Biochemical and Biophysical Research Communications **263,** 135–138 (1999)
Article ID bbrc.1999.1319, available online at http://www.idealibrary.com on IDEAL®

Human S100A11 Exhibits Differential Steady-State RNA Levels in Various Tissues and a Distinct Subcellular Localization

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Received July 25, 1999

In order to analyze the steady-state RNA levels of S100A11 in different tissues, a cDNA fragment of human S100A11 was isolated from a cDNA library. The obtained fragment was labeled and hybridized to RNA isolated from various tissues. The Northern blot analysis revealed that S100A11 RNA levels varied from high in placenta, through intermediate in heart, lung, kidney, and most muscle samples, to barely detectable in brain. An efficient purification method for recombinant S100A11 yielding high quantities was developed. Furthermore, to examine the subcellular localization of this protein, the human polypeptide S100A11 antibodies were raised in rabbit. S100A11 was found to have a localization distinct from other S100 proteins examined, and is mostly localized in the nucleus, with slight variations among different glioblastoma cell types.

Ca^{2+}-binding S100 proteins became of major interest because of their close association with several human diseases (1, 2) and their use as prognostic markers in different tumor types (3, 4). S100A11 (previously named S100C or calgizzarin) is a novel and less known member of this large protein family, exhibiting several unique properties. S100A11, first purified and partially characterized from porcine heart (5, 6) and chicken gizzard (7), was subsequently found to be highly expressed in colorectal cancer (8). The low expression in normal colon tissue suggested a role in cell transformation. This would be consistent with the localization of the S100A11 gene on human chromosome 1q21 (9, 10), a region most frequently amplified in tumor tissues. S100A11 was found to regulate cytoskeletal function via Ca^{2+}-dependent interaction with annexin I (5, 11), targeting S100A11 to early endosomes (12). Recently, S100A11 was discovered to be a component of the keratinocyte cornified envelope (13) post-translationally modified by transglutaminase. The purpose of this study was to investigate the S100A11 RNA steady-state levels in a number of human tissues and to determine the distinct intracellular localization of S100A11 by confocal laser scanning microscopy.

Nomenclature of S100 proteins: Schäfer *et al.* (1995) *Genomics* **25,** 638–643; Wicki *et al.* (1996) *Cell Calcium* **20,** 459–464; Wicki *et al.* (1996) *Biochem. Biophys. Res. Commun.* **227,** 594–599.

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MATERIALS AND METHODS

Screening of a human cDNA library. A cDNA fragment of human S100A11 was amplified from a human cDNA library (Stratagene) by the polymerase chain reaction (PCR). The oligonucleotide primers for PCR were based on the 5′- and 3′-ends of the open reading frame of the porcine S100A11 cDNA (5). The nucleotide sequence was determined using the automated DNA Sequencer (ABI, model 377). Database searches in GenBank and sequence analysis were performed by the DNA Data Bank of Japan (DDBJ, National Institute of Genetics, Mishima, Japan).

Northern blot analysis. The Northern blot membrane containing human poly(A)+ RNA from various tissues was purchased from Clontech. The filter was hybridized with a human S100A11 cDNA open-reading frame fragment labeled with [γ-^{32}P]dCTP (Amersham), according to a Megaprime protocol (Amersham). Hybridization was carried out at 68°C in ExpressHyb buffer (Clontech, according to the manufacturer's instructions). The membrane was exposed to X-ray film (Kodak) for 2 or 16 h at −80°C.

Expression, purification of GST human S100A11 fusion protein, production of anti-human S100A11 peptide polyclonal antibody, and Western blot analysis. A cDNA fragment containing the open reading frame of the human S100A11 coding sequence was cloned into the BamHI and the EcoRI sites of the pGEX-2T (Pharmacia) expression vector. The GST human S100A11 protein was expressed in *E. coli* NM522 and lysates were prepared as described earlier (14). One liter of culture was incubated at 37°C until the absorbance at 600 nm reached 0.7. At this point the culture was supplemented with IPTG (1 mM final concentration) and incubated for additional 20 h at 25°C. The cells were centrifuged at 6000 *g* for 10 min. The pellets were resuspended in 30 ml of TBS (25 mM Tris-HCl, 135 mM NaCl, pH 7.4) containing 1% Triton X-100 (v/v). The recombinant protein was

0006-291X/99 $30.00

purified by glutathione Sepharose 4B column chromatography (Pharmacia).

A peptide corresponding to 92-105 amino acids of human S100A11 (HDSFLKAVPSQKRT) was synthesized. Antibodies against human S100A11 peptide were raised in rabbits through series of injections containing the peptides and Freund's complete adjuvant (15). In order to analyze the efficiency of the purification protocol and the purity of the S100A11 recombinant protein, different fractions from the purification process plus the final product were electrophoresed on 12.5% SDS-polyacrylamide gel. Proteins were stained with Coomassie Blue or electroeluted onto nitrocellulose membranes, immunoblotted with anti-S100A11 followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG, and visualized by enhanced chemiluminescence.

Cell culture and immunofluorescent staining. Human glioblastoma cell lines: U-373MG, U-87MG (HTB-17, HTB-14, respectively, available from ATCC), LN215, and LN444 (a generous gift from Erwin G. Van Meir) were maintained in 10% fetal calf serum (FCS) in Dulbecco's modified Eagle's medium (DMEM) with penicillin-streptomycin. In order to stain the cells, they were rinsed with DMEM and fixed with 3.7% formaldehyde in DMEM. Cells were permeabilized with methanol, washed in 5% horse serum (HS)-DMEM, and incubated with specific antibodies (anti-human S100A11, described above, dilution 1:500, and anti-human S100A6 raised in goat, described in (18), dilution 1:1000) for 1 h at 37°C. After washing the cells twice in 5% HS-DMEM, they were incubated with secondary Cy3-conjugated antibodies (goat anti-rabbit IgG (H + L) or mouse anti-goat IgG (H+L), respectively, Jackson Immuno-Research Laboratories, Inc.) for 45 min at 37°C. The cells were washed twice in 5% HS-DMEM, once in PBS (pH 9), and mounted in Molwiol (Hoechst) containing 0.75% n-propyl-gallate as an anti-bleaching agent. Mounted slides were left to dry for 24 h at room temperature in the dark, and stored in the dark at 4°C until viewed. As a control, the cells were incubated with pre-immune sera.

Confocal laser scanning microscopy. Cells were scanned with a Zeiss Axioplan fluorescence microscope (100× oil objective) equipped with a confocal unit MRC-600 (Biorad) and an argon-krypton laser with an excitation wavelength of 568 nm, and a long pass filter of 585 nm. Subsequently, the images were processed using Imaris (Bitplane) and Photoshop (Adobe System) software on a SGI-Indigo 2 workstation (Silicon Graphics) described in (16, 17).

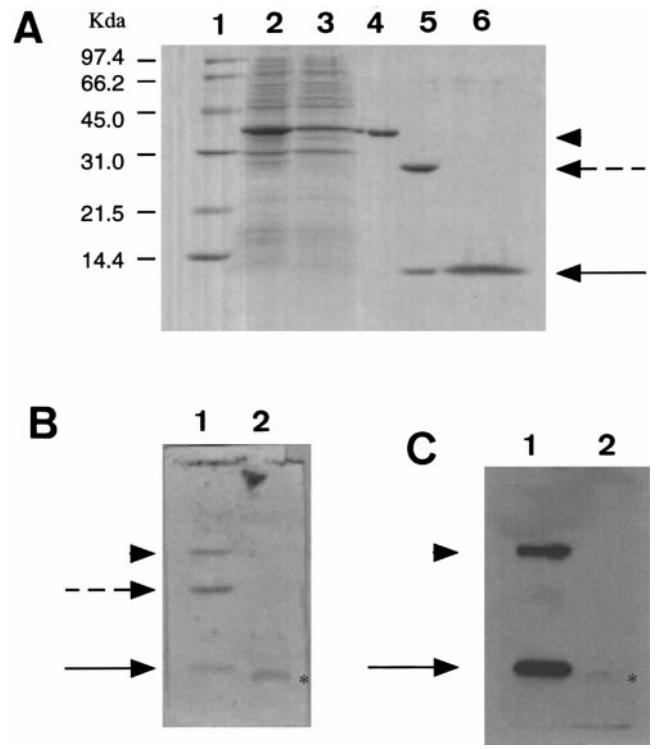

FIG. 1. Expression and purification of recombinant human S100A11. Reducing SDS–polyacrylamide gel (15%) was stained with Coomassie blue. (A) Lane 1, marker; lane 2, crude extract of *E. coli;* lane 3, supernatant fraction of *E. coli* extract; lane 4, eluate from glutathione Sepharose 4B column; lane 5, thrombin cleavage fraction; lane 6, eluate from SP Sepharose fast flow. (B) Lane 1, purified human GST-S100A11 fusion protein; lane 2, porcine S100A11 analyzed by SDS–PAGE (12.5%) and stained with Coomassie blue. (C) Proteins transferred onto nitrocellulose membrane and visualized by enhanced chemiluminescence, using anti-human S100A11 and horseradish peroxidase-conjugated anti-rabbit IgG; lane 1, GST-S100A11 fusion protein; lane 2, porcine S100A11. Arrowhead, GST human S100A11 fusion protein; dotted arrow, GST; arrow, recombinant human S100A11; asterisk, porcine S100A11.

RESULTS AND DISCUSSION

The glutathione Sepharose 4B column chromatography yields high quantity of pure S100A11. In order to purify large quantities of S100A11, the cDNA was subcloned into the pGEX-2T expression vector and expressed in *E. coli.* The GST human S100A11 fusion protein was successfully purified using glutathione Sepharose 4B column chromatography (Fig. 1). The analysis by SDS-PAGE and enhanced chemiluminescence revealed two bands: human S100A11 and GST human S100A11 fusion protein. The antibody against human S100A11 did not cross-react with porcine S100A11 protein.

S100A11 steady-state RNA levels vary between different tissues. Using Northern blot analysis, we examined steady-state RNA levels of human S100A11 in various adult (Figs. 2A and 2B) and some fetal (Fig. 2C) human tissues. A single-species transcript of expected size (about 0.5 kb) was detected at high levels in placenta. Intermediate levels were found in adult heart, adult and fetal lung and kidney, as well as adult pancreas. Low levels were detected in adult skeletal muscle, as well as adult and fetal liver. S100A11 was barely detectable in fetal or adult brain. Further analysis of various adult muscle tissues showed that although S100A11 is present in all of the samples examined, its levels vary greatly from rather low in skeletal muscle and colon to quite high in bladder.

S100A11 localizes mostly in the nucleus of glioblastoma cell lines. To examine the localization of S100A11, the U-373 MG and U-87 MG glioblastoma cells (LN215, LN444, data not shown) were stained with S100A11-specific polyclonal peptide antibody and fluorescent secondary antibody. Figure 3A shows that S100A11 is predominantly localized in the nucleus and less in the cytoplasm of U-373 MG cells. Figure 3B shows localization of S100A11 in the nucleus and in cytoplasm. In contrast, S100A6 (Fig. 3C) localizes

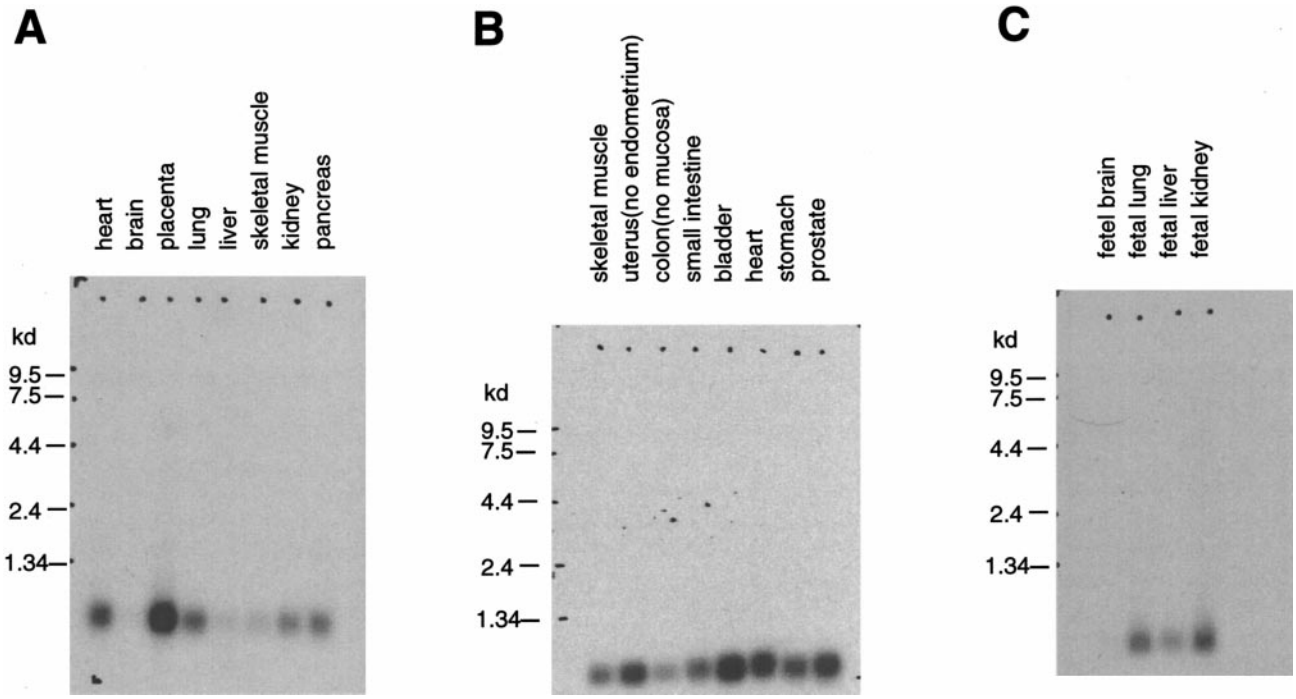

FIG. 2. Steady-state levels of S100A11 mRNA in various human tissues. Commercially available Northern blots were hybridized with the ^{32}P-labeled human S100A11 cDNA fragment. (A) Human multiple-tissue Northern blot. (B) Human muscle multiple-tissue Northern blot. (C) Human fetal multiple-tissue Northern blot.

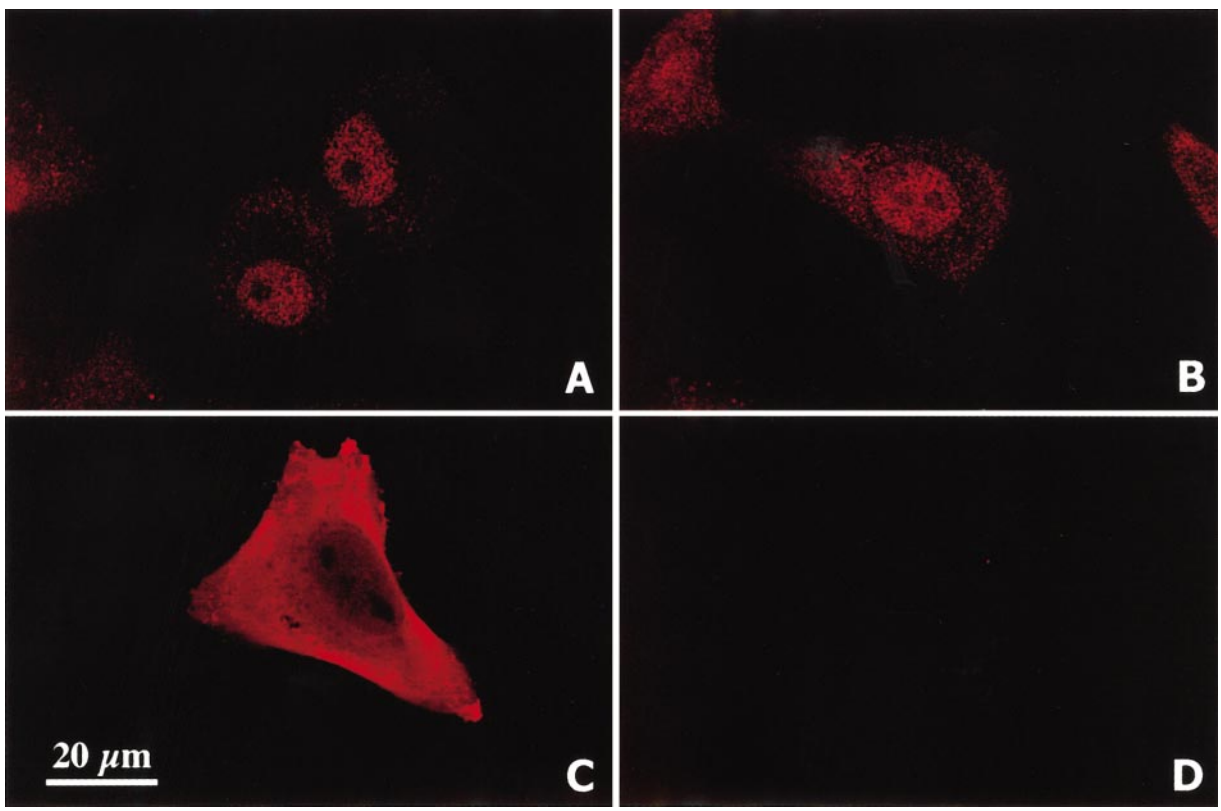

FIG. 3. Subcellular localization. The glioblastoma cells were stained with primary rabbit anti-human S100A11 (dilution 1:500; A, U-373 MG cells; B, U-87 MG cells). (C) Staining with goat anti-human S100A6 (dilution 1:1000). (D) Control staining for S100A11 using corresponding pre-immune serum. All samples were stained with a corresponding secondary Cy3-coupled antibody (anti-rabbit or anti-goat, dilution 1:500).

mostly in the cytoplasm of these cells. These results demonstrate a distinct subcellular localization of S100A11 different from that of other S100 proteins. Furthermore, S100A11, in contrast to other S100 proteins, was found to be expressed at varying levels and locations in different glioblastoma cell lines exhibiting distinct invasiveness and motility rates (data not shown). Considering that S100A11 RNA levels were barely detectable in normal brain tissue, these results suggest that S100A11 might regulate specific cellular processes involved in tumor progression. This is consistent with the preliminary findings suggesting that overexpression of S100A11 in colon cancers might be of importance in the tumor progression process (8).

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas, Scientific Research (A)and(B), International Grants Scientific Research (Joint Research) and Exploratory Research from the Ministry of Education, Science, Sports and Culture, and the Grant for Pediatric Diseases from the Ministry of Health and Welfare, Japan. It was also supported in part by grants for Research Project on Muscle Regulation and on Cerebral Vasospasm from Mie University School of Medicine, a Grant-in-Aid (1996) from the Mie Medical Research Foundation, and the Swiss National Science Foundation (31-50510.97). We thank M. Killen for critical reading of the manuscript, as well as Matthias Höchli and Thomas Bächi for their help with confocal microscopy.

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