Journal of Cellular Biochemistry 71:1–10 (1998)

ARTICLES

Dual Cytoplasmic and Nuclear Distribution of the Novel Arsenite-Stimulated Human ATPase (hASNA-I)

Buran Kurdi-Haidar,[1]* Doreen K. Hom,[1] David E. Flittner,[1] Dennis Heath,[1] Lynn Fink,[1] Peter Naredi,[2] and Stephen B. Howell[1]

[1]Department of Medicine and the UCSD Cancer Center, University of California at San Diego, La Jolla, California 92093–0058
[2]Department of Surgery I, University Hospital, Umea, Sweden

Abstract The arsenite-stimulated human ATPase (hASNA-I) protein is a distinct human ATPase whose cDNA was cloned by sequence homology to the *Escherichia coli* ATPase arsA. Its subcellular localization in human malignant melanoma T289 cells was examined to gain insight into the role of hASNA-I in the physiology of human cells. Immunocytochemical staining using the specific anti-hASNA-I monoclonal antibody 5G8 showed a cytoplasmic, perinuclear, and nucleolar distribution. Subcellular fractionation indicated that the cytoplasmic hASNA-I was soluble and that the perinuclear distribution was due to association with the nuclear membrane rather than with the endoplasmic reticulum. Its presence in the nucleolus was confirmed by showing colocalization with an antibody of known nucleolar specificity. Further immunocytochemical analysis showed that the hASNA-I at the nuclear membrane was associated with invaginations into the nucleus in interphase cells. These results indicate that hASNA-I is a paralogue of the bacterial ArsA protein and suggest that it plays a role in the nucleocytoplasmic transport of a nucleolar component. J. Cell. Biochem. 71:1–10, 1998.

Key words: immunocytochemistry; breast cancer; monoclonal antibody; subcellular localization; confocal microscopy

Resistance to metal salts in bacteria is mediated by specific plasmid-borne multicomponent ATP-dependent efflux systems [Kaur and Rosen, 1992b; Silver and Ji, 1994]. In *Escherichia coli*, resistance to arsenite, arsenate, and antimonite is mediated by the well-characterized *ars* operon [Rosen et al., 1990] that contains two regulatory (*arsR* and *arsD*) and three structural (*arsA, B*, and *C*) genes [Broer et al., 1993; Kauer and Rosen, 1992a]. The *arsA* gene codes for an oxyanion-dependent ATPase that associates with the product of the *arsB* gene, which is a putative channel-forming transmembrane protein. The ATP-binding cassette of *arsA* identifies it as belonging to a superfamily of genes with a modified nucleotide binding motif that is distinct from that present in other ATPases including the cation-translocating transporters [Koonin, 1883; Silver et al., 1993]. The *arsA* gene codes for a 583-amino-acid catalytic subunit with two ATP-binding domains, whereas the *arsB* gene codes for a 429-amino-acid inner membrane protein with 12 transmembrane spanning domains that is postulated to serve as the anion channel and as an anchor for the arsA protein [Rosen et al., 1993]. Together these two proteins transport arsenite and antimonite out of the cell.

We previously reported the cloning of the arsenite-stimulated human ATPase (*hASNA-I*) cDNA on the basis of its DNA sequence homology to the *arsA* gene of *E. coli* [Kurdi-Haidar et al., 1996]. We documented that, like the bacterial ArsA, the human hASNA-I protein also has ATPase activity, although its biochemical characteristics point to it being functionally different from that of the arsA protein [Kurdi-Haidar et al., 1996; unpublished results]. By analogy to the role of ArsA in bacteria, we have hypothesized that hASNA-I may be part of a transporter. Owing to the vast evolutionary distance, we examined the role this distinct arsenite-

Contract grant sponsor: National Institute of Health; Contract grant number: CA69004; Contract grant sponsor: California Breast Cancer Research Program; Contract grant number: 2RB-0125.

*Correspondence to: Buran Kurdi-Haidar, Ph.D., UCSD Cancer Center, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093–0058. E-mail: bhaidar@ucsd.edu

Received 24 March 1998; Accepted 26 March 1998

stimulated ATPase plays in the physiology of human cells by analyzing the hASNA-I subcellular location, its tissue-specific distribution, and correlation of its expression with a disease state. In the present study, we describe the generation of a specific anti-hASNA-I mouse monoclonal antibody and the hASNA-I subcellular localization by using immnocytochemical staining and Western blot analysis of subcellular fractions.

MATERIALS AND METHODS

Cell Line

The human malignant melanoma T-289 cell line [Taetle et al., 1987] was chosen for this study based on the fact that its drug-selected derivative subline [McClay et al., 1993] showed resistance to arsenite. In addition, when analyzed by Western blotting using a rabbit anti-hASNA-I polyclonal antibody, it was found to overproduce hASNA-I (Kurdi-Haidar and Howell, unpublished results). T289 cells were maintained in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 nM hydrocortisone, 5 μg/ml insulin, 5 μg/ml human transferrin, 10 nM estradiol, and 5 ng/ml selenium (Sigma Chemical Co., St. Louis, MO).

Recombinant hASNA-I-Containing Proteins

Two recombinant hASNA-I-containing proteins were used in the course of this study: a previously described glutathione-S-transferase (GST)-hASNA-I fusion protein [Kurdi-Haidar et al., 1996] and a new 6xHis-tagged hASNA-I (6xHis-hASNA-I) protein. The latter was generated by using the pPRO EX HT prokaryotic expression system (Life Technologies, Bethesda, MD). The fragment B26/$B8_a$, generated by polymerase chain reaction, that includes the coding region of the full-length cDNA of *hASNA-I* [Kurdi-Haidar et al., 1996] was amplified by using primers B26 and $B8_a$ synthesized by using the published sequence of hASNA-I [Kurdi-Haidar et al., 1996]. B26 (5′-TTACAGAATCAGAGCGAAGATGTTGTTGGATGT-3′) extends from position −9 to +14, spanning the initiation codon, and is preceded by an *Eco*RI recognition site. $B8_a$ (5′-GCATCTCGAGTGGAGGGTGAGTGTGAAATG-3′) extends from position +1044 to +1024, lies downstream of the stop codon, and is preceded by an *Xho*I recognition site. Fragment B26/$B8_a$ was digested with *Eco*RI and *Xho*I and subcloned in frame into *Eco*RI/*Xho*I-digested pPRO EX HTa plasmid. The coding frame in the resulting plasmid HT-*hASNA-I* was verified by sequencing with primer B6, a previously described antisense *hASNA-I*-specific primer [Kurdi-Haidar et al., 1996]. Plasmid HT-*hASNA-I* was electroporated into DH5α cells, and the 6xHis-tagged hASNA-I was induced and purified by using the nickel-nitrilo-tri-acetic acid (Ni-NTA) affinity resin, as recommended by the manufacturer (QIAGEN, Chatsworth, CA).

Generation of Anti-hASNA-I Monoclonal Antibody

Five female Balb/c mice were immunized with GST-hASNA-I protein emulsified in Freund's adjuvant. Sera from immunized mice were screened by enzyme-linked immunosorbent assay (ELISA) by using the GST-hASNA-I protein. Two mice with high serum titers were selected and splenectomized. Lymphocytes were isolated and fused with Balb/s-derived P3X63AgA.53 myeloma cells by using polyethylene glycol. Hybridomas were selected in HAT medium, and individual colonies were isolated. Because the GST-hASNA-I used for the immunization and serum screening steps contains a GST portion, hybridomas producing specific anti-hASNA-I antibodies were identified by indirect ELISA in plates coated with the 6xHis-hASNA-I protein. Positive hybridoma clones were subcloned by using two series of limited dilution, and individual clones were obtained. Mouse immunizations, serum screening, fusions with myeloma cells, screening of hybridomas, and subcloning of anti-hASNA-I-producing hybridomas were carried out by using the customized monoclonal services of Berkeley Antibody Company (Richmond, CA).

Antibodies

Anti-glutathione-S-transferase rabbit IgG was purchased form Molecular Probes (Eugene, OR). Anti-His-tag mouse monoclonal IgG was purchased from Oncogene Research Products/Calbiochem (Cambridge, MA). Calnexin-N rabbit polyclonal antibody, purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada), was raised against a synthetic peptide from the amino terminus of canine calnexin. The cross reactivity of the rabbit serum to human calnexin had been documented [Wada et al., 1991; Le et al., 1994]. Mouse monoclonal

1277 antibody against human nucleoli was purchased from Chemicon International (Temecula, CA). Texas Red-conjugated sheep anti-mouse Ig, whole antibody, and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit Ig, whole antibody, were obtained from Amersham (Arlington Heights, IL). FITC-conjugated donkey anti-mouse IgM, μ-chain specific, and Texas Red-conjugated goat anti-mouse IgG, Fcγ fragment specific, were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Western Blot Analysis of Bacterial and Human Cellular Lysates

Analysis of cellular lysates and recombinant proteins were performed as previously described [Kurdi-Haidar et al., 1996] by using anti-His-tag mouse monoclonal and anti-glutathione-S-transferase rabbit antibody in addition to the anti-hASNA-I mouse monoclonal 5G8 produced in the course of this study. Peroxidase-linked sheep anti-mouse Ig and peroxidase-linked donkey anti-rabbit Ig (Amersham) were used as appropriate, and Western blots were developed by using the enhanced Chemiluminescence signal detection system (Amersham).

Indirect Antibody and Lectin Labeling of Cells

Cells on coverslips were washed with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) at room temperature and then fixed in cold methanol at −20°C for 5 min. Coverslips were briefly washed and stored in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) [Sobue et al., 1988]. All immunocytochemical manipulations were performed at room temperature. Blocking was carried out for 1.5 h in 2.5% normal sheep, donkey, or goat serum in PHEM buffer before the primary antibody was added for 1 h, followed by three 15-min washes in PHEM buffer. Incubation in the appropriate secondary antibody was carried out for 45 min, followed by three 30-min washes with PHEM buffer. Coverslips were mounted in 90% glycerol in PBS containing 1 mg/ml *p*-phenylenediamine (Sigma). For lectin labeling, cell-coated coverslips were incubated for 30 min at room temperature in PHEM buffer containing FITC-conjugated concanavalin A (FITC-Con A) purchased from Vector Laboratories (Burlingame, CA) at a concentration of 5 μg/ml [Fricker et al., 1997]. Coverslips were washed three times with PHEM and mounted for visualization, as described above.

Confocal Microscopy

A Bio-Rad MRC 1000 confocal microscope equipped with a krypton-argon laser and coupled to a ZeissAxiovert 135 M microscope was used to examine fluorochrome-labeled cells. Images were collected by using BioRad 1024 and Comos software. Composites were assembled by using Adobe Photoshop 4.0 and printed on a Tektronix Phaser IISDX.

Preparation of Subcellular Fractions Enriched for Smooth and Rough Microsomes

Enrichment for smooth and rough microsomal fractions was carried out by using the subcellular fractionation scheme previously described [Graham, 1984]. Cells were disrupted by using a Dounce homogenizer, and the homogenate was centrifuged at 10,000*g* to obtain the postmitochondrial P_{10}. The rough microsomal fraction (rough endoplasmic reticulum; RER) was separated from the smooth microsomal fraction (smooth endoplasmic reticulum; SER) by using a discontinuous sucrose-Tris-CsCl gradient.

Preparation and Analysis of Membrane Fractions

T289 cells were harvested by scraping into ice-cold PBS containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml pepstatin, 20 μg/ml leupeptin, and 5 mM EDTA). Cells were lysed and processed essentially as described elsewhere [De Vries et al., 1996]. Lysis was carried out by 10 passages through a 30-gauge needle, and the postnuclear supernatant (PN) was recovered after centrifugation at 600*g* for 3 min at 4°C in a microcentrifuge. The soluble cytosolic fraction (S) was separated from the crude membrane fraction (P) by centrifugation of the postnuclear supernatant at 100,000*g* for 25 min in a Beckman TL100 rotor. The crude membrane fraction was resuspended in a volume of protease inhibitor-containing ice-cold PBS matching that of the soluble fraction (S). Equal volumes of the PN, P, and S fractions were analyzed by Western blotting by using the anti-hASNA-I mouse monoclonal antibody 5G8.

RESULTS

Antibody Specificity

The IgM monoclonal antibody 5G8 was raised against the GST-hASNA-I fusion protein and

identified by ELISA screening by using purified 6xHis-hASNA-I protein. The specificity of hASNA-I was established by Western blot analysis by using two purified recombinant hASNA-I proteins produced in prokaryotic cells, the GST-hASNA-I fusion protein and the 6xHis-tagged hASNA-I. Figure 1A shows that the GST-hASNA-I was detected by using both an anti-GST-antibody (lane 1) and the 5G8 antibody (lane 3). Similarly, the 6xHis-hASNA-I protein was detected by using both an anti-His-tag (Ab-1) antibody (lane 2) and 5G8 (lane 4). Cellular lysates from T289 cells were analyzed by the same technique. The 5G8 antibody detected a single 37-kDa protein, consistent with the estimated size of the protein encoded for by the *hASNA-I* cDNA (Fig. 1B). These results established that the 5G8 was a hASNA-I-specific antibody that detected a single protein in cellular lysates.

Immunocytochemical Distribution of hASNA-I

The subcellular location of hASNA-I in human T289 cells was visualized by indirect immunofluorescence by using the 5G8 monoclonal antibody and a Texas Red-linked sheep anti-mouse Ig. Figure 2A shows that staining was observed in three subcellular locations: the cytoplasm, the perinuclear region, and the nucleolus. The same pattern of staining was also obtained for two breast cell lines, MCF7 [Zugmaier et al., 1991] and MCF10A (data not shown) [Soule et al., 1990]. No staining was obtained when 5G8 was omitted (data not shown). With the exception of the condensed subnuclear staining, the dual cytoplasmic and perinuclear staining was reminiscent of proteins localized to the ER. The subcellular location of ER proteins in these cells was determined by using lectin staining with FITC-Con A. Figure 2B shows that the FITC-Con A produced distinct perinuclear and a diffuse cytoplasmic staining with no intranuclear fluorescence. Concanavalin A binds to mannose-terminated oligosaccharides and has been shown to label the endoplasmic reticulum and the nuclear envelope [Fricker et al., 1997]. Thus, outside the nucleus, hASNA-I was distributed in a pattern similar to that of ER proteins. However, this double immunocytochemical staining did not clearly distinguish whether the hASNA-I was associated with the perinuclear ER versus the nuclear membrane, or whether the cytoplasmic hASNA-I was actually bound to the ER.

Lack of Association With the Endoplasmic Reticulum

The amino acid sequence of the hASNA-I protein [Kurdi-Haidar et al., 1996] does not

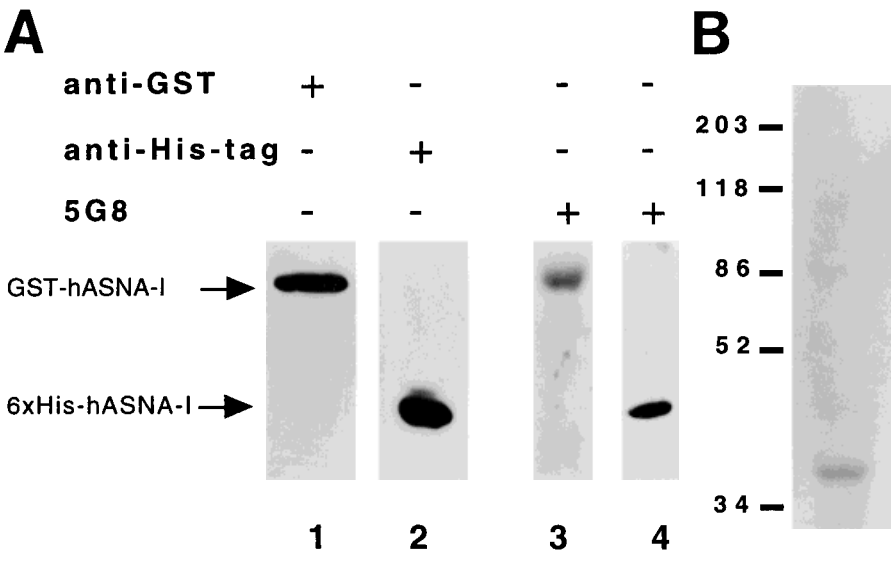

Fig. 1. Western blot analysis of purified recombinant hASNA-I and total cellular lysates from T289 cells. **A:** Purified GST-hASNA-I (**lanes 1 and 3**) and 6xHis-hASNA-I (**lanes 2 and 4**) proteins were analyzed by using the primary antibody depicted above the lanes. **B:** Cellular lysates from T289 cells were analyzed by using anti-hASNA-I mouse monoclonal 5G8 antibody. The numbers on the side indicate the size (in kilodaltons) of the molecular weight markers (prestained sodium dodecylsulfate–polyacrylamide gel electrophoresis standard; Bio-Rad, Richmond, CA).

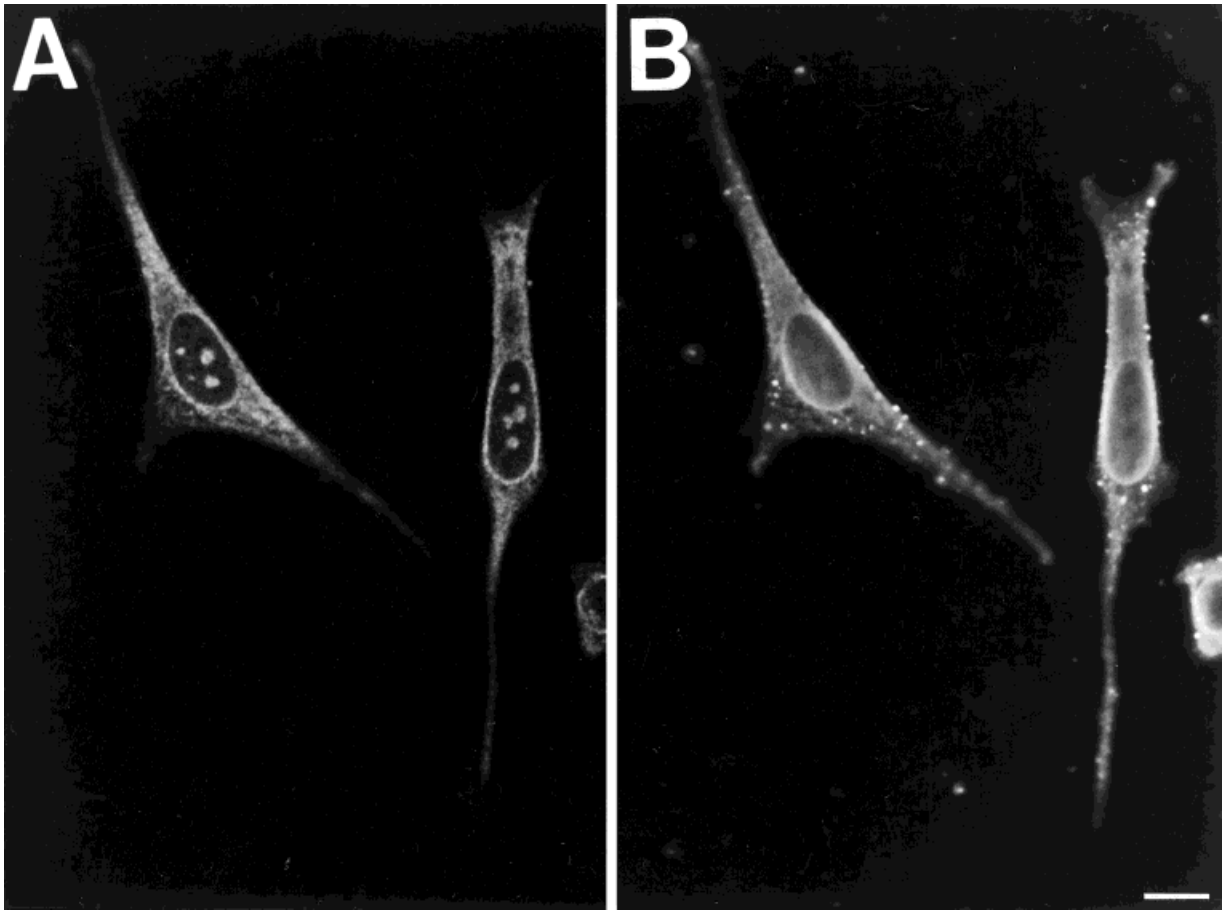

Fig. 2. Immunolocalization of hASNA-I. Human T289 cells were doubly stained with anti-hASNA-I monoclonal antibody 5G8 and FITC-Con A to visualize the ER. Coverslips were incubated with mouse monoclonal antibody 5G8 (1/100), followed by Texas Red-linked sheep anti-mouse (1/200) antibody and FITC-Con A (5 µg/ml) conjugate. **A:** Texas Red visualization of hASNA-I in the cytoplasm, at the perinucleus, and within the nucleolus. **B:** FITC visualization of ER staining that is limited to the cytoplasm and the perinucleus. Both A and B were visualized at the same confocal optical plane close to the nuclear equator. Scale Bar = 10 µm.

possess the carboxy-terminal motif (KDEL) or the double-lysine peptide (KKXX or KXKX) characteristic of proteins retained in the lumen of the ER [Jackson et al., 1990; Pelham, 1990]. This suggested that hASNA-I was not a resident ER protein but did not exclude the possibility that it could be bound to the ER membrane. This issue was addressed by fractionating the human T289 cells to remove the nuclei and examining the distribution of hASNA-I by Western blot analysis of the crude membrane and the soluble fractions (100,000*g* pellet and supernatant, respectively). The results presented in Figure 3A show that hASNA-I was present in the soluble fraction (lane S) but not in the membrane fraction (lane P). As a control, the fractions were examined for the distribution of calnexin, known to be an integral ER membrane protein [Wada et al., 1991]. Calnexin was found in the membrane (lane P) but not in the soluble fraction (lane S). To further establish that hASNA-I is not bound to the ER membranes, ER microsomal fractions enriched for smooth (SER) and rough (RER) microsomes were analyzed for enrichment of hASNA-I and calnexin by Western blotting. Figure 3B shows that no enrichment of hASNA-I was obtained in either fraction when compared with the total cellular lysate (lanes 1, 2, and 3, respectively). However, the RER was clearly enriched for calnexin (lane 3). These results support the conclusion that hASNA-I does not associate selectively with either the SER or the RER. Therefore, the observed cytoplasmic staining seen in Figure 2A represents a soluble pool of hASNA-I, and the perinuclear staining results

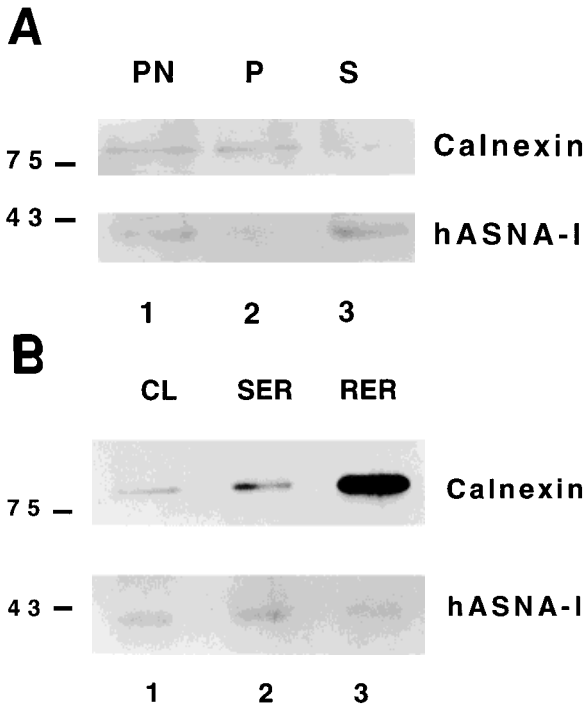

Fig. 3. Immunoblot analysis of subcellular fractions. **A:** A postnuclear supernatant (PN; **lane 1**) from T289 cells was centrifuged at 100,00*g* to seperate the crude membrane fraction (P; **lane 2**) from the soluble fraction (S; **lane 3**). **B:** Immunoblotting of subcellular fractions from T289 cells enriched for smooth (SER) and rough (RER) microsomes. Whole cell lysate (CL; **lane 1**), SER (**lane 2**), and RER (**lane 3**). Fractions were analyzed by immunoblotting using 5G8 and anti-calnexin rabbit polyclonal antibody detected by enhanced chemiluminesence. The numbers on the sides indicate the molecular weight markers.

from association with the nuclear membrane rather than the ER.

Nucleolar Localization

Immunocytochemical staining of T289 cells showed that in the nucleus hASNA-I localized primarily within multiple subnuclear structures. Although the size of the subnuclear structures suggested that it was the nucleolus, other subnuclear structures known as speckles that represent spliceosomes have been described [Spector et al., 1991; Bregman et al., 1994; Fabrizio et al., 1997]. Therefore, double immunofluorescent staining was carried out by using both the 5G8 monoclonal anti-hASNA-I antibody and the 1277 monoclonal antibody specific for human nucleoli. Although both are mouse antibodies, we were able to detect 5G8 and 1277 independently during colocalization studies by making use of the fact that the two monoclonal antibodies belong to different classes of immunoglobulins. The 5G8 is an IgM and the 1277 is an IgG_1. An FITC-conjugated donkey anti-mouse IgM, μ-chain specific, was used to detect 5G8, and a Texas Red-conjugated goat anti-mouse IgG, Fcγ fragment specific, was used to detect 1277. Figure 4 shows that 1277 stained only the nucleolus and that the signal from 5G8 colocalized with that of 1277 in the nucleolus. Thus, hASNA-I is clearly present in the nucleolus and apparently is bound in such a way that it is unable to diffuse freely throughout the nucleoplasm.

Association of hASNA-I With Invaginations of the Nuclear Envelope

It has been demonstrated that interphase nuclei contain dynamic nuclear envelope invaginations into the nucleoplasm presumed to play a role in the nucleocytoplasmic transport [Bourgeois et al., 1979; Fricker et al., 1997]. Some of these have been identified as "nucleolar canals" because they connect the nuclear envelope directly to the nucleolus in different cell types [Bourgeois et al., 1979]. In addition, a second type of dynamic cell type-specific nuclear channel has been described, only a subset of which connect to the nucleolus [Fricker et al., 1997]. The latter channels have been shown to be derived from the ER and to include both membranes of the nuclear envelope. As shown in Figure 5, confocal examination of T289 cells doubly immunolabeled with 5G8 and the anti-calnexin antibody demonstrated an irregular pattern of nuclear staining at the level coinciding with the top of the nucleus. Furrows extending the length of the nucleus were observed, whose sides stained with both antibodies. The fact that the central portion of these furrows was devoid of calnexin staining was consistent with the interpretation that these furrows are invaginations that involve the two components of the nuclear envelope, the ER and the underlying nuclear membranes and the ER membranes. Thus, hASNA-I is found in nuclear invaginations in T289 cells that may be related to channels of either the type that connects directly to the nucleolus [Bourgeois et al., 1979] or other intranuclear structures [Fricker et al., 1997].

DISCUSSION

We previously cloned the cDNA of the *hASNA-I* and determined that the hASNA-I protein is a distinct arsenite-stimulated ATPase

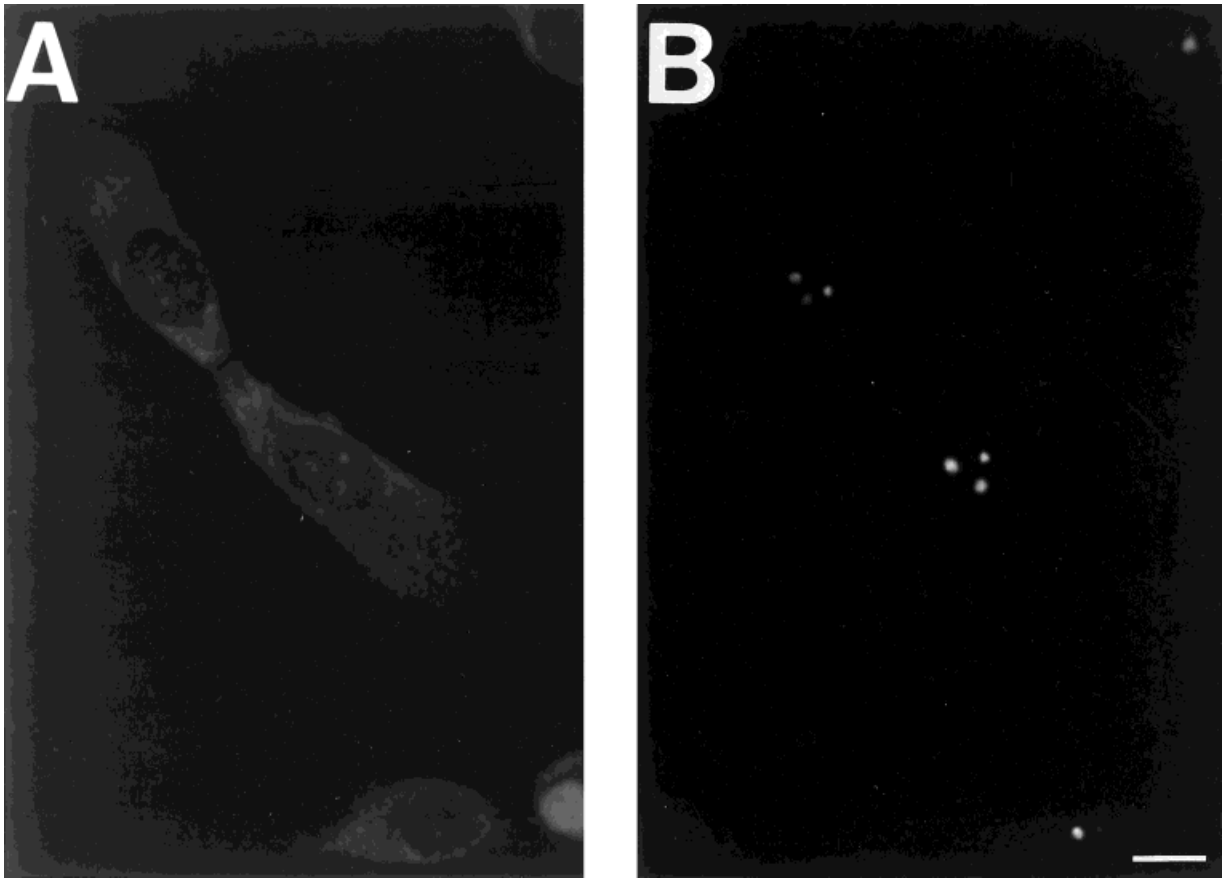

Fig. 4. Immunolocalization of hASNA-I within the nucleus to the nucleolus. Human T289 cells were doubly stained with anti-hASNA-I 5G8 and 1277 antibodies. Coverslips were incubated with anti-hASNA-I IgM mouse monoclonal 5G8 antibody (1/100) and the anti-human nucleolus IgG mouse monoclonal 1277 antibody (1/50), followed by FITC-linked donkey anti-mouse IgM, μ-chain specific (1/200), and Texas Red-linked goat anti-mouse IgG, Fcγ fragment specific (1/200), secondary antibodies. **A:** The hASNA-I staining with 5G8 is the same as that described in Figure 2. **B:** Staining with 1277 is limited to the nucleolar condensations. Both A and B were visualized at the same confocal optical plane close to the nuclear equator. Bar = 10 μm.

[Kurdi-Haidar et al., 1996; unpublished results]. Because cloning of *hASNA-I* was based on DNA sequence homology between distantly related species, the human protein could represent either an orthologue of the bacterial ArsA that carries out a similar plasma membrane transport function or a paralogue with an independent cellular function. As a first step toward elucidating the physiologic role of hASNA-I in human cells, we raised an anti-hASNA-I-specific monoclonal antibody and used it to determine the subcellular localization of hASNA-I.

Immunocytochemical analysis of independent cell lines showed hASNA-I to be localized to two subcellular compartments: the cytoplasm and the nucleus. Subfractionation of the cytoplasm into the soluble and membrane-bound fractions established that hASNA-I is a soluble cytosolic protein. In the nucleus, however, both the nuclear membrane and the nucleolus were clearly enriched for hASNA-I, with no detectable protein in the nucleoplasm. Such a distribution suggested that the nuclear hASNA-I is bound and does not diffuse freely. Therefore, we deduced that human cells contained two pools of hASNA-I, one soluble in the cytoplasm and another bound at the nuclear membrane and within the nucleolus.

Analysis of the amino acid sequence of hASNA-I indicated that it does not have a sequence that matches any of the known nuclear localization signal motifs [reviewed in Gorlich and Mattaj, 1996] or sequences extremely rich in basic amino acids such as those that have been identified as nucleolar localization signals for some viral proteins [Siomi et al., 1988; Dang and Lee, 1989; Cochrane et al., 1990]. As such, we concluded that the distinct perinuclear and

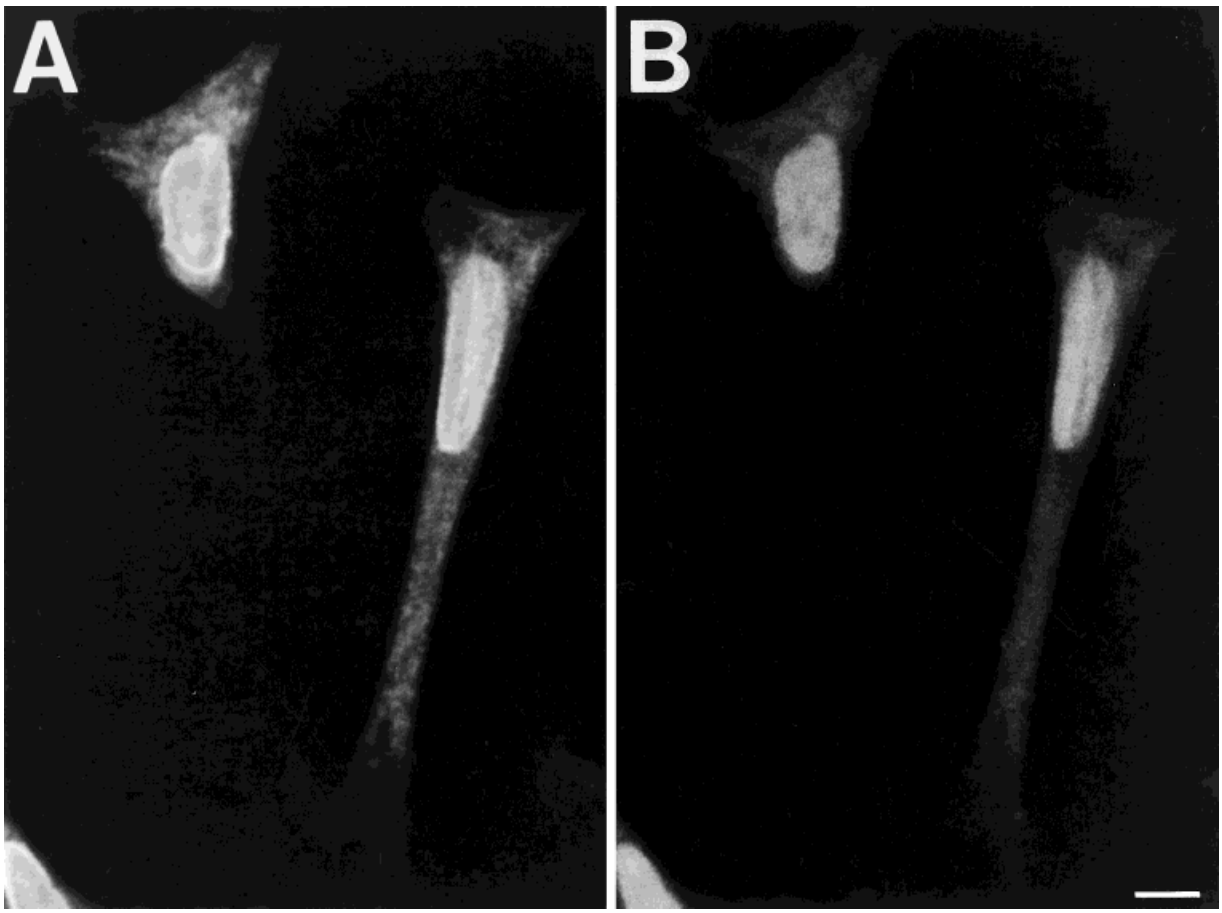

Fig. 5. Immunocytochemical localization of hASNA-I to nuclear invaginations in interphase cells. Human T289 cells were doubly stained with mouse anti-hASNA-I 5G8 (1/100; **A**) and rabbit anti-calnexin (1/200; **B**) antibodies, followed by Texas Red-linked sheep anti-mouse (1/200) and FITC-linked donkey anti-rabbit (1/200) secondary antibodies. Both A and B were visualized at the same confocal optical plane close to the top of the nucleus. Scale Bar = 10 μm.

nucleolar localization of hASNA-I is not signal dependent and most likely results from stable binding to constituents of the nuclear membrane and nucleolus, as has been suggested for other proteins [Maeda et al., 1992; Schmidt-Zachmann and Nigg, 1993; Yan and Melese, 1993]. The steady-state distribution between the two subcellular compartments is probably governed by the rate of nuclear export, import of hASNA-I, and its relative affinities for cytoplasmic and nuclear binding partners [Kambach and Mattaj, 1992; Schmidt-Zachmann et al., 1993]. Some caution about the conclusion that hASNA-I localization is not signal dependent is appropriate because three yeast ribosomal proteins have been reported to have nonclassical nuclear localization signal motifs composed of clusters of basic amino acids including Pro, Arg, and Lys [Moreland et al., 1985; Underwwod and Fried, 1990; Schaap et al., 1991], and although hASNA-I does not contain such clusters, we cannot exclude the possibility that it may contain an undefined signal sequence that awaits future elucidation.

The distinctive localization of hASNA-I to the nucleolus strongly suggested its association with a constituent of this subnuclear compartment. In addition, its presence at the nuclear membrane and in the cytoplasm suggests that it shuttles between the nucleus and the cytoplasm. Several such shuttle proteins have been reported, including three other nucleolar proteins, nucleolin, NO38 [Borer et al., 1989], and Nopp140 [Meier and Blobel, 1992], members of the 70-kDa heat shock proteins [Mandell and Feldherr, 1990], the heterogeneous nuclear RNA-packaging protein A1 [Pinol-Roma and Dreyfuss, 1992], the U1 snRNP-specific protein U1A [Kambach and Mattaj, 1992], and the progesterone receptor [Guiochon-Mantel et al.,

1991]. The movement of these proteins between the nucleus and the cytoplasm is believed to be part of the bidirectional energy-requiring process of nucleocytoplasmic transport [Laskey and Dingwall, 1993; Schmidt-Zachmann et al., 1993], which in turn affects the regulation of cytoplasmic and nuclear activities [Schmidt-Zachmann et al., 1993]. The presence of hASNA-I in a soluble-free form in the cytoplasm, at the nuclear membrane, and in the nucleolus is consistent with the interpretation that it plays a role in a transport process such as the nucleocytoplasmic movement of a nucleolar or ribosomal constituent, as is the fact that it is an ATPase [Kurdi-Haidar et al., 1996; unpublished results]. This notion is fortified by the presence of hASNA-I at invaginations of the nuclear envelope, which are also believed to function in nucleocytoplasmic transport.

Recent immunohistochemical analysis of a variety of human tissues has shown detectable expression of hASNA-I in select cells within certain tissues excluding breast epithelium [Kurdi-Haidar et al., 1998]. In addition, unlike normal breast epithelium, elevated levels of hASNA-I expression were found in a number of tested breast adenomas and carcinomas, pointing to its potential role in breast cancer [Kurdi-Haidar et al., 1998]. Taken together, our results indicate that hASNA-I is a paralogue rather than an orthologue of the bacterial ArsA that in human cells may play a role in nucleocytoplasmic transport rather than in the transport of oxyanions across the plasma cell membrane.

ACKNOWLEDGMENTS

We thank M.G. Farquhar for helpful discussions; M. Mcgrail, H. Matthies, and A.V. Philp for help with confocal microscopy, image collection, and composite assembly; and L.H. Rome (UCLA) for providing the protocol for immunocytochemistry.

REFERENCES

Borer RA, Lehner CF, Eppenberger HM, Nigg EA (1989): Major nucleolar proteins shuttle between nucleus and cytoplasm. Cell 56:379–390.

Bourgeois CA, Hemon D, Bouteille M (1979): Structural relationship between the nucleolus and the nuclear envelope. J Ultrastruct Res 68:328–340.

Bregman DB, Du L, Li Y, Ribisi S, Warren SL (1994): Cytostellin distributes to nuclear regions enriched with splicing factors. J Cell Sci 107:387–396.

Broer S, Ji G, Broer A, Silver S (1993): Arsenic efflux governed by the arsenic resistance determinant of *Staphylococcus aureus* plasmid pI258. J Bacteriol 175:3480–3485.

Cochrane AW, Perkins A, Rosen CA (1990): Identification of sequences important in the nucleolar localization of human immunodeficiency virus Rev: Relevance of nucleolar localization to function. J Virol 64:881–885.

Dang CV, Lee WM (1989): Nuclear and nucleolar targeting sequences of c-erb-A, c-myb, N-myc, p53, HSP70, and HIV tat proteins. J Biol Chem 264:18019–18023.

De Vries L, Elenko E, Hubler L, Jones TL, Farquhar MG (1996): GAIP is membrane-anchored by palmitoylation and interacts with the activated (GTP-bound) form of G αi subunits. Proc Natl Acad Sci USA 93:15203–15208.

Fabrizio P, Laggerbauer B, Lauber J, Lane WS, Luhrmann R (1997): An evolutionarily conserved U5 snRNP-specific protein is a GTP-binding factor closely related to the ribosomal translocase EF-2. EMBO J 16:4092–4106.

Fricker M, Hollinshead M, White N, Vaux D (1997): Interphase nuclei of many mammalian cell types contain deep, dynamic, tubular membrane-bound invaginations of the nuclear envelope. J Cell Biol 136:531–544.

Gorlich D, Mattaj IW (1996): Nucleocytoplasmic transport. Science 271:1513–1518.

Graham J (1984): Isolation of subcellular organelles and membranes. In Rickwood D (ed): "Centrifugation." Oxford: IRL Press, pp 161–182.

Guiochon-Mantel A, Lescop P, Christin-Maitre S, Loosfelt H, Perrot-Applanat M, Milgrom E (1991): Nucleocytoplasmic shuttling of the progesterone receptor. EMBO J 10: 3851–3859.

Jackson MR, Nilsson T, Peterson PA (1990): Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J 9:3153–3162.

Kambach C, Mattaj IW (1992): Intracellular distribution of the U1A protein depends on active transport and nuclear binding to U1 snRNA. J Cell Biol 118:11–21.

Kaur P, Rosen BP (1992a): Mutagenesis of the C-terminal nucleotide-binding site of an anion-translocating ATPase. J Biol Chem 267:19272–19277.

Kaur P, Rosen BP (1992b): Plasmid-encoded resistance to arsenic and antimony. Plasmid 27:29–40.

Koonin EV (1993): A superfamily of ATPase with diverse functions containing either classical or deviant ATP-binding motif. J Mol Biol 229:1165–1174.

Kurdi-Haidar B, Aebi S, Heath D, Enns RE, Naredi P, Hom DK, Howell SB (1996): Isolation of the ATP-binding human homolog of the *arsA* component of the bacterial arsenite transporter. Genomics 36:486–491.

Laskey RA, Dingwall C (1993): Nuclear shuttling: The default pathway for nuclear proteins? Cell 74:585–586.

Le A, Steiner JL, Ferrell GA, Shaker JC, Sifers RN (1994): Association between calnexin and a secretion-incompetent variant of human alpha 1-antitrypsin. J Biol Chem 269:7514–7519.

Maeda Y, Hisatake K, Kondo T, Hanada K, Song CZ, Nishimura T, Muramatsu M (1992): Mouse rRNA gene transcription factor mUBF requires both HMG-box1 and an acidic tail for nucleolar accumulation: Molecular analysis of the nucleolar targeting mechanism. EMBO J 11:3695–3704.

Mandell RB, Feldherr CM (1990): Identification of two HSP70-related *Xenopus* oocyte proteins that are capable of recycling across the nuclear envelope. J Cell Biol 111:1775–1783.

McClay EF, Albright KD, Jones JA, Christen RD, Howell SB (1993): Tamoxifen modulation of cisplatin cytotoxicity in human malignancies. Int J Cancer 55:1018–1022.

Meier UT, Blobel G (1992): Nopp140 shuttles on tracks between nucleolus and cytoplasm. Cell 70:127–138.

Moreland RB, Nam HG, Hereford LM, Fried HM (1985): Identification of a nuclear localization signal of a yeast ribosomal protein. Proc Natl Acad Sci USA 82:6561–6565.

Pelham HR (1990): The retention signal for soluble proteins of the endoplasmic reticulum. Trends Biochem Sci 15:483–486.

Pinol-Roma S, Dreyfuss G (1992): Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. Nature 355:730–732.

Rosen BP, Hsu CM, Karkaria CE, Kaur P, Owolabi B, Tisa LS (1990): A plasmid-encoded aniontranslocating ATPase. Biochim Biophys Acta 1018:203–205.

Rosen BP, Dey S, Dou D, Ji G, Kau P, Yu M, Silver S, Wu J (1993): Evolution of an ion-translocating ATPase. Ann NY Acad Sci 671:257–272.

Schapp PJ, van't Riet J, Woldringh CL, Raue HA (1991): Identification and functional analysis of the nuclear localization signals of ribosomal protein L25 from *Saccharomyces cerevisiae*. J Mol Biol 221:225–237.

Schmidt-Zachmann MS, Nigg EA (1993): Protein localization to the nucleolus: A search for targeting domains in nucleolin. J Cell Sci 105:799–806.

Schmidt-Zachmann MS, Dargemont C, Kuhn LC, Nigg EA (1993): Nuclear export of proteins: The role of nuclear retention. Cell 74:493–504.

Silver S, Ji G (1994): Newer systems for bacterial resistances to toxic heavy metals. Environ Health Perspec Suppl 3 102:107–113.

Silver S, Ji G, Broer S, Dey S, Dou D, Rosen BP (1993): Orphan enzyme or patriarch of a new tribe: The arsenic resistance ATPase of bacterial plasmids. Mol Microbiol 8:637–642.

Siomi H, Shida H, Nam SH, Nosaka T, Maki M, Hatanaka M (1988): Sequence requirements for nucleolar localization of human T cell leukemia virus type I pX protein, which regulates viral RNA processing. Cell 55:197–209.

Sobue K, Fujio Y, Kanda K (1988): Tumor promoter induces reorganization of actin filaments and calspectin (fodrin or nonerythroid spectrin) in 3T3 cells. Proc Natl Acad Sci USA 85:482–486.

Soule HD, Maloney TM, Wolman SR, Peterson WD Jr, Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF, Brooks SC (1990): Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 50:6075–6086.

Spector DL, Fu XD, Maniatis T (1991): Associations between distinct pre-mRNA splicing components and the cell nucleus. EMBO J 10:3467–3481.

Taetle R, Jones OW, Honeysett JM, Abramson I, Bradshaw C, Reid S (1987): Use of nude mouse xenografts as preclinical screens. Characterization of xenograft-derived melanoma cell lines. Cancer 60:1836–1841.

Underwood MR, Fried HM (1990): Characterization of nuclear localizing sequences derived from yeast ribosomal protein L29. EMBO J 9:91–99.

Wada I, Rindress D, Cameron PH, Ou WJ, Doherty JJD, Louvard D, Bell AW, Dignard D, Thomas DY, Bergeron JJ (1991): SSR alpha and associated calnexin are major calcium binding proteins of the endoplasmic reticulum membrane. J Biol Chem 266:19599–19610.

Yan C, Melese T (1993): Multiple regions of NSR1 are sufficient for accumulation of a fusion protein within the nucleolus. J Cell Biol 123:1081–1091.

Zugmaier G, Knabbe C, Fritsch C, Simpson S, Ennis B, Lippman M, Dickson RB (1991): Tissue culture conditions determine the effects of estrogen and growth factors on the anchorage independent growth of human breast cancer cell lines. J Steroid Biochem Mol Biol 39:681–685.