulin in the MIN mouse [Cole et al., 2000]. Unfortunately these results were from the total cell extracts and a differentiation between the nuclear and the cytoplasmic components were not made.

With the increase of calreticulin in other malignant tissues it may have a limited use as a serum based assay for the early detection of the disease. It does appear that the increase of calreticulin is involved in the development of malignancy and could be used either as a single marker or its combination with other markers as a promising tool for the detection of colon cancer in the future. It can be additionally useful as a tissue marker for making decisions regarding the malignant potential of tissue biopsies and can be clinically helpful not just for colon biopsies.

In fact, of the numerous described functions of calreticulin, the role of calreticulin in the nuclear matrix remains unclear and more so does its elevation in colon cancer. Calreticulin could be a potential therapeutic target in the transcriptional activation by glucocorticoid, androgen, retinoic acid, and vitamin D3.

Further investigations are necessary to explore this particularly with regard that this could be a therapeutic target in colon cancer or other malignancies.

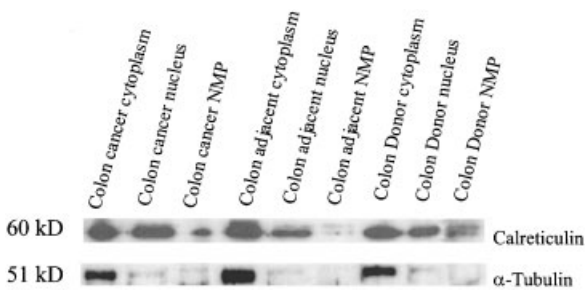

Fig. 3. Representative of one-dimensional immunoblot analysis for human calreticulin and α-tubulin (specific for cytoplasmic), in nuclear matrix fraction, nuclear fraction, and cytoplamic fraction extracted from human colon cancer, human normal adjacent colon cancer, and human normal donor tissues.

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