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ARTICLES

Cell Cycle-Dependent Nuclear Location of the Matricellular Protein SPARC: Association With the Nuclear Matrix

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Abstract Secreted protein acidic and rich in cysteine (SPARC) is a matricellular protein that inhibits cellular adhesion and proliferation. In this study, we report the detection of SPARC in the interphase nuclei of embryonic chicken cells in vivo. Differential partitioning of SPARC was also noted in the cytoplasm of these cells during discrete stages of M-phase: cells in metaphase and anaphase exhibited strong cytoplasmic immunoreactivity, whereas cells in telophase were devoid of labeling. Immunocytochemical analysis of embryonic chicken cells in vitro likewise showed the presence of SPARC in the nucleus. Furthermore, elution of soluble proteins and DNA from these cells indicated that SPARC might be a component of the nuclear matrix. We subsequently examined cultured bovine aortic endothelial cells, which initially appeared to express SPARC only in the cytoplasm. However, after elution of soluble proteins and chromatin, we also detected SPARC in the nuclear matrix of these cells. Embryonic chicken cells incubated with recombinant SPARC were seen to take up the protein and to translocate it to the nucleus progressively over a period of 17 h. These observations provide new information about SPARC, generally recognized as a secreted glycoprotein that mediates interactions between cells and components of the extracellular matrix. The evidence presented in this study indicates that SPARC might subserve analogous functions in the nuclear matrix. J. Cell. Biochem. 74:152–167, 1999.

Key words: nucleus; osteonectin; nuclear matrix components; mitotic cycle; nuclear translocation; BM-40

Secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin and BM-40, is a Ca^{2+}-binding glycoprotein that was initially identified in bone. It was subsequently isolated from the culture medium of endothelial cells and from a basement membrane-producing tumor [reviewed in Lane and Sage, 1994]. In situ hybridization and immunocytochemical studies have shown that SPARC is expressed at high levels in embryonic tissues [Lane and Sage, 1994], as well as by invasive malignant tumors [Porter et al., 1995]. SPARC is also prominent in healing wounds, in areas of bone growth, and in regions of tissue morphogenesis, which correlates with its proposed function as a mediator of tissue remodeling [Reed et al., 1993; Iruela-Arispe et al., 1996, 1995; Reed and Sage, 1996; Wu et al., 1996]. Although the mechanisms by which it exerts its effects have not been fully elucidated, SPARC is generally characterized as a secreted counteradhesive protein that modulates the interaction of cells with components of the extracellular matrix, in part through disassembly of focal adhesion complexes [Sage et al., 1989; Murphy-Ullrich et al., 1995]. It is thus functionally similar to tenascin C and thrombospondin 1 [Murphy-Ullrich and Hook, 1989; Murphy-Ullrich et al., 1991; Sage and Bornstein, 1991]. Studies in vitro have demonstrated that SPARC mediates cell-matrix attachments by interaction with collagen (types I–V), thrombospondin 1, vitronectin [Lane and Sage, 1994; Rosenblatt et al., 1997], vascular endothelial growth factor (VEGF) [Kupprion et al., 1998], and platelet-derived growth factor (PDGF) [Raines et al., 1992]. Consistent with its proposed function as a modulator of cell-matrix interactions, SPARC has also been shown to affect the synthesis of certain matrix-related proteins. Exogenous SPARC, for example, promotes increased levels

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of mRNAs for type I plasminogen activator inhibitor (PAI-1) and several metalloproteinases, but decreased levels of fibronectin and thrombospondin 1 mRNAs [Hasselaar et al., 1991; Lane et al., 1992; Tremble et al., 1993]. Furthermore, SPARC introduced into the culture media of endothelial cells, smooth muscle cells, and fibroblasts attenuates cell cycle progression in G_1 [Funk and Sage, 1991, 1993; Sage et al., 1995].

SPARC is believed to control mRNA levels and synthesis of DNA through interaction with cognate membrane receptor(s)/binding proteins or through the antagonism of receptor-ligand recognition sites (e.g., integrin-collagen). Although there is evidence that intracellular signaling events that ultimately affect cell cycle and adhesion are mediated by a membrane-bound receptor [Yost and Sage, 1993], we consider the possibility that, in some cases, SPARC could exert its influence from within the cell, rather than from the extracellular matrix. Consistent with such an hypothesis are studies in vivo that have detected a predominance of intracellular SPARC in kidney, adrenal gland, liver [Mundlos et al., 1992; Porter et al., 1995], Leydig and Sertoli cells [Vernon and Sage, 1989], and retina [Yan et al., 1998], as well as in healing wounds [Reed et al., 1993]. By contrast, extracellular SPARC has been confined largely to bone matrices, organ capsules, and basement membranes [reviewed by Lane and Sage, 1994].

We have studied the expression of SPARC in embryonic chicken tissue and present evidence that the distribution of endogenous, intracellular SPARC varies according to specific stages of the mitotic cycle. A novel observation was the identification of SPARC within the nucleus of many interphase cells throughout the embryo. Subsequent in vitro investigation showed that SPARC exists not only in the nuclei of embryonic chicken cells, but also in those of bovine aortic endothelial (BAE) cells. Sequential extraction of soluble proteins and chromatin from the nuclei of these cells also showed that SPARC is an integral component of the nuclear matrix. In addition, when embryonic chicken cells were incubated in medium containing recombinant SPARC (rSPARC), the recombinant protein traversed the plasma membrane of many cells and was translocated to the nucleus, an observation that is particularly provocative in view of recent reports that other secreted proteins (e.g., lysyl oxidase and angiogenin) undergo a similar nuclear translocation [reviewed by Henderson, 1997].

SPARC is generally perceived as a secreted protein that functions extracellularly to modulate interactions between cells and the extracellular matrix. However, we present evidence that the signals generated by SPARC might not be limited to cell surface targets, but might also involve SPARC localized to the nuclear matrix. These observations thus contribute new insight into the mechanisms underlying the documented effects of SPARC on cell behavior.

MATERIALS AND METHODS

Preparation of Chicken Embryo Whole Mounts

Early chicken embryos at 48 h of development (2–19 somite stage) were isolated by the ring method of Drake et al. [1992] as follows. Overlying albumin was cleared from the exposed embryos and the surrounding yolk. A ring of Whatman 52 filter paper (inner and outer dimensions of 8 mm and 12 mm, respectively) was placed on the vitelline membrane to enclose the embryo and the surrounding area that gives rise to the extraembryonic vascular plexus. After at least 5 min to allow the ring to adhere to the vitelline membrane, the ring containing the embryo was excised around the outside rim. The embryo ring assemblies were flooded in situ with methyl Carnoy's fixative (60% methanol, 30% chloroform, and 10% glacial acetic acid), embedded in paraffin, sectioned to 8 mm, and processed for immunocytochemistry. Paraffin was dissolved in xylene, and the embryos were subsequently hydrated and rinsed in 0.1 M phosphate-buffered saline (PBS), pH 7.5. Endogenous peroxidase was blocked with 0.1% H_2O_2 for 15 min, and the tissue was subsequently rinsed in 0.1 M PBS. We used the ABC (avidin-biotin complex) Blocking Kit from Vector Laboratories (Burlingame, CA) to block endogenous avidin and biotin. The tissue was rinsed and incubated in 4% normal goat serum to block nonspecific labeling by the secondary antibody. A monoclonal anti-osteonectin primary antibody from Haematologic Technologies (Essex Junction, VT) was used at a concentration of 1:200 (5.3 mg/ml stock solution) for one h, followed by three rinses in 0.1 M PBS. An anti-mouse IgG_1 secondary antibody from Sigma Chemical Company (St. Louis, MO) was used at a concentration of 2.5 μg/ml for 30 min. After several rinses in PBS, the tissue was incubated with complexed avidin and biotin for 30 min

(ABC Kit, Vector Laboratories). The tissue was treated with diaminobenzidine and was counterstained with toluidine blue to demarcate the nuclei. Incubation with the conjugated secondary antibody in the absence of the primary antibody validated the specificity of the latter reagent.

Culture of Cells In Vitro and Immunocytochemistry

Chicken embryos at 2 days were removed from their Whatman paper frames, or at 10 days were isolated from their yolk sacs, and were rinsed in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY) containing 100 U/ml penicillin, 100 µg/ml streptomycin SO_4, and 2.5 µg/ml amphotericin-B (Sigma). Subsequent to rinsing in 2% ethylenediaminetetraacetic acid (EDTA), embryos were digested in a solution of 0.1% trypsin and 2% EDTA. The cell preparation was pelleted in DMEM with 10% fetal calf serum (FCS) and antibiotics, followed by resuspension in the DMEM/10% FCS supplemented with 100 ng/ml basic fibroblast growth factor (bFGF) [a gift from J. Abraham; Scios Corporation, Mountain View, CA]. Cells were subsequently plated on gelatin-coated coverslips and were incubated at 37°C in Medium 199 (Gibco-BRL) supplemented with 10% FCS, antibiotics, and 100 ng/ml bFGF. BAE cells were isolated according to the method of Sage et al. [1979] and were plated on coverslips. They were incubated at 37°C in DMEM supplemented with 10% FCS and antibiotics.

For immunocytochemistry, subconfluent cells were fixed briefly in 2% paraformaldehyde or methanol, rinsed, and blocked with 4% normal goat serum. Cells were incubated with either (1) the monoclonal anti-osteonectin IgG (Haematologic Technologies); (2) a guinea pig polyclonal IgG directed against synthetic peptide 2.3, corresponding to a sequence in the follistatin domain of SPARC [Iruela-Arispe et al., 1995; Hohenester et al., 1997]; (3) an anti-human bone osteonectin monoclonal IgG purified from mouse ascites fluid (Biodesign, Kennebunk, ME); or (4) a rabbit anti-murine SPARC polyclonal IgG (5944A) [Sage et al., 1989]. Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies from Sigma were used at 1:200.

Sequential Extraction of Intact Cells In Vitro

Cells plated on coverslips were rinsed twice in ice-cold PBS. Elution of soluble cytoplasmic and nuclear proteins was accomplished by a 3-min rinse in cytoskeleton (CSK) buffer (10 mM PIPES [piperazine-N,N-bis(2-ethanesulfonic acid)]/pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 4 mM vanadyl nucleoriboside complex [Sigma], 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF [Sigma]) containing 0.5% Triton X-100, at 4°C. Cells were fixed in 4% paraformaldehyde in CSK buffer for 40 min at 4°C and were rinsed extensively in cold CSK buffer, followed by incubation in DNA digestion buffer (10 mM PIPES/pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM AEBSF, and 200 mg/ml RNase-free DNase I [Promega, Madison, WI]) at 37°C for 50 min. Cells were rinsed twice in CSK buffer followed by 1 rinse in 250 mM ammonium sulfate. Cells were processed, as described above, for immunocytochemistry with anti-SPARC antibodies. Staining with diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) was performed to determine whether digestion of DNA was complete.

Cellular Uptake of SPARC In Vitro

Cells of early chicken embryos were dissociated and grown to semiconfluence on gelatin-coated coverslips as described above. rSPARC with a C-terminal tag of 6 histidines (His_6) [Bassuk et al., 1996] was incubated with the cultured cells. After various intervals of incubation with rSPARC, cells were fixed in 100% methanol and rinsed in PBS. Subsequently, they were incubated in 4% normal goat serum. Immediately after this step, the cells were incubated with an anti-His primary antibody (Invitrogen Scientific Products, St. Louis, MO) for 2 h, followed by incubation with an FITC goat anti-mouse IgG. Cells were counterstained with both BODIPY phalloidin (Molecular Probes) to delineate actin filaments, and DAPI, to distinguish DNA. Controls included (1) Sytox, a vital stain (Molecular Probes, Eugene, OR), applied to cells to verify that uptake of SPARC occurred in viable cells; (2) cell cultures devoid of rSPARC, processed with the anti-His-tag primary antibody and an FITC secondary antibody; (3) cells incubated with labeled rSPARC, processed for immunocytochemistry without the anti-His antibody.

Isolation and Purification of Cell Nuclei

BAE cells or cells from embryonic chickens were grown in vitro on 2% gelatin, passaged 1–12 times, and grown to semiconfluence. Cells were rinsed twice in cold PBS with 10 mM EDTA and incubated in cold PBS with 10 mM EDTA for 5 min. Cells were scraped into 14-ml tubes on ice and vortexed vigorously for 10 s. Cells were pelleted by centrifugation at 2,000 rpm for 3 min at 4°C. The pelleted cells were suspended in cold nuclear isolation buffer (0.65% Nonidet P-40, 10 mM Tris HCl/pH 7.8, 150 mM NaCl, 1.5 mM $MgCl_2$), vortexed vigorously for 10 s, and kept on ice for 1 min. A total of 10 µl of the nuclear suspension was placed on a slide and examined under an inverted-phase microscope to determine whether nuclei were free of cytoplasmic debris. The nuclei were subsequently pelleted by centrifugation (2,000 rpm) at 4°C. The supernate (containing the cytoplasmic fraction) was precipitated in 9 vol cold acetone and was collected by centrifugation at 3,000 rpm for 20 min, and at 4°C. The cytoplasmic precipitates and pelleted nuclei were solubilized in 2× electrophoresis buffer (2% sodium dodecyl sulfate [SDS], 0.1 M Tris-HCl, 20% glycerol, 1 M urea; pH 6.8) containing protease inhibitors. A stock solution of 1 tablet of Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN) in 2 ml dH_2O was used at a ratio of 1:25.

Western Blot Analysis of Purified Nuclei from Embryonic Chicken and BAE Cells

Nuclear and cytoplasmic isolates were suspended in SDS sample buffer and were boiled for 5 min with or without 50 mM dithiothreitol (DTT). The samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to nitrocellulose membranes. The blots were blocked with 5% nonfat dry milk and 0.1% Tween-20. Blots were subsequently incubated with anti-SPARC antibodies and appropriate secondary antibodies. Immunoreactivity was visualized by enhanced chemiluminescence (Amersham Technologies; Arlington Heights, IL). Nuclear preparations were evaluated for purity by exposure to an antibody specific for the α-subunit of prolyl 4-hydroxylase [Bassuk et al., 1989], an enzyme within the lumen of the endoplasmic reticulum.

RESULTS

Immunolocalization of SPARC Within the Nuclei of Embryonic Chicken Cells

Initial experiments on sections of early chicken embryos incubated with anti-SPARC IgG showed staining in the nuclei of cells throughout both embryonic and extraembryonic regions (Fig. 1A). Nuclear localization of SPARC was prominent in many cells within (1) the neural tube, (2) the cephalic region, (3) the somites, (4) the area opaca, and (5) the vascular endothelium of the area pellucida. The staining within nuclei was punctate and was never evident within the nucleolus. Immunoreactivity for SPARC was also particularly strong near the nuclear lamina in a few of the cells, especially those of the neural tube (Fig. 1B) and somites. At no time, within the same cell, did nucleoplasmic labeling by anti-SPARC IgG occur concomitantly with labeling at the nuclear lamina. Cells in interphase exhibited comparatively low levels of SPARC in the cytoplasm, whereas the cytoplasm of cells in metaphase and anaphase was highly immunoreactive (Fig. 1C). In contrast, cells in telophase were devoid of cytoplasmic immunolabeling (Fig. 1D). These observations were consistent in cells throughout all embryos.

Omission of primary antibodies produced no staining reaction in vivo or in vitro. We also tested the fidelity of the monoclonal IgG from Haematologic Technologies, which is claimed to recognize domain 1 of SPARC, by absorption of the IgG with synthetic peptides corresponding to sequences within the first domain of SPARC. Immunoreactivity was negligible when the anti-SPARC IgG was preabsorbed with a synthetic peptide corresponding to amino acids 4–24 (peptide 1.1) of SPARC (Fig. 1E). However, when the anti-SPARC antibody was incubated with peptides corresponding to amino acids 16–35 (peptide 1.2) (Fig. 1F) or amino acids 32–51 (peptide 1.3) [not shown], immunoreactivity was comparable in intensity to sections exposed to the anti-SPARC antibody alone. These results confirmed the specificity of the monoclonal antibody. Of the four antibodies used in these experiments, three detected SPARC within nuclei: a mouse anti-human monoclonal antibody from Haematologic Technologies, a guinea pig polyclonal antibody against synthetic peptide 2.3 from the follistatin domain of SPARC [Iruela-Arispe et al., 1995] (not shown), and an anti-

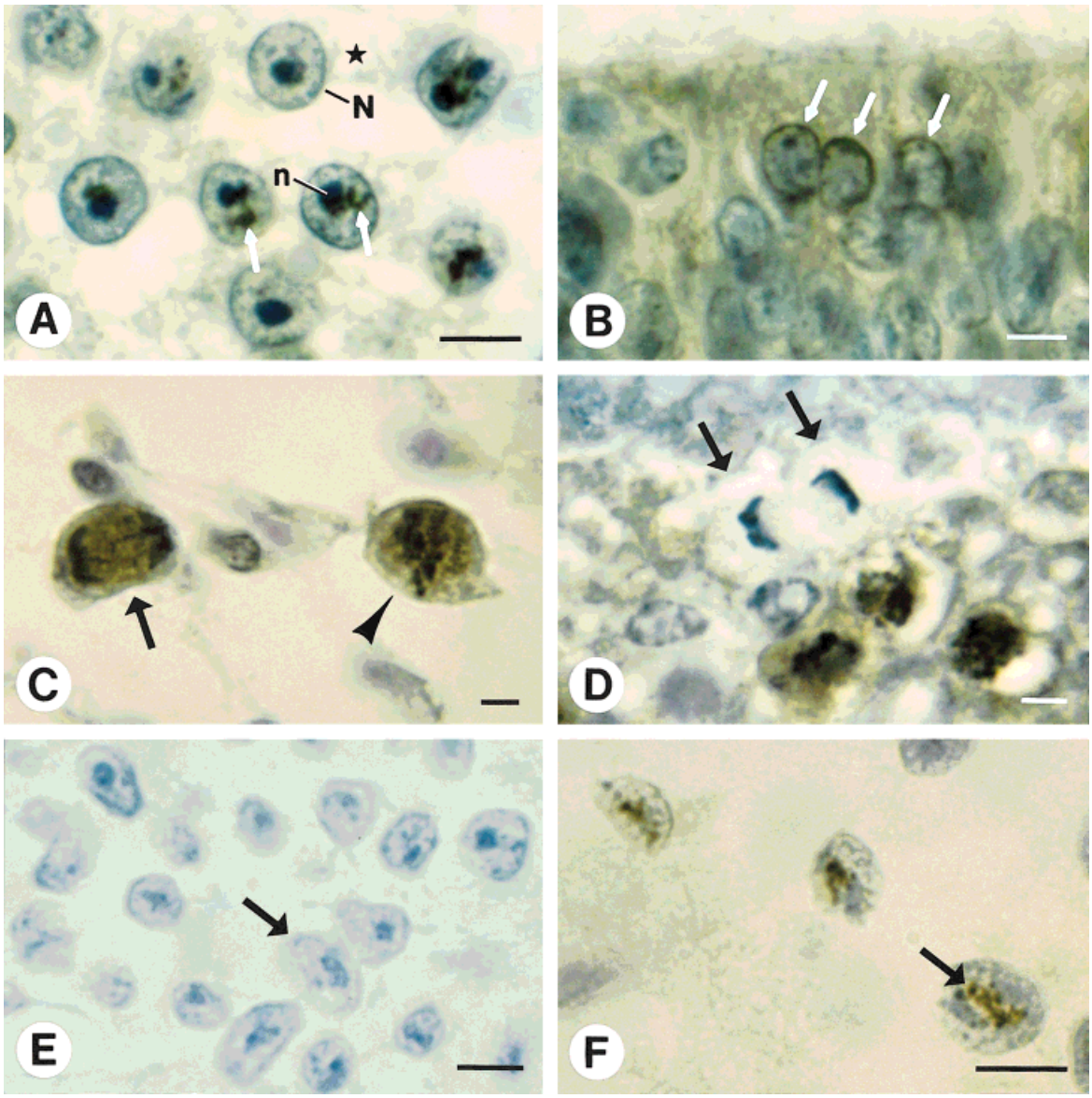

Fig. 1. SPARC is expressed at specific stages of the mitotic cycle in early-stage chicken embryos. **A:** After incubation with a monoclonal anti-SPARC/osteonectin IgG from Haematologic Technologies, cells in interphase exhibited SPARC immunoreactivity (arrows) within nuclei stained with toluidine blue; the cytoplasm (asterisk) of cells was devoid of staining. These cells were located in the primitive streak region but were representative of cells throughout the embryo. **B:** Before rounding and subsequent docking at the lumen, cells of the neural tube exhibited SPARC immunoreactivity in the region of the nuclear lamina (arrows). **C:** Cells of the area pellucida displayed strong immunoreactivity with anti-SPARC IgG during metaphase (arrowhead) and anaphase (arrow). **D:** A cell undergoing cytokinesis in the neural tube was devoid of SPARC immunoreactivity (arrows). **E:** Labeling was attenuated (arrow) when the anti-SPARC IgG was absorbed with a synthetic peptide (1.1) that corresponds to the N-terminal region of SPARC. **F:** Conversely, immunolabeling was not blocked (arrow) when the anti-SPARC IgG was incubated with a peptide (1.2) that corresponds to a different sequence of similar charge and composition in SPARC. N, nucleus; n, nucleolus. Scale bars = 10 μm.

human bone osteonectin antibody that was purified from mouse ascites fluid (Biodesign). A rabbit polyclonal antibody against murine SPARC, 5944A, exhibited cytoplasmic but not nuclear staining.

Analysis of embryonic chicken cells in vitro yielded results consistent with those in vivo, with the possible exception that cells undergoing cytokinesis in vitro exhibited labeling of the cytoplasm. Typical of cells in vivo, however,

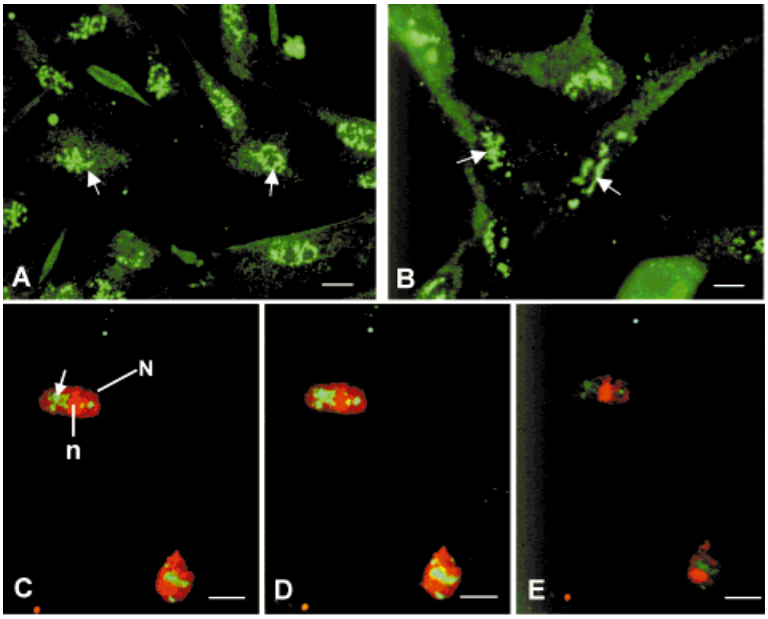

Fig. 2. Expression of SPARC is prominent in nuclei of cultured embryonic chicken cells. Nuclei were stained with a monoclonal anti-SPARC IgG (arrows) from Haematological Technologies (**A**) and by an anti-SPARC monoclonal antibody from Biodesign (arrows) (**B**), followed by FITC secondary antibodies. A series of images on the Z axis by confocal microscopy confirms that SPARC is present throughout the nucleus (**C,D,E**). SPARC is labeled with fluorescein (green arrows); chromatin is stained with propidium iodide (red). Micrographs proceed from the top surface of the nucleus (**C**). **D:** Approximate midline of the nucleus. N, nucleus; n, nucleolus. Scale bars = 10 μm.

2 cultured embryonic cells exhibited nuclear labeling that was not restricted to one cell type. SPARC detected within the cytoplasm of cultured cells appeared minimal and diffuse, whereas nuclear immunoreactivity appeared to be highly concentrated and predominant (Fig. 2A,B).

To obtain a more definitive localization of SPARC within the nucleus, we sequentially extracted soluble proteins and chromatin from cross-linked nuclei, before immunocytochemical analyses. This extraction technique preserves the integrity of the matrix and thus permits a more accurate assessment of nuclear components [Nickerson et al., 1997; Nickerson and Penman, 1992; Fey et al., 1984] (Fig. 3). The nuclear matrix consists of an outer tegument of various proteins and of an inner core of dense matter composed of proteins and heterogeneous nuclear RNA (hnRNA) [He et al., 1990]. The objective of this experiment was to determine whether SPARC is a detergent-soluble constituent of the nucleoplasm or a component of the nuclear matrix. For removal of soluble proteins, cells plated on coverslips were treated with CSK buffer containing Triton X-100 (Fig. 3A). The remaining detergent-insoluble structures were cross-linked with paraformaldehyde

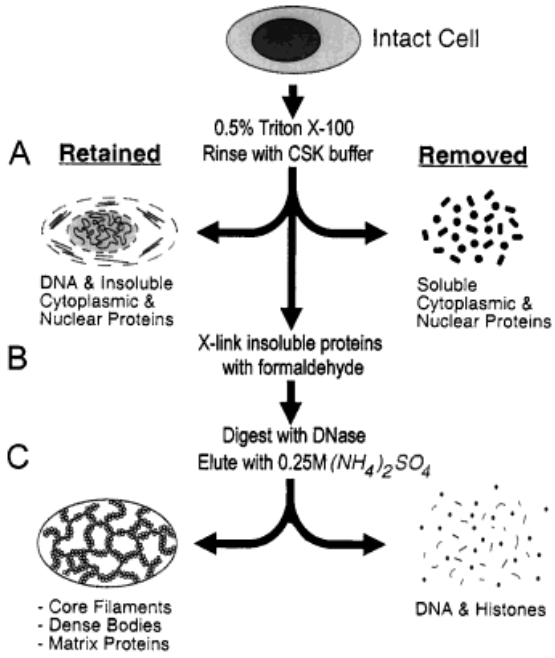

Fig. 3. Extraction of cellular components for localization of nuclear SPARC. **A:** Detergent-soluble proteins were removed by incubating cells in CSK buffer containing Triton X-100. **B:** The remaining cellular proteins were crosslinked by 4% paraformaldehyde. **C:** DNA was removed by DNase I digestion. This treatment yielded intact, cross-linked nuclear matrices that were analyzed by immunocytochemistry.

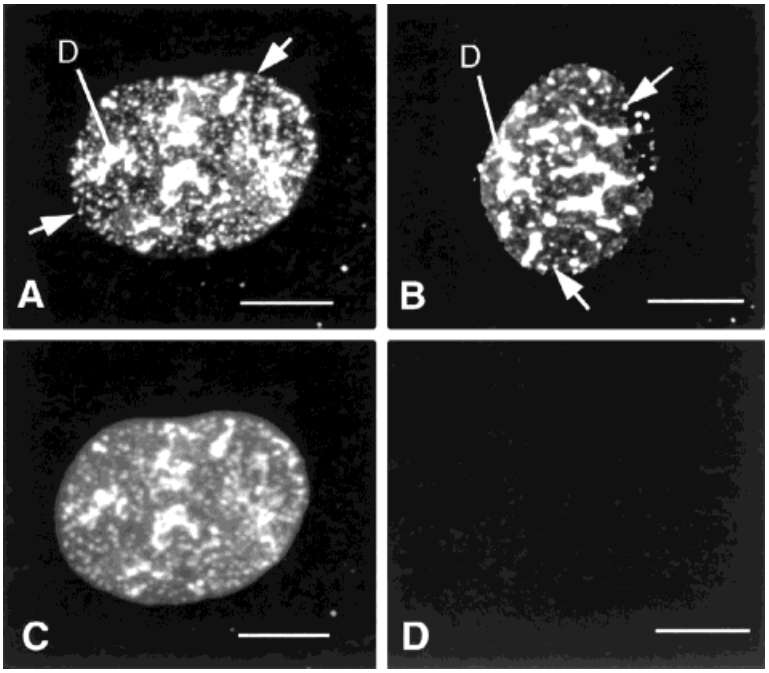

Fig. 4. SPARC was shown in the nuclear matrix after solubilized proteins were eluted from cultured embryonic chicken cells (**A**). Staining with a monoclonal IgG (Haematologic Technologies) consisted of small punctate dots (arrows) and larger granular densities. **B:** Labeling of densities and punctate matter (arrow) was still evident after removal of chromatin. **C:** Chromatin was apparent in nuclei from which soluble proteins had been eluted, as seen in this double exposure of DAPI staining and SPARC immunolabeling. **D:** The absence of DAPI staining after treatment with DNase confirmed that chromatin removal was complete. D, dense bodies. Scale bars = 5 μm.

(Fig. 3B). Chromatin was subsequently digested with DNase-I and was eluted with CSK buffer containing 250 mM ammonium sulfate (Fig. 3C). According to Fey et al., [1984] this treatment would be expected to yield intact, cross-linked nuclear matrices composed of thick polymorphic fibers. Immunocytochemical analysis subsequent to the removal of soluble proteins revealed that SPARC was present within the nucleus as a detergent-insoluble fraction (Fig. 4A). Moreover, such labeling was retained after the digestion of chromatin by DNase. SPARC thus appeared to be a component of the nuclear matrix (Fig. 4B). SPARC expressed in the nuclear matrix exhibited a pattern of punctate spots and granular densities that extended throughout the nucleus. As observed in vivo, the nucleoli of cultured embryonic chicken cells expressed no immunoreactivity with SPARC IgGs. To confirm that the nuclear matrix was free of residual chromatin, we used DAPI (a chromatin stain) on both DNase-treated and untreated cells [Nickerson et al., 1997]. DAPI was retained in nuclei from which soluble proteins had been eluted (Fig. 4C), whereas nuclei treated with DNase were devoid of DAPI staining (Fig. 4D).

SPARC Immunoreactivity in BAE Cells

We wanted to ascertain whether SPARC was associated with the nuclear matrix of cells derived from other species. The adult BAE cell was a compelling candidate, inasmuch as these cells produce copious amounts of SPARC in response to culture shock [Sage et al., 1986]. Furthermore, the bovine represents a class divergent from the avian and could thus contribute insight into the scope and specificity of SPARC expression in the nuclear matrix.

Previous efforts in this laboratory to determine the subcellular distribution of SPARC in BAE cells proved difficult because the strong labeling of cytoplasmic SPARC obscured visualization of the nucleus (Fig. 5A). We were therefore interested in examining the distribution of SPARC immunoreactivity after the elution of soluble proteins and the subsequent removal of chromatin. Cells subjected to protein extraction procedures exhibited punctate SPARC immunoreactivity throughout the cytoplasm, with more pronounced labeling both around and within nuclei (Fig. 5B). Elution of chromatin demonstrated further that SPARC was indeed associated with the nuclear matrix of this cell type

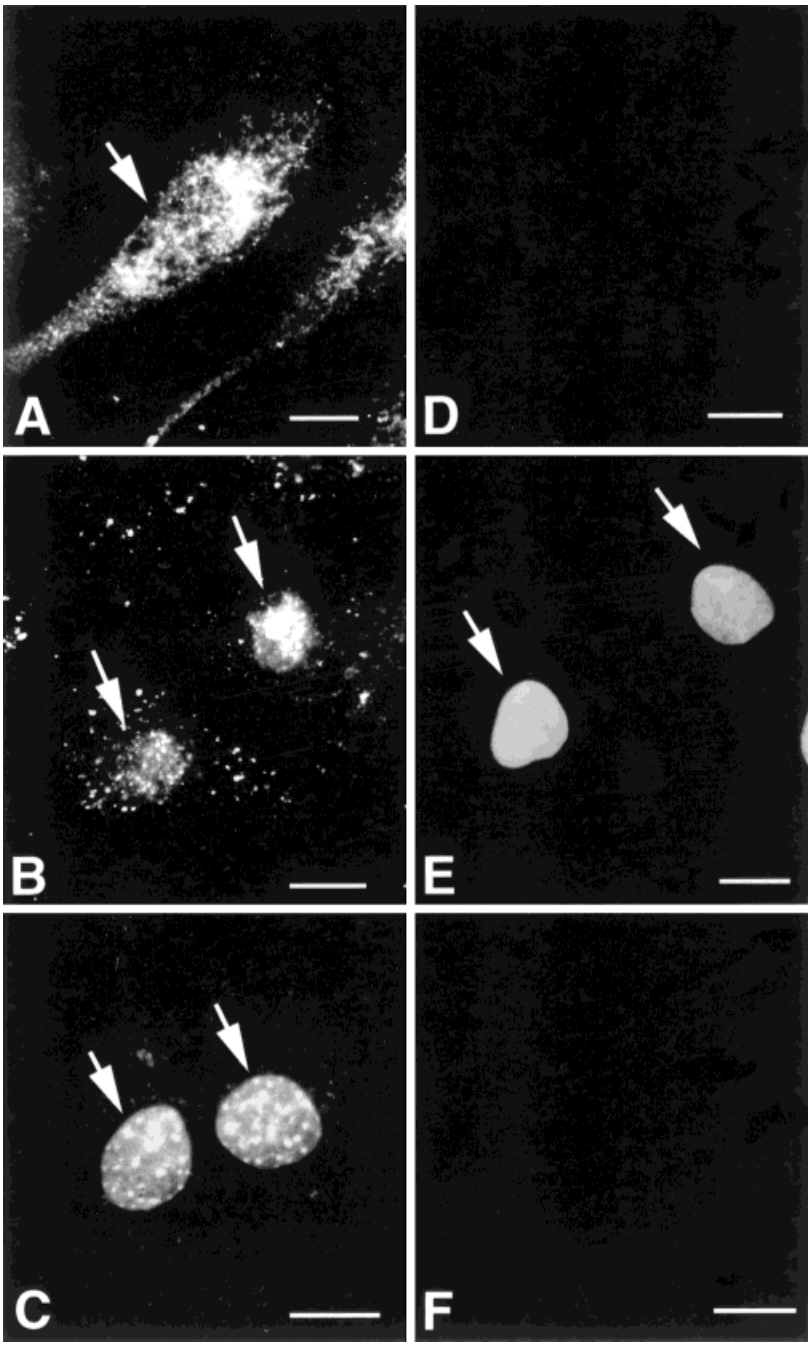

Fig. 5. SPARC is associated with the nuclear matrix of endothelial cells. **A:** BAE cells expressed strong cytoplasmic immunoreactivity with anti-SPARC IgG (Haematologic Technologies) (arrows) that obscured visualization of nuclei. **B:** Cells that were treated with detergent displayed cytoplasmic and somewhat nebulous nuclear staining by anti-SPARC IgG (arrows). **C:** Nuclei in which chromatin had been removed exhibited granular staining (arrows). **D:** Labeling was at background levels in cells that had been treated with DNase and processed without the primary anti-SPARC IgG. **E:** The nuclei in B exhibited strong chromatin staining, as detected by DAPI (arrows). **F:** Conversely, the DNase-treated nuclei of panel C were devoid of DAPI staining. Scale bars = 10 μm.

(Fig. 5C). The pattern of SPARC expression in BAE cells was reminiscent of that observed in the embryonic chicken nucleus, which exhibited similar punctate structures and dense bodies. Control cells, in which the primary anti-SPARC antibody was omitted, expressed only background levels of reactivity (Fig. 5D). Cells treated with DNase-I were devoid of staining after treatment with DAPI, whereas cells from which solubilized proteins were extracted exhibited strong staining for chromatin (cf. Fig. 5E and F).

Western Blot Analysis of Purified Nuclear Extracts

To confirm that the protein detected by immunocytochemical analyses was indeed SPARC, we probed both cytoplasmic and nuclear fractions of embryonic chicken cells and BAE cells by Western blotting. The antibody from Haematologic Technologies identified a single 43-kD band in the nuclear fraction of chicken cells, whereas the cytoplasmic fractions expressed doublets of approximately 43 kD (Fig. 6A). The shift in relative molecular weight with reduction of disulfide bonds is characteristic of the mobility of SPARC on SDS-PAGE. A monoclonal antibody from Biodesign detected a diffuse 43-kD band in both nuclear and cytoplasmic fractions derived from embryonic chicken cells (Fig. 6B). Both nuclear and cytoplasmic fractions of BAE cells expressed single bands at 43 kD, as detected by the anti-SPARC IgG from Haematologic Technologies and the monoclonal anti-SPARC antibody from Biodesign, respectively (Fig. 6C). These molecular weights correspond to those reported for SPARC on SDS-PAGE after reduction of disulfide bonds. SPARC derived from a murine teratocarcinoma cell line served as the positive control (SP, Fig. 6A,B).

To address the possibility that nuclear fractions were contaminated with SPARC from the

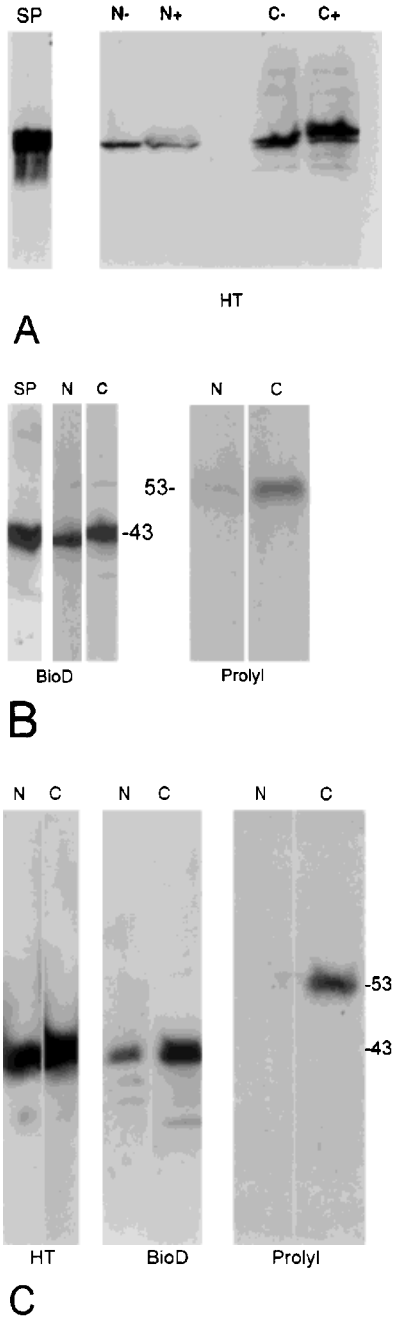

Fig. 6. Western blot analyses of nuclear (N) and cytoplasmic (C) extracts prepared from embryonic chicken cells (A,B) and bovine aortic endothelial (BAE) cells (C). Fractions were probed with either the anti-SPARC IgG from Biodesign (BioD) or a mouse anti-SPARC IgG from Haematologic Technologies, Inc. (HT). SPARC (SP) derived from murine teratocarcinoma cells (43 kD) served as the positive control. Nuclear and cytoplasmic fractions were also probed with an antibody specific for the α-subunit of prolyl 4-hydroxylase (Prolyl). **A:** Embryonic chicken cell nuclei (reduced, N+; nonreduced, N−) exhibited 43-kD bands when probed with the HT IgG. Cytoplasmic extracts (reduced, C+; nonreduced, C−) expressed doublets of approximately 43 kD when probed with the HT IgG. **B:** Nuclear and cytoplasmic extracts from embryonic chicken cells produced bands of 43 kD when probed with the IgG from Biodesign. Probing the nuclear and cytoplasmic fractions for the presence of prolyl 4-hydroxylase confirmed that the nuclear fractions were devoid of cytoplasmic contamination. **C:** BAE cell extracts were probed with the HT and the BioD antibodies. Prominent 43-kD bands were apparent in both nuclear and cytoplasmic fractions. Prolyl 4-hydroxylase immunoreactivity appeared exclusively in the cytoplasmic fractions. Fractions were reduced with DTT (except where noted) and were resolved on 4–20% gradient polyacrylamide gels.

endoplasmic reticulum, we used an antibody against the α-subunit of prolyl-4-hydroxylase [Bassuk et al., 1989] to probe nuclear fractions on Western blots. As shown in Fig. 6B and 6C, the cytoplasmic fractions exhibited reactivity with the anti-α-subunit antibody (Prolyl) at M_r 53,000. Conversely, the nuclear extracts were essentially devoid of this subunit, which resides nearly exclusively in the endoplasmic reticulum, because of its association with protein disulfide isomerase [Bassuk and Berg, 1989]. These results verify that our nuclear fractions were relatively free of SPARC from the endoplasmic reticulum and that the SPARC detected was derived from the nuclear compartment. In addition, 4–20% gradient gels were used to show that the histones were present exclusively in the nuclear extracts (data not shown).

Uptake of Recombinant SPARC into Nuclei of Embryonic Chicken Cells

In view of reports that several secreted proteins undergo endocytosis and subsequent translocation to the nucleus, we wanted to ascertain whether SPARC displayed a similar behavior. We therefore incubated cultured embryonic chicken cells in media containing 15 μg/ml rSPARC. The immunolocalization of rSPARC was tracked, at various time points, by an anti-His antibody that recognizes the His_6 tag of the recombinant protein. The results demonstrated that rSPARC traversed the plasma membrane of most cells, before 15 min, and that it was subsequently distributed throughout the cytoplasm (Fig. 7A). The location of rSPARC at 30 min was predominantly cytoplasmic, although a substantial amount of rSPARC was evident within the nucleus of many cells. Thereafter, the concentration of rSPARC within nuclei increased incrementally, as seen at 1 h (Fig. 7B), 3 h (not shown), 5 h (Fig. 7C), and 17 h (Fig. 7D).

Control experiments verified that rSPARC was transported across the plasma membrane and ultimately entered the nucleus: (1) Sytox, a vital stain, confirmed that labeled cells were viable; (2) cell cultures devoid of rSPARC were processed with the anti-His primary antibody and a fluoresceinated secondary antibody; an absence of labeling confirmed that the reaction product was not an artifact of the primary antibody (Fig. 7E); and (3) an absence of labeling was noted when the primary antibody was omitted from immunocytochemical procedures; thus, the reaction product was not an artifact contributed by the secondary antibody (Fig. 7F).

DISCUSSION

Three conclusions can be drawn from the experiments described in this study: (1) extraction of nuclear chromatin from the cells of two diverse classes, avian and bovine, indicated that SPARC is a component of the nuclear matrix during interphase; (2) immunocytochemical analyses of embryonic chicken cells in vivo demonstrated that the expression of intracellular SPARC is correlated with specific stages of the cell cycle; and (3) extracellular rSPARC is taken up and is translocated to the nuclei of cultured embryonic chicken cells.

SPARC and the Nuclear Matrix

We wanted to determine whether SPARC existed in a soluble state within the nucleoplasm or was a component of the nuclear matrix. The protocols of Nickerson et al. [1997] produced nuclear matrices composed of detergent-insoluble proteins clustered around the 9- to 13-nm dense core [as defined by He et al., 1990]. Immunocytochemical analyses subsequently showed that SPARC was associated with the nuclear matrix in both embryonic chicken and adult BAE cells.

The detection of SPARC in the nuclear matrix is especially provocative, considering the central role of this structure in cellular biology. In recent years, investigators have both defined the nature of the nuclear matrix [Penman, 1995; Nickerson and Penman, 1992; Dworetzky et al., 1990; Berezney and Coffey, 1975] and compiled a body of evidence establishing the prominent role of this structure in both gene transcription and cell cycle regulation [Stein et al., 1996, 1998; Scovassi et al., 1997; Ornelles and Penman, 1990; Chaly et al., 1984]. Far from being static in nature, the nuclear matrix is a highly dynamic structure into which specific complexes of protein are incorporated, according to the biological state of the cell [reviewed by Stein et al., 1998, and by Nickerson, 1998; Ornelles and Penman, 1990]. The nuclear matrix is the scaffolding on which steroid recep-

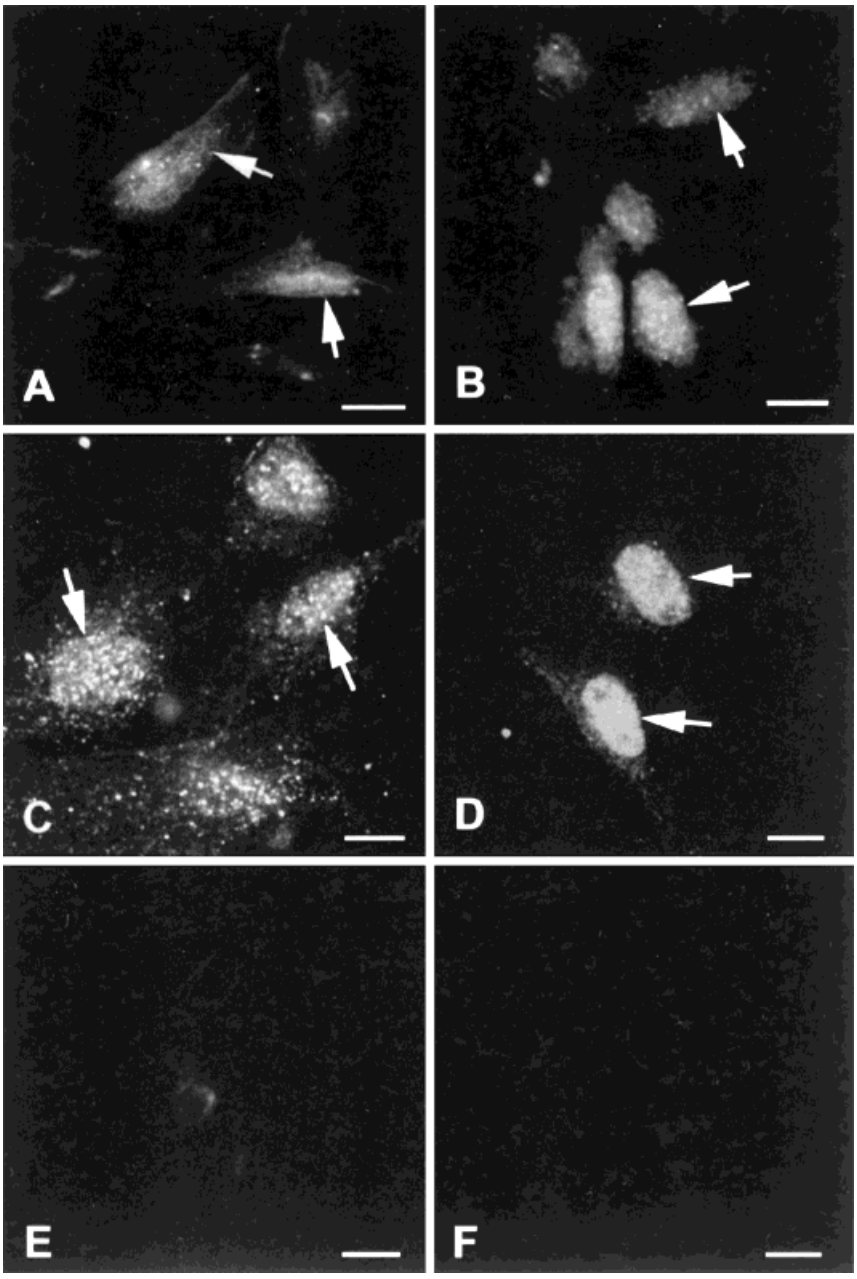

Fig. 7. Extracellular SPARC is translocated to the nucleus of embryonic chicken cells in vitro. Recombinant (r) SPARC, containing a C-terminal His_6, was incubated with cells for (**A**) 15 min, (**B**) 1 h, (**C**) 5 h, and (**D**) 17 h; rSPARC was visualized by an anti-His antibody. **E:** Control cultures, in which rSPARC was omitted, were also incubated with the anti-His antibody. **F:** Cells were incubated with rSPARC for 17 h and were subjected to immunocytochemical analysis in which the primary antibody was omitted. Scale bars = 10 μm.

tors bind, chromatin loop attachments are stabilized, and DNA replication occurs. For example, the retinoblastoma gene product (pRb) inhibits DNA replication through association with the nuclear matrix during specific stages of the cell cycle, as a function of its phosphorylation state [Mancini et al., 1994]. Mutations that abrogate either the binding of pRb to the nuclear matrix (or the binding of matrix-associated oncoproteins to pRb) are hypothesized to result in uncontrolled proliferation and tumorigenesis. In addition to its involvement in cell cycle control, there is also evidence that the nuclear matrix regulates transcription by serving as a repository for transcriptional regulatory factors. In support of this concept, Bidwell et al.

[1994] compared the nuclear matrix proteins of normal rat osteoblasts with those of ROS 17/2.8 cells and detected classes of proteins that were exclusive to normal or transformed cells. The protein content of the nuclear matrix is thus a potential tool for the diagnosis of some forms of cancer [reviews by Nickerson, 1998; Replogle-Schwab et al., 1996]. In conjunction with the localization of regulatory proteins to the nuclear matrix, actively transcribed genes are also selectively relegated to this structure. Ciejek et al. [1983] demonstrated that the ovalbumin gene was preferentially associated with the nuclear matrix upon hormone stimulation, whereas withdrawal of the hormone terminated this association.

In view of the data presented in this report, we consider it possible that SPARC belongs to a growing roster of nuclear matrix proteins that have been shown to influence both DNA synthesis and levels of mRNA. This concept is compatible with mechanisms currently attributed to SPARC, especially those associated with the replication of DNA [Funk and Sage, 1991, 1993] and the regulation of mRNAs for PAI-1, fibronectin, thrombospondin 1, and certain matrix metalloproteinases [Murphy-Ullrich et al., 1995; Lane et al., 1992; Tremble et al., 1993; Hasselaar et al., 1991]. It is therefore conceivable that SPARC acts both extracellularly (to influence cell-matrix interactions) as well as intracellularly (to influence cell cycle dynamics and synthesis of certain secreted proteins).

SPARC and the Cell Cycle

In the embryonic chicken, the intracellular expression of SPARC in vivo was correlated with specific stages of the cell cycle. Cells in interphase displayed comparatively lower levels of labeling within the cytoplasm, whereas the cytoplasm of cells in metaphase and anaphase was highly immunoreactive. Moreover, clusters of cells in interphase exhibited either punctate labeling within nuclei or laminar staining contiguous with the nuclear membrane. Interestingly, cells in telophase were devoid of immunolabeling.

The cytoarchitecture of the neural tube is conducive to assessment of cell cycle progression, because the cells of this structure reposition their nuclei as they progress through the cell cycle [Burt, 1993]—a phenomenon that allowed the expression of SPARC to be temporally monitored in the embryonic chicken. Figure 8 represents one possibility that seems particularly compatible with our observations of SPARC expression in cells of the neural tube. The cytoplasm of each cell within the neural tube extends from the abluminal to the luminal side of this structure. As the cell progresses through the mitotic cycle, its nucleus migrates toward the lumen. Once the nucleus docks near the lumen, the cell becomes round, the nuclear lamina depolymerizes, and the cell enters M-phase. The spatiotemporal distribution of SPARC in the neural tube of the chicken embryo thus permits certain inferences to be made concerning SPARC and the cell cycle. First, the punctate intranuclear expression of SPARC (Fig. 8A–C) occurs before its appearance at the nuclear lamina (Fig. 8D). Second, the shift in SPARC immunoreactivity to the nuclear lamina is manifested when the nucleus is contiguous with the area of the neural tube that accommodates the breakdown of the lamina in prophase. In view of these observations, it seems plausible that SPARC resides in the nucleus during interphase; as the cell approaches M-phase, SPARC translocates to the nuclear lamina (Fig. 8D). As the cell enters M-phase, the nuclear membrane breaks down and SPARC disperses throughout the cytoplasm (as do other nuclear proteins, such as transcription factors and nuclear lamins). Immunoreactivity for SPARC is apparent in the cytoplasm from metaphase (Fig. 8E) through anaphase (Fig. 8F), but it is

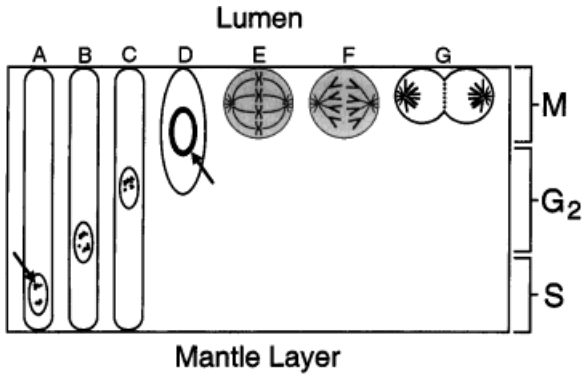

Fig. 8. A–C: Nuclear expression of SPARC (arrow) in the neural tube of the embryonic chicken occurs before its appearance at the nuclear lamina. **D:** SPARC immunoreactivity shifts to the nuclear lamina (arrow) when the nucleus is contiguous with the area of the neural tube that accommodates the breakdown of the lamina in prophase. Immunoreactivity for SPARC is manifested in the cytoplasm from metaphase **(E)** through anaphase **(F)** but is not detected when the cell enters telophase **(G)**. Stages of the cell cycle are represented on the left side as S, synthesis phase; G_2, gap-2 phase; and M, mitotic phase.

strikingly absent when the cell enters telophase (Fig. 8G). The disappearance of SPARC immunoreactivity during cytokinesis is an interesting phenomenon that could be explained relative to the disposition of other nuclear proteins during this phase of the cell cycle. It is recognized that, during telophase, nuclear proteins bind to the chromosomes and are subsequently sequestered from the cytoplasm as the lamins coalesce around the chromosomes. The lack of SPARC immunoreactivity during this later stage of cytokinesis could therefore be due to the trapping and masking of SPARC within the reconstituting nuclear membrane.

Nuclear Translocation of SPARC

Recombinant SPARC, derived from *Escherichia coli,* translocates to the nucleus in vitro. It thus appears to be analogous to other proteins that are secreted and subsequently taken up by the cell and transported to the nucleus (e.g., lysyl oxidase, insulin, bFGFs) [Henderson, 1997; R. Li et al., 1997; Shah et al., 1995; Mello et al., 1995; Moroianu and Riordan, 1994]. This discovery is especially interesting, given an earlier investigation by Funk and Sage [1991] that demonstrated the abrogation of the G1- to S-phase transition when SPARC was introduced into the medium of BAE cells. That exogenous SPARC inhibited ^{3}H-thymidine incorporation into DNA during S-phase prompts further questions regarding the role of SPARC in the cell cycle. For example, did the exogenous SPARC perturb the G1/S transition by initiation of a receptor-mediated cascade at the cell surface? Alternatively, could SPARC have entered the cell via endocytosis and perturbed DNA synthesis at the level of the nuclear matrix?

A number of secreted proteins have recently been shown to mediate effects at the nuclear level [Henderson, 1997]; these include FGFs (and their receptors), portions of chondroitin sulfate and heparan sulfate proteoglycans, insulin [Shah et al., 1995], lysyl oxidase [W. Li et al., 1997], and angiogenin [R. Li et al., 1997; Moroianu and Riordan, 1994]. Several of these proteins are considered bifunctional, in the sense that they can induce intracellular signaling via a cell-surface receptor, as well as through a second pathway involving endocytosis, nuclear translocation, and direct association with nuclear substrates [Henderson, 1997]. Angiogenin, for example, is a secreted potent inducer of angiogenesis that initiates transduction of intracellular signaling cascades and thereby affects cell migration, differentiation, and proliferation. For angiogenin to induce angiogenesis, both signal transduction at the plasma membrane and nuclear translocation must occur [Moroianu and Riordan, 1994]. In addition, insulin binds to receptor tyrosine kinases and initiates signal transduction at the cell surface, but there is also evidence that the insulin-receptor complex is taken up through endocytosis and accumulates in the nucleus [Shah et al., 1995]. Lysyl oxidase, a secreted protein that cross-links lysine residues on extracellular collagen and elastin, is taken up via endocytosis and subsequently translocates to the nucleus of transformed cells, where it represses the oncogenic activity of *ras* through its effect on the organization of chromatin [Mello et al., 1995]. This enzyme was also recently discovered in the nuclei of rat vascular smooth muscle cells and 3T3 cells [W. Li et al., 1997].

For a secreted protein to enter the nucleus, it translocates to the nuclear pore by virtue of specific amino acid sequences, called nuclear localization signals (NLS). Such signal sequences are usually characterized by clusters of either basic or acidic residues that are recognized by cognate sequences embedded in cytoplasmic transporter proteins [Gorlich and Mattaj, 1996; reviewed by Nigg, 1997, reviewed by Boulikas, 1996]. Sequence analysis of mouse SPARC by Mason et al. [1986] reported basic amino acid clusters at positions 56–59 (KHGK), 113–117 (KKGHK), and 169–176 (KQKLRVKK) that could potentially be recognized by cognate acidic residues on cytoplasmic transporter proteins. Alignment of chicken SPARC with that of mouse exhibited 100% identity with the sequences KHGK and KKGHK, whereas the mouse sequence KQKLRVKK was slightly different in chicken SPARC (KQKLKVKN) [Bassuk et al., 1993]. Interestingly, the KKGHK sequence is also present in RIP 140, a nuclear protein that interacts with the estrogen receptor and is proposed to be a general initiator of hormone-associated transcription [Cavailles et al., 1995]. The KQKLKN sequence of chicken SPARC corresponds to a classic NLS, KXKXK [Boulikas, 1996]. There are also numerous acidic sequences in mouse SPARC that could complex with the basic constructs of transporter proteins, e.g., amino acids 9–15 (EEIVEEE) and 30–44 (EMGEFEDGAEETVEE) in domain 1;

there are homologous sequences of similar charge in chicken SPARC.

The expression of nuclear SPARC in vivo establishes that its presence within the nucleus is physiologically relevant and is not merely a result of culture shock [Sage et al., 1986]. SPARC is a component of the nuclear matrix in highly proliferating systems, such as embryonic avian and cultured adult BAE cells, implying that the protein subserves roles in development as well as in cell cycle control. The concept that SPARC performs such functions at the level of the nuclear matrix, although novel, is compatible with data from laboratories that have investigated SPARC and its various functions. Rather than provide definitive answers regarding the mechanisms by which SPARC acts, our observations prompt additional questions regarding the biological activity of this glycoprotein: (1) does SPARC exert its physiological effects at the level of the extracellular matrix (as is currently perceived) or at the level of the nuclear matrix? Or, are both paradigms employed? and (2) does the translocation of rSPARC to the nucleus of cells in culture imply that SPARC acts in an autocrine/paracrine manner? Or conversely, does SPARC in vivo translocate to the nucleus directly after translation, without leaving the cell? Future experiments will address these questions and will attempt to elucidate the mechanisms that mediate translocation of SPARC into the nucleus, the functions of intracellular SPARC at all stages of the mitotic cycle, and potential nuclear ligands for SPARC.

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